DEVELOPMENTAL METHYLMERCURY TOXICITY IN A 6-HYDROXYDOPAMINE PARKINSONIAN RAT MODEL: EVALUATING *SEARSIA CHIRINDENSIS* AS A POTENTIAL NEUROPROTECTANT

By

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Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD) in the Discipline of Human Physiology, in the College of Health Science at the University of KwaZulu-Natal – Westville Campus

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(Zulfiah Mohamed Moosa)

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(Professor M.V. Mabandla)

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LIST OF ABBREVIATIONS

$^{\bullet}OH$ - hydroxyl radical

6-OHDA - 6-hydroxydopamine

atg12 - ATG12 autophagy related 12 homolog

Al - aluminium

AMPA - 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid

ANOVA - analysis of variance

cdkn1a - Cyclin-dependent kinase inhibitor 1A

Ca^{2+} - calcium ion

Cd - cadmium

Cu - copper

DAT - dopamine transporter

DPPH - 2.2-Diphenyl-1-picrylhydrazyl

DNA - deoxyribonucleic acid

EDTA - ethylene-diamine-tetra-acetic acid

ELISA - enzyme linked immunosorbent assay

ERK - extracellular signal regulated kinases

fth1 - Ferritin, heavy polypeptide 1

Fe - iron

Fe^{2+} - ferrous ion

Fe^{3+} - ferric ion

GA - gallic acid

GABA - gamma-amino butyric acid

GIT - gastrointestinal tract

GLAST - glutamate aspartate transporter

GLT1 - glutamate transporter 1

GLU - glutamate

GND - gestational day

Gpx - glutathione peroxidase

GR - glutathione reductase

GSH - oxidized glutathione

GSSG - reduced glutathione

hmox1 - Heme oxygenase (decycling) 1

H_2O - water

H_2O_2 - hydrogen peroxide

HCl - hydrochloric acid

Hg - mercury

$HgCl_2$ - mercury (II) chloride

ICP-OES - inductively coupled plasma optical emission spectrometry

IL1β - interleukin 1 beta

IL-6 - Interleukin 6

K^+ - potassium ion

KZN - KwaZulu-Natal

MAO - monoamine oxidase

MDA - malondialdehyde

MeHg - methylmercury

MeOH - methanol

MD - Minamata disease

Mn - manganese

Na^+- sodium ion

nbn1 - Nibrin

nqo1 - NAD(P)H dehydrogenase quinone 1

NMDA - N-Methyl-D-aspartate

nNOS - neuronal nitric oxide synthase

NO - nitric oxide

O_2 - oxygen

O_2^- - superoxide anion

OH^- - hydroxide ion

OA - oleanolic acid

Pb - lead

PCR - polymerase chain reaction

PD - Parkinson's disease (PD)

PND - postnatal day

Q - quercetin

RNA - ribonucleic acid

ROS - reactive oxygen species

sqstm1 - Sequestosome 1

-SH - thiol

Se - selenium

SEM - standard error of means

SC - *Searsia chirindensis*

TAC - total antioxidant capacity

TBA/BHT - thiobarbituric acid/ butylated hydroxytolene

TBARS - thiobarbituric acid reacting substances

THPA - Traditional Health Practitioners Act, No 22 of 2007

TNFα - tumor necrosis factor alpha

TrxR - thioredoxin reductases

ulk - Unc-51 like kinase 1

Zn - zinc

ABSTRACT

Methylmercury (MeHg) pollution in South Africa has escalated due to increased demand from industrial sources such as coal-fired power stations. This had led to a growing interest in the effects of this metal toxin on human health. Prenatal MeHg exposure has been suggested to be a silent neurotoxicant, which may display its effects when triggered by a further neurotoxic insult. MeHg exposure during the perinatal period leads to neurodevelopmental deficits resulting in motor and cognitive dysfunction. This suggests that developmental MeHg exposure may predispose to the development of neurodegenerative diseases such as Parkinson's disease (PD). In this study, we investigate the effects of prenatal MeHg exposure at adolescence and furthermore when subjected to an additional neurotoxic insult in a parkinsonian rat model. Behavioural tests were conducted to assess motor deficits with neurochemical assessment of trace element levels, total antioxidant capacity, dopamine and cytokine concentrations as well as gene expression profiling. We also investigated a novel plant extract *Searsia chirindensis (SC)* as a potential neuroprotectant by alleviating neurotoxicity. Overall the results of our study show that prenatal MeHg exposure disrupts trace element homeostasis at adolescence asymptomatically however, these imbalances are exaggerated following a further neurotoxic insult leading to motor deficits. Treatment with *SC* reduced motor deficits in MeHg-exposed offspring as reflected by higher dopamine levels. Contrastingly, treatment in the absence of MeHg exacerbated motor deficits with higher copper levels and upregulation of antioxidant genes *fth1* and *nqo1* in response to the neurotoxic effect. Therefore the overall total antioxidant capacity was not affected by *SC*. We also investigated the effect of *SC* on normal body parameters to assess for toxicity. Our findings showed that *SC* did not affect either liver or renal function and therefore does not affect the homeostasis of other body systems. Therefore conclusively our study showed that developmental MeHg exposure results in altered trace element homeostasis which may predispose to the development of neurodegenerative diseases such as Parkinson's. We also showed that *SC* stem-bark extract reduced motor deficits caused by 6-hydroxydopamine in MeHg-exposed offspring but exacerbated neurotoxicity in its absence. *SC* also did not have any adverse effect on the homeostasis of other body systems. Overall, this suggests that *SC* has potential as a neuroprotectant however further studies must be conducted to fully elucidate the mechanisms involved in its effect.

LIST OF PUBLICATIONS ARISING FROM THIS THESIS

The publications that constitute this thesis and the contribution I made to each of the manuscripts are presented here.

Publication 1:

Moosa, Z.M., Daniels, W.M.U., Mabandla, M.V., 2014. The effects of prenatal methylmercury exposure on trace element and antioxidant levels in rats following 6-hydroxydopamine-induced neuronal insult. Metabolic Brain Disease 29, 459-469. *In Print*

Author Contributions:
I designed the study together with the co-authors; I collected and analyzed all data as well as compiled and wrote the manuscript. The co-authors reviewed the manuscript and provided critical feedback.

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Moosa, Z.M., Mabandla, M., 2017. Searsia chirindensis stem-bark extract exacerbates 6-hydroxydopamine neurotoxicity in control rats but prevents motor deficits in offspring prenatally exposed to methylmercury. Neurotoxicology and Teratology. *Under review*

Author Contributions:
I designed the study, collected and analyzed all data, compiled and wrote the manuscript. The co-author guided the design, reviewed the manuscript and provided critical feedback.

Publication 3:

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Author Contributions:
I designed the study, collected and analyzed all data, compiled and wrote the manuscript. The co-author reviewed the manuscript and provided critical comments.

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Chapter 1

Introduction

The following chapter includes the literature review providing a background to the thesis, the problem statement, the study objectives as well as an outline of the overall study design.

1.1 Literature review

1.1.1 Metals

Heavy metals are elements with high atomic weights and are most commonly found in the industrial sector (Gupta et al., 2015, Järup, 2003). They are especially prevalent in the environment and are used typically in the food, mining, medical and pharmaceutical industries (Giacoppo et al., 2014, Järup, 2003). Heavy metal emission via the industry is particularly hazardous due to contamination via air, water and soil (Gupta et al., 2015, Järup, 2003).

Some of these metals have been shown to be toxic to human health (Formigari et al., 2007, Valko et al., 2005, Zahir et al., 2005). Metal-induced health defects can develop via two different mechanisms. It can occur as a result of abnormal regulation of essential metals or by toxic accumulation of non-essential metals (White, 2016, Wright and Baccarelli, 2007, Valko et al., 2005). Essential metals such as copper (Cu), iron (Fe) and zinc (Zn) are used by the body for normal physiological processes such as enzymatic and protein function and may become toxic to the body as a result of defective homeostatic mechanisms (Chen et al., 2016, Cristóvão et al., 2016, Kozlowski et al., 2009). Toxic metals are those which are not used by the body but are rather found in the environment and include lead (Pb), cadmium (Cd), mercury (Hg) and aluminium (Al) (Chen et al., 2016, Giacoppo et al., 2014, Franco et al., 2009b). Cd exposure has been associated with kidney and skeletal damage while Pb and Hg exposure leads to neurological deficits (Chen et al., 2016, Järup, 2003). It was also found that Cd, Pb and Hg are commonly associated with oxidative stress and apoptosis therefore leading to many pathological conditions (Franco et al., 2009b, Valko et al., 2005).

Essential metals (Trace elements)

Trace elements are essential micronutrients which are used by the body for normal physiological processes such as enzymatic and protein function (Formigari et al., 2007, Kozlowski et al., 2009). These metals can become toxic to the body as a result of defective homeostasis mechanisms. Imbalances in Cu, Fe and Zn concentrations have been strongly associated with oxidative stress which can lead to apoptosis and the development of many pathological conditions (Torres-Vega et al., 2012, Formigari et al., 2007, Kozlowski et al., 2009). Trace element imbalances in the brain have been strongly linked to the development of neurological disorders such as depression and epilepsy as well as the neurodegenerative diseases Alzheimer's and Parkinson's disease (Torres-Vega et al., 2012, Zatta et al., 2003). Some of these trace elements will now be discussed with regard to their biological function as well as their toxicity in the brain.

- *Iron (Fe)*

Fe functions primarily for the transport of oxygen in the body as a component of haemoglobin and is essential for proper neurodevelopment especially *in utero* and during the early postnatal period (Torres-Vega et al., 2012, Lozoff and Georgieff, 2006). Fe deficiency can affect the displacement of other metals such as Cu and Zn, and in doing so promote their toxicity (Oladiji, 2003). Fe deficiency is also associated with lower neurotransmitter levels since Fe forms a component of the enzymes involved in the synthesis of dopamine and serotonin (Lozoff et al., 2006b). Excessive Fe concentrations also lead to neurotoxicity by promoting the formation of reactive oxygen species (ROS) during the Fenton and Haber-Weiss reactions (Figure 1) (Levenson, 2005, Valko et al., 2005).

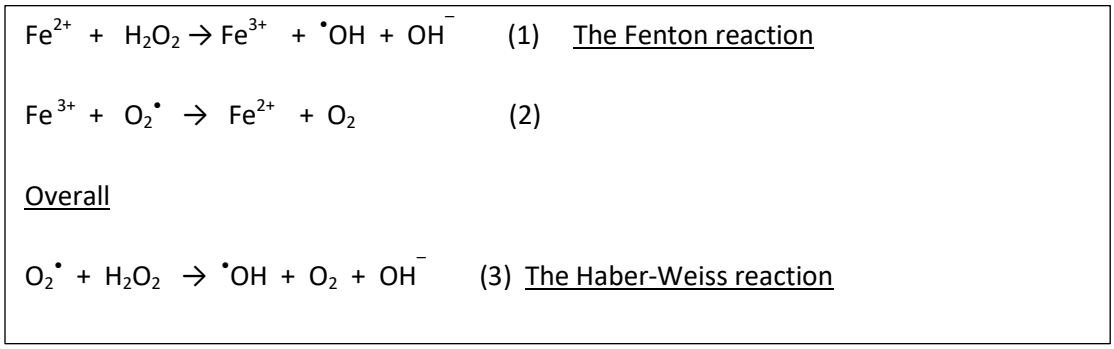


Figure 1: Reactions explaining the mechanism by which Fe promotes free radical formation (Valko et al., 2005).

Briefly, transition metal ions such as Fe form a hydroxyl radical and an oxidized metal ion when it reacts with hydrogen peroxide (Wright and Baccarelli, 2007, Valko et al., 2005). The oxidized metal ion can in turn react with the superoxide free radical (Figure 1). The net reaction can thus result in the formation of the hydroxide ion and the highly reactive hydroxyl radical which promote oxidative stress leading to apoptosis.

- Zinc (Zn)

Zn is found primarily in the brain and is vital for synaptic transmission, enzymatic function and also forms part of the transcription factors in the zinc-finger protein family (Torres-Vega et al., 2012, Formigari et al., 2007, Levenson, 2005). It is imperative to maintain Zn homeostasis since both Zn deficiencies as well as Zn toxicity have been shown to lead to oxidative stress and neuronal cell death (Bitanihirwe and Cunningham, 2009, Chen and Liao, 2003). Extremely high Zn concentrations have been associated with the inhibition of the electron transport chain and decreased cellular energy production via

the production of mitochondrial ROS (Bitanihirwe and Cunningham, 2009, Dineley et al., 2005). Zn has also been directly linked to apoptosis in primary cell cultures and has been shown to stimulate pro-apoptotic molecules such as p38 (Adamo et al., 2010, Bitanihirwe and Cunningham, 2009). Zn deficiency has been associated with the development of neurodegeneration via apoptosis (Formigari et al., 2007). Adamo et al., 2010 showed that Zn deficiency promotes apoptosis by reducing the expression of anti-apoptotic proteins as well as promoting the pro-apoptotic cascade. They also showed that Zn deficiency inhibits nuclear factor-kappa B (NFκB) and the pro-survival extracellular signal regulated kinases (ERK). These events promote apoptosis thus inducing neurodegeneration.

- Copper

The key function of Cu in the body is to act as a co-factor for the functioning of many essential enzymes in the body (Torres-Vega et al., 2012, Formigari et al., 2007, Levenson, 2005). Accumulation of Cu in the brain can be toxic as it readily promotes the formation of ROS thereby leading to neuronal cell death (Levenson, 2005, Gaetke and Chow, 2003). Cu acts similarly to Fe and promotes the formation of free radicals by the Fenton reaction (Figure 1) (Valko et al., 2005). Cu can also displace other metals thereby disrupting their homeostasis leading to toxicity and hence promoting apoptosis (Formigari et al., 2007).

- Manganese

Under normal physiological conditions manganese (Mn) is utilized as a co-factor for enzymatic function and is also important in carbohydrate, lipid and protein metabolism (Weiss, 2011, Takeda, 2003). Mn is especially important in neurodevelopment and has been implicated as a risk factor for neurological disorders in adulthood (Cordova et al., 2013, Weiss, 2011). Mn accumulates readily in the brain and can be neurotoxic by impairing neurotransmitter systems leading to motor and cognitive dysfunctions (Weiss, 2011, Takeda, 2003, Zatta et al., 2003).

- Selenium

Selenium (Se) is a micronutrient which is important for brain function. Se is an essential component of many selenoproteins such as glutathione peroxidase (Gpx), thioredoxin reductases (TrxR) as well as selenoprotein P (Schweizer et al., 2004, Chen and Berry, 2003). Gpx and TrxR both have antioxidant functions and help maintain redox balance to prevent oxidative stress (Branco et al., 2012, Schweizer et al., 2004). Selenoprotein P binds readily to heavy metals and therefore it is important in regulating metal toxicity (Chen and Berry, 2003). Se has been shown to have a protective role in the brain by reducing the toxic effects of metals such as methylmercury (MeHg) (Meinerz et al., 2011, Usuki et al., 2011).

Heavy metals: MeHg

MeHg is an organometallic compound which is used in a variety of human applications (Ceccatelli et al., 2010, Crespo-López et al., 2009). It is used in the manufacture of thermometers, in dental tooth fillings and in the agricultural and pharmaceutical industries (Farina et al., 2011b, Ceccatelli et al., 2010, Crespo-López et al., 2009). MeHg exposure can occur via many different routes (Holmes et al., 2009). It can be formed by the biomethylation of natural inorganic Hg released from chemical industrial plants and via the mining industry (Farina et al., 2011b, Crespo-López et al., 2009, Holmes et al., 2009). These chemicals are often released into nearby water sources such as rivers and dams and can be transferred to marine species. Human exposure to MeHg occurs primarily by ingestion of contaminated fish from these environments (Clarkson et al., 2007, Johansson et al., 2007). This is highly toxic due to the inclination of MeHg to biomagnify within the aquatic food chain leading to massive accumulation in the foetal brain after exposure in pregnant women (Crespo-López et al., 2009). When ingested, MeHg readily enters the gastrointestinal tract (GIT) and can be converted to its inorganic form by microflora which is then excreted from the body (Figure 2) (Ceccatelli et al., 2010, Clarkson et al., 2007). MeHg has a high affinity for erythrocytes where it binds to the cysteine residues in haemoglobin and therefore enters the bloodstream (Clarkson et al., 2007). There, it is rapidly transported to all parts of the body but accumulates preferentially in the liver, hair and the brain (Figure 2).

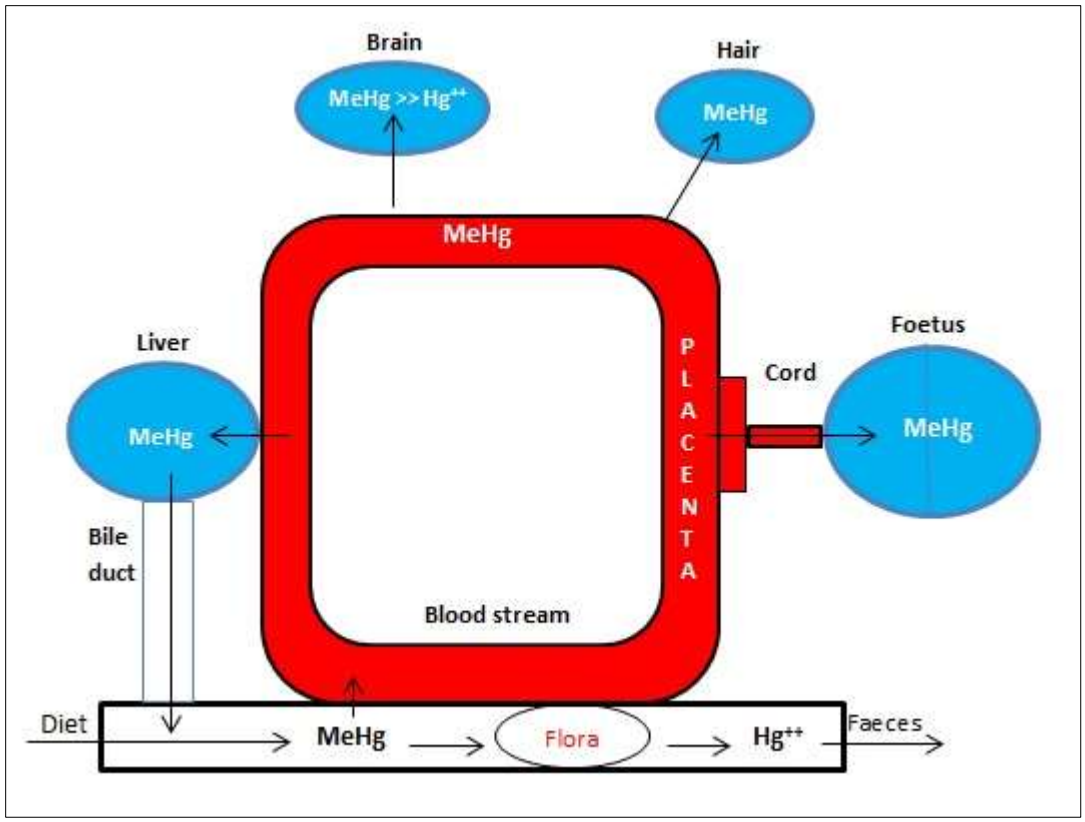


Figure 2: Diagram showing metabolism and distribution of methylmercury during pregnancy [Adapted from (Clarkson et al., 2007)].

1.1.2 Hg and MeHg exposure in South Africa

Hg pollution in South Africa stems primarily from coal-fired power stations, artisanal gold mining as well as cement production (Masekoameng et al., 2010, Leaner et al., 2009). Many of these sources are located close to rivers and dams which are accessed by local communities for daily use as sanitation, food and water resources (Walters et al., 2011). Hg pollution in South Africa had not been extensively studied previously however in the early 1990's a Hg processing plant in KwaZulu-Natal (KZN) was held liable for occupational exposure leading to several deaths as well as severe cases of poisoning (Oosthuizen and Ehrlich, 2001). Following campaigning by many environmentalists the processing plant was decommissioned however, despite this, the Hg remained stored for two decades afterwards thus remaining a threat to the local communities (Papu-Zamxaka et al., 2010a). Although extensive water testing showed that Hg levels were below the detection limit, concerns remain since Hg readily transfers to sediments. Papu-Zamxaka *et al* (2010) found elevated hair Hg levels in villagers living in these areas. Hg levels were also measured in fish as well as in sediments from a nearby river where concentrations

were also elevated (Papu-Zamxaka et al., 2010b). Assessment of Hg and MeHg in South Africa identified the highest concentrations in areas near coal-fired power stations (Walters et al., 2011). Areas most affected by MeHg include the provinces Limpopo, Gauteng and KZN with the highest levels seen in the Mpumalanga and Free State provinces (Walters et al., 2011).

1.1.3 Mechanisms of MeHg toxicity

The mechanism underlying MeHg toxicity has been shown to affect multiple pathways/factors which may work in synergy to generate its toxic effect. These factors will be briefly discussed.

1.1.3.1 Oxidative stress and the glutathione antioxidant system

The primary route targeted by methylmercury is the glutathione antioxidant system. MeHg binds directly to glutathione (GSH) due to its high affinity for thiol (-SH) groups (Figure 3) (Farina et al., 2011a, Nascimento et al., 2008). Binding to MeHg reduces the amount of free glutathione available for antioxidant function therefore reducing the antioxidant balance (Farina et al., 2011a). MeHg also inhibits complex II and III of the mitochondrial respiratory complexes in the electron transport chain promoting the generation of reactive oxygen species such a superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Figure 3) (Mori et al., 2011). In the presence of ferrous ions (Fe^{2+}), H_2O_2 is converted to the hydroxyl radical ($^{\bullet}OH$) via the Fenton reaction (Figure 1) (Leonard et al., 2004). Excessive accumulation of ROS leads to the development of oxidative stress which promotes cellular damage and apoptosis (Circu and Aw, 2010). The activity of the Gpx enzyme is also inhibited by MeHg (Farina et al., 2011b, Franco et al., 2009a). Gpx is involved in removing free radicals such as peroxides by catalyzing the conversion of reduced glutathione (GSSG) to its oxidized form (GSH) to exert its antioxidant function (Figure 3) (Franco et al., 2009a). Therefore MeHg exerts a direct and indirect mechanism of impaired antioxidant status by firstly acting directly on GSH and its antioxidant enzymes but also by increasing the generation of reactive oxygen species and the development of oxidative stress (Farina et al., 2011a, Franco et al., 2009a).

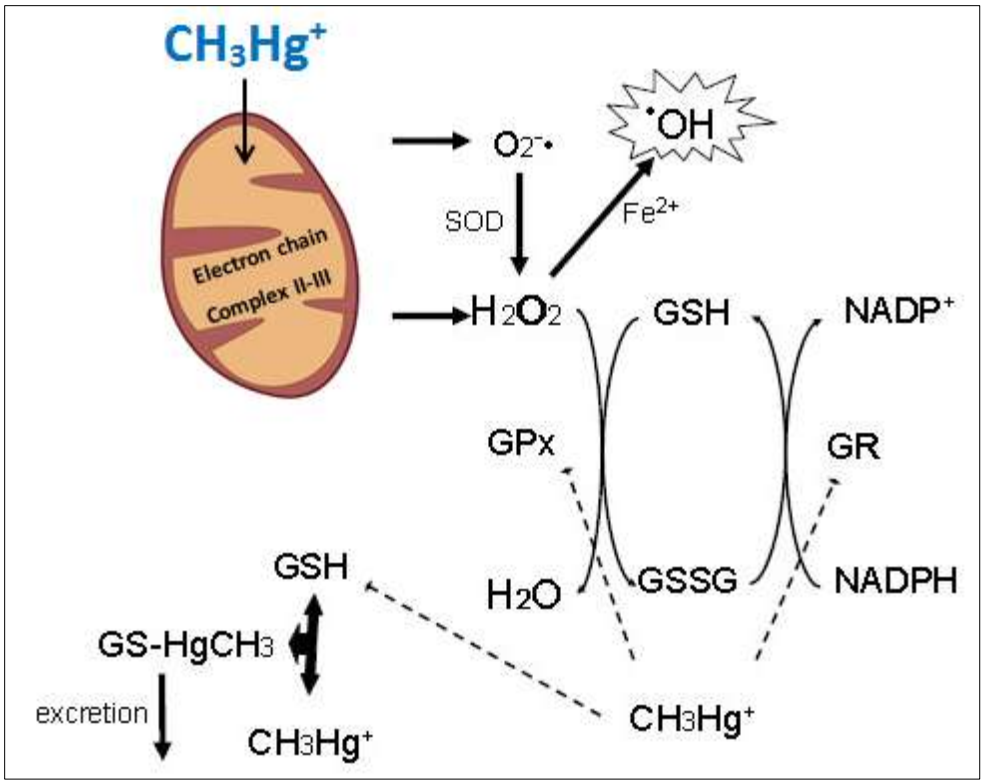


Figure 3: Diagram showing the effect of MeHg on the glutathione antioxidant system [Adapted from (Farina et al., 2011b)].

1.1.3.2 Glutamate excitotoxicity

Glutamate is an excitatory neurotransmitter which mediates learning, memory and cognitive functions (Liu et al., 2014b). In dopaminergic neurons, glutamate binds to N-Methyl-D-aspartate (NMDA) and 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid (AMPA) receptors resulting in an influx of calcium ions, which activates the signaling pathways for learning and memory (Lau and Tymianski, 2010). Excessive accumulation of extracellular glutamate can lead to excitotoxicity which promotes cell death (Liu et al., 2014b, Aschner et al., 2007). MeHg neurotoxicity has been linked to increased extracellular glutamate (Aschner et al., 2007, Juarez et al., 2002). Glutamate is released from astrocytes with MeHg exposure and its reuptake is inhibited (Figure 4) (Aschner et al., 2007, Farina et al., 2011b). The glutamate transporters glutamate aspartate transporter (GLAST) and glutamate transporter 1 (GLT1) are responsible for maintaining glutamate homeostasis (Farina et al., 2011b). In conditions of excess extracellular glutamate, the glutamate transporters promote glutamate re-uptake by astrocytes. MeHg stimulates cytoplasmic phospholipase A_2 in astrocytes which releases arachidonic acid into the synaptic cleft (Aschner and Aschner, 2007, Aschner et al., 2007). Arachidonic acid in turn blocks the GLAST/GLT1 transporter (Aschner et al., 2007). This increases extracellular glutamate concentrations.

MeHg also acts at neurons where it inhibits glutamate re-uptake into vesicles and it stimulates the release of glutamate into the synaptic cleft (Figure 4, event 2 & 3). This leads to excessive concentrations of glutamate in the synaptic cleft which hyper-activates the N-Methyl-D-aspartate (NMDA) receptors on the post-synaptic neuron (Farina et al., 2011a, Nascimento et al., 2008). Over-stimulation of NMDA receptors leads to high intracellular levels of Ca^{2+} which promotes mitochondrial dysfunction and activates neuronal nitric oxide synthase (nNOS) (Figure 4, event 4, 5, 6 & 7) (Farina et al., 2011a, Nascimento et al., 2008). Neuronal nitric oxide synthase synthesizes nitric oxide (NO) which promotes oxidative damage primarily by interacting with O_2^- to form peroxynitrite, an extremely effective oxidant (Aschner and Aschner, 2007, Farina et al., 2011a). Therefore glutamate excitotoxicity plays an essential role in MeHg neurotoxicity.

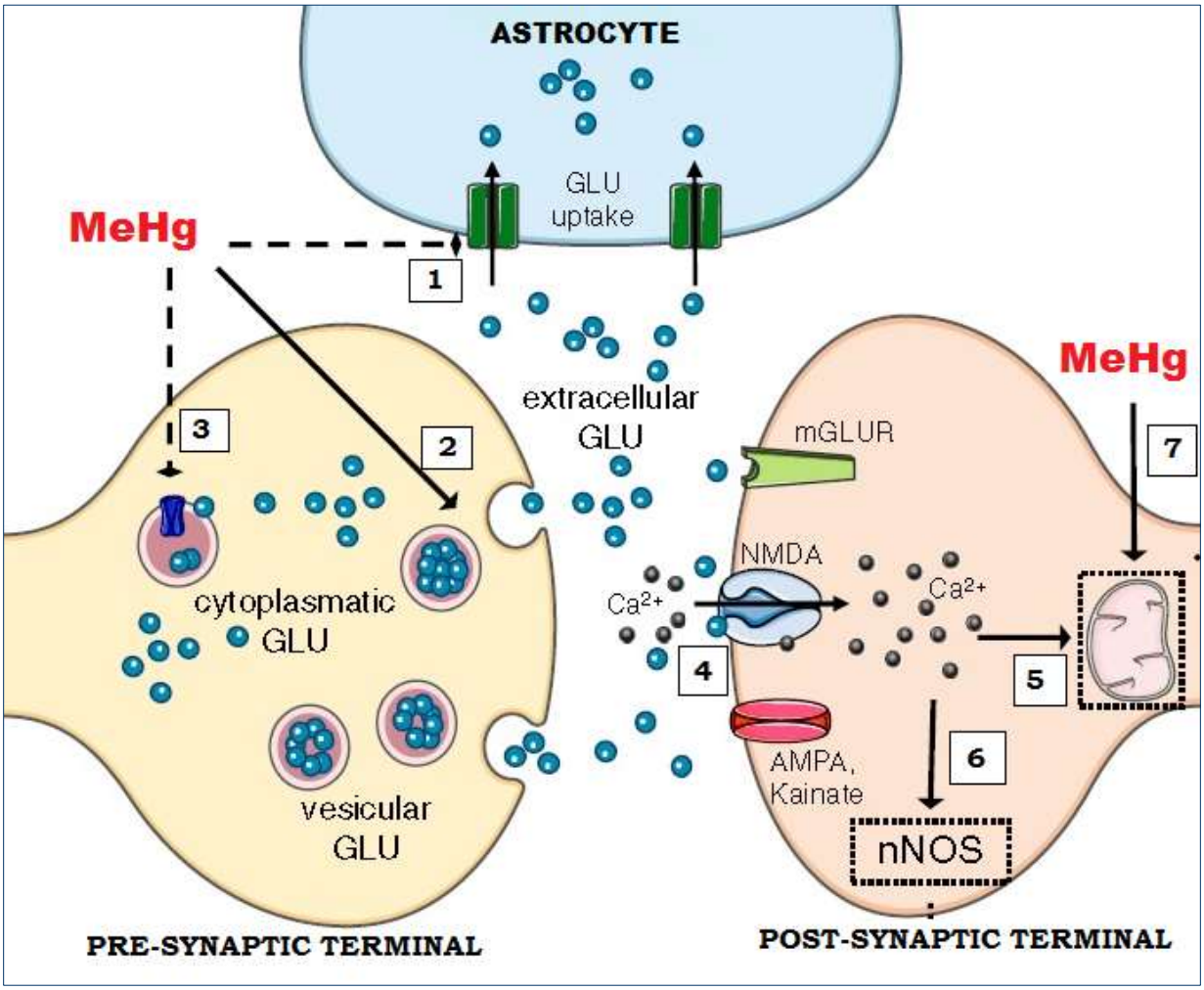


Figure 4: Mechanisms of MeHg neurotoxicity [Adapted from (Farina et al., 2011a)]

1.1.3.3 Genotoxicity and Cytoskeletal structure

MeHg may also cause apoptosis by genotoxicity and by damage to the cellular cytoskeleton (Aggarwal et al., 2014, Crespo-López et al., 2007). MeHg has been shown to induce DNA damage at low concentrations (100µg/day) which may be mediated partly by the generation of ROS (Grotto et al., 2009). ROS can damage DNA directly by binding to nucleic acids resulting in genetic mutations or indirectly by impairing the proteins necessary for the DNA repair processes (Figure 5, event 1) (Crespo-López et al., 2009). ROS can also disrupt mitotic spindle formation preventing DNA synthesis. MeHg may interact directly with DNA molecules binding to nucleotides which can affect the secondary DNA structure (Figure 5, event 2)(Crespo-López et al., 2009). DNA repair enzymes are also compromised with MeHg toxicity due to the binding of MeHg to the sulfhydryl (thiol) groups within the enzymes (Crespo-López et al., 2009). This changes the structural conformation of the enzymes which renders them inactive (Figure 5, event 3). MeHg also binds to the thiol group of tubulin in the cytoskeleton preventing tubulin polymerization and inhibiting tubulin synthesis (Aggarwal et al., 2014, Crespo-López et al., 2009, Miura, 2000). This disrupts cytoskeletal structure, prevents cell division and leads to apoptosis (Figure 5, event 4) (Miura, 2000).

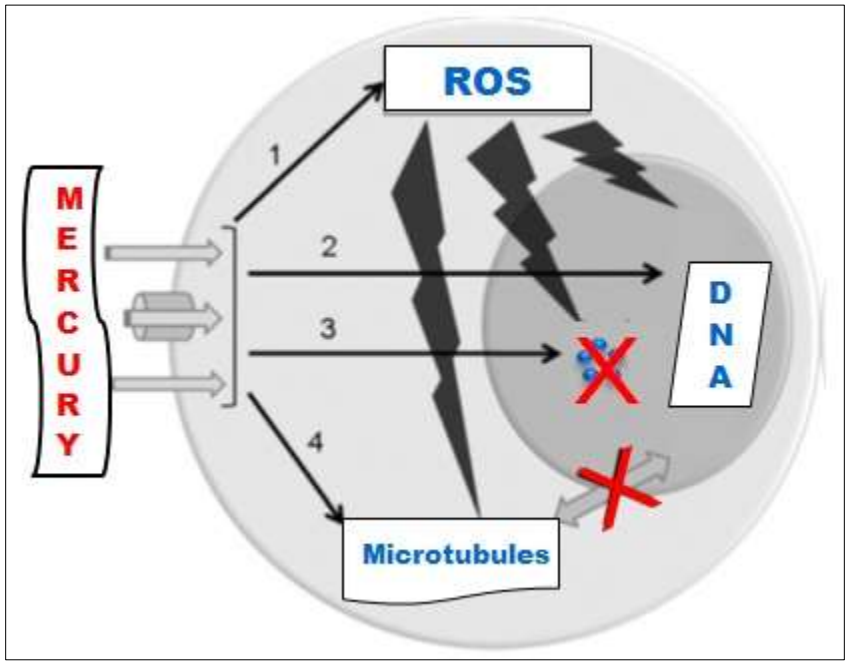


Figure 5: Effects of MeHg on DNA and microtubular structure [Adapted from (Crespo-López et al., 2009)].

1.1.3.4 Trace element imbalances

Hg compounds have been shown to displace other trace elements leading to imbalance in their concentrations which promotes their toxicity (Zhang et al., 2007, Feng et al., 2004). Prenatal treatment with mercury (II) chloride can cause Fe deficiency in offspring brain as well as increased Zn and Cu levels (Zhang et al., 2007). Muto et al. (1991) also showed an increase in Zn and Cu levels when adult rats were treated with MeHg. The effect of prenatal MeHg treatment on trace element balance of offspring has not been well established.

1.1.4 Metals and neurodegenerative diseases: MeHg

There has been increasing evidence implicating metal-based toxicity in neurodegenerative diseases (Chen et al., 2016, Cristóvão et al., 2016, Crichton et al., 2008, Bush, 2000). Several studies have suggested that metals such as Hg, Zn, Al, Pb and Fe are directly linked to the neurodegenerative process (Chen et al., 2016, Cristóvão et al., 2016, Giacoppo et al., 2014). This metal-mediated neurodegeneration has been proposed to occur primarily via the accumulation of ROS (Figure 1) resulting in the development of oxidative stress (Valko et al., 2005, Oteiza et al., 2004, Ercal et al., 2001). Oxidative stress can be mediated by either damage to the antioxidant defense system (depletion of antioxidants/antioxidant enzymes) or by the direct generation of ROS (Leonard et al., 2004, Ercal et al., 2001). This results in the generation and accumulation of reactive oxygen species (ROS) which promotes cellular damage leading to apoptosis.

MeHg was discovered to have severe neurotoxic effects following episodes of mass environmental exposure in Japan and Iraq (Nascimento et al., 2008, Sakamoto et al., 2002). MeHg poisoning in Japan during the 1950's has led to Minamata disease (MD) which is characterized by neurological dysfunction, cognitive deficits and has resulted in death in severe cases (Yorifuji et al., 2011, Ekino et al., 2007). Foetal cases of MD resulted in mental disturbances including motor and psychiatric dysfunction (Eto et al., 2010, Ekino et al., 2007). The developing brain is particularly susceptible to MeHg toxicity due to the metal's ability to penetrate the placenta and the blood-brain barrier (Ceccatelli et al., 2010, Clarkson et al., 2007, Sakamoto et al., 2002). Prenatal exposure to MeHg resulted in offspring with major neurodevelopmental deficits such as mental retardation as well as motor and cognitive dysfunction (Ferraro et al., 2009, Johansson et al., 2007, Daré et al., 2003). Previous studies have indicated that MeHg may have adverse effects on the dopaminergic system (Huang et al., 2016, Shao and Chan, 2015, Martinez-Finley et al., 2013, Dreiem et al., 2009). MeHg was shown to decrease dopamine synthesis,

dopamine receptor binding as well increase alpha synuclein expression (Huang et al., 2016, Shao and Chan, 2015, Daré et al., 2003). This suggests that MeHg may promote dopaminergic neurodegeneration which may lead to the development of Parkinson's disease.

1.1.5. Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disease of the basal ganglia that is characterized by the progressive loss of dopamine neurons in the nigrostriatal pathway (Blesa et al., 2012, Dauer and Przedborski, 2003). PD is diagnosed clinically only when there is more than 80% dopamine neuron degeneration (Le et al., 2014, Deumens et al., 2002). The late onset of motor symptoms is thought to be due to compensatory mechanisms within the brain that maintain dopamine levels despite the progressive loss of dopamine neurons (Deumens et al., 2002). Symptoms characteristic of PD include bradykinesia (slowness of movement), muscle rigidity, postural instability and tremors at rest (Choukairi et al., 2013, Blesa et al., 2012). Other non-motor symptoms include disruption in sleep patterns, olfactory deficits as well as depression (Blesa et al., 2012, Solayman et al., 2016). PD is mainly an idiopathic disease however, studies have shown that it may have a genetic (5-10% prevelance) or an environmental origin (Blesa et al., 2012, Le et al., 2014).

1.1.5.1 The 6-Hydroxydopamine (6-OHDA) model of PD

The 6-OHDA model is the most commonly used animal model of Parkinson's disease (Le et al., 2014, Blandini et al., 2008). When injected into the brain, 6-OHDA can selectively enter catecholamine neurons via the dopamine transporter (DAT) because of its structural similarity to dopamine (Blandini et al., 2008, Choukairi et al., 2013, Jackson-Lewis et al., 2012). It causes neuronal cell death by either entering the mitochondria or by accumulating within the cytosol (Blum et al., 2001). In the mitochondria, 6-OHDA inhibits mitochondrial complexes I and IV of the mitochondrial respiratory enzymes thus impairing neuron function. This has been shown to lead to neuron death by apoptosis (Figure 6) (Deumens et al., 2002, Blandini et al., 2008). In the cytosol, 6-OHDA undergoes auto-oxidation resulting in the formation of reactive oxidative species and oxidative stress (Figure 6) (Choukairi et al., 2013, Blum et al., 2001). Blum et al., (2001) suggest that the oxidative stress could be mediated via monoamine oxidase (MAO) (Figure 6). MAO is the enzyme involved in the breakdown of dopamine and can similarly act on 6-OHDA. 6-OHDA is deaminated by MAO to form H_2O_2, which induces oxidative stress leading to apoptosis (Figure 6) (Blum et al., 2001).

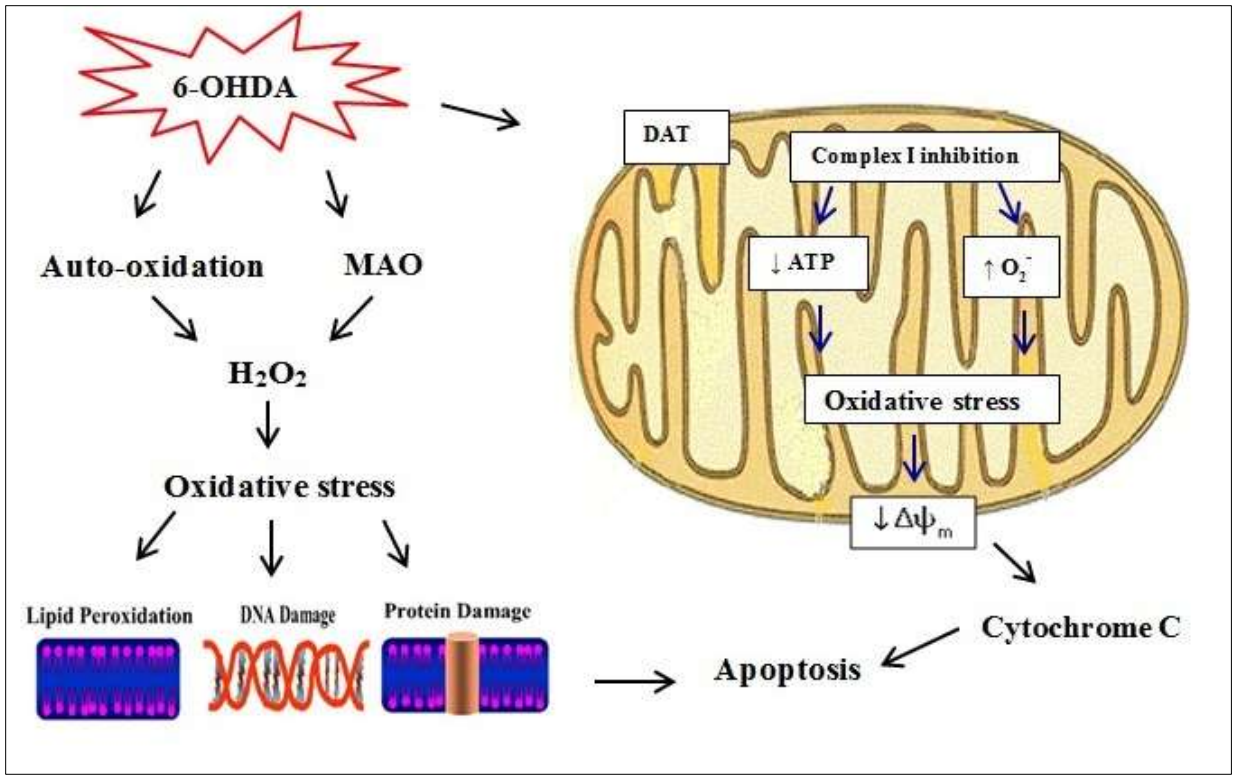


Figure 6: Proposed mechanism of 6-OHDA toxicity [Adapted from (Blandini et al., 2008, Blum et al., 2001).

1.1.6 Traditional medicine

Many communities in Africa rely primarily on treatment from traditional healers via the use of plant extracts. It is approximated that 80% of the South African population consult traditional healers for the treatment of various ailments (Moagi, 2009). Considering this, the post-apartheid government of South Africa implemented the Traditional Health Practitioners Act, No 22 of 2007 (THPA), in an attempt to recognize the previously marginalized practices of the African culture (Moagi, 2009). The aim of this act is to acknowledge traditional healers and their practices as a form of remedial medicine in the diagnosis, treatment and prevention of physical and mental disorders (Truter, 2007). Mental disorders in particular are stigmatized, consequently directing more people to herbalists instead of conventional medical treatment (Egbe et al., 2014). Plant extracts have shown many beneficial effects in human health which has led to an increase in plant-derived drug discovery. Although the passing of the THPA indicates progressive thinking, the governing of the safety regulations to ensure safe, efficient and quality traditional health care services is not well implemented (Moagi, 2009). Plant extracts are rich in phytochemical constituents which are highly variable in concentration and therefore these different constitutes of the plant may display differential effects in different body systems and may have unwanted

side effects. Therefore it is essential to understand the mechanism of action of these extracts to fully elucidate their effectiveness and to identify and isolate their active components rather than treat with whole plant extracts. The study of the effects of crude extracts is also necessary as this could provide valuable information to rural communities and advise on possible adverse effects of treatment. Additionally, this may also assist in determining the optimal dosage for the treatment of different disorders because administration of the incorrect dosage may mask the beneficial properties.

1.1.6.1 Searsia chirindensis

Figure 7: Diagram showing the *Searsia chirindensis* tree

Searsia chirindensis (*SC*) (Baker F.) (Anacardiaceae) is a semi-deciduous tree which is widely distributed in southern Africa and found predominantly in the KwaZulu-Natal, Western Cape and Limpopo provinces of South Africa (Moffett, 2007, Ojewole, 2007). *SC* is used commonly in KwaZulu-Natal by African traditional health practitioners for the treatment and management of inflammatory conditions as well as diabetes mellitus (Ojewole, 2007). Following these claims, Ojewole (2007) showed that *SC* has hypoglycaemic, analgesic and anti-inflammatory properties. Furthermore, *SC* was also found to be an effective anticonvulsant, increasing latency and reducing the duration of seizures (Qulu et al., 2016, Ojewole, 2008). Qulu et al (2016) also showed that *SC* stem-bark extract alleviated febrile seizure-induced increases in interleukin-1β concentrations thereby counteracting neuroinflammation. Leaf extracts of *SC* also exhibited antibacterial activity against Gram-negative and Gram-positive bacterial strains and can therefore be effective in the treatment of diarrhea (Madikizela et al., 2013).

1.2 Problem statement

Although awareness regarding Hg pollution in South Africa has improved significantly, the risks and health effects of Hg and MeHg exposure has not been extensively publicized. Exposure to MeHg during the perinatal period in particular has been shown to result in cognitive dysfunction which persists into adulthood (Debes et al., 2016, Yorifuji et al., 2015, Debes et al., 2006). It has also been suggested that early life neurotoxicity may lead to the development of neurodegenerative diseases (Bellinger et al., 2016, Kraft et al., 2016). Therefore prenatal MeHg toxicity may pose as a risk for neurodegeneration.

Recent trends have shown a surge in the use of plant extracts and their phytochemical constituents for the treatment of neurological and neurodegenerative conditions (Beppe et al., 2014, Xu et al., 2012, Stafford et al., 2008). Therefore, plant extracts may constitute a pertinent treatment for MeHg-induced neurodegeneration.

This study aims to increase awareness of MeHg poisoning in South Africa, especially amongst pregnant women. We hope that the outcome of this research will further elucidate the mechanisms involved in MeHg neurotoxicity during pregnancy and their effects on the cognitive function of their children. This will educate pregnant woman about the dangers of eating fish and using water from sources close to industrial plants. We also aim to provide clarity on the use of plant extracts for MeHg neurotoxicity and the potential benefits and/or detriments of these extracts on normal bodily function.

1.3 Study Objectives

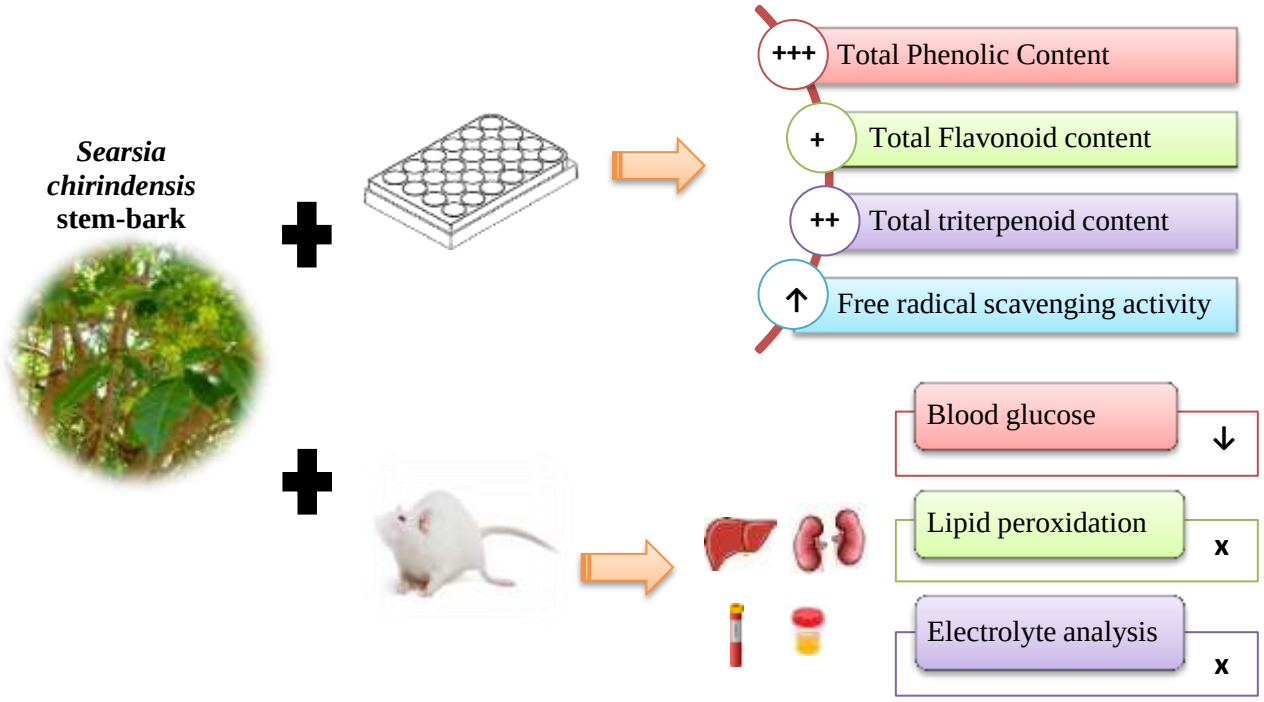
In this thesis, we studied MeHg exposure during pregnancy and examined the effect on the brain of offspring in adolescence and adulthood. We also used the neurotoxin 6-OHDA to create an animal model of Parkinson's disease. The *SC* plant extract was also evaluated as a potential neuroprotectant. The resultant studies (chapters 2, 3 and 4) therefore specifically aim to:

- determine the effect of prenatal MeHg exposure on trace element levels and total antioxidant capacity in adolescent offspring.
- investigate whether prenatal exposure to MeHg exacerbates the neurotoxic effects of 6-OHDA in a parkinsonian rat model.
- evaluate the phytochemical profile of crude stem-bark extract of *SC* and assess its effect on normal body homeostasis

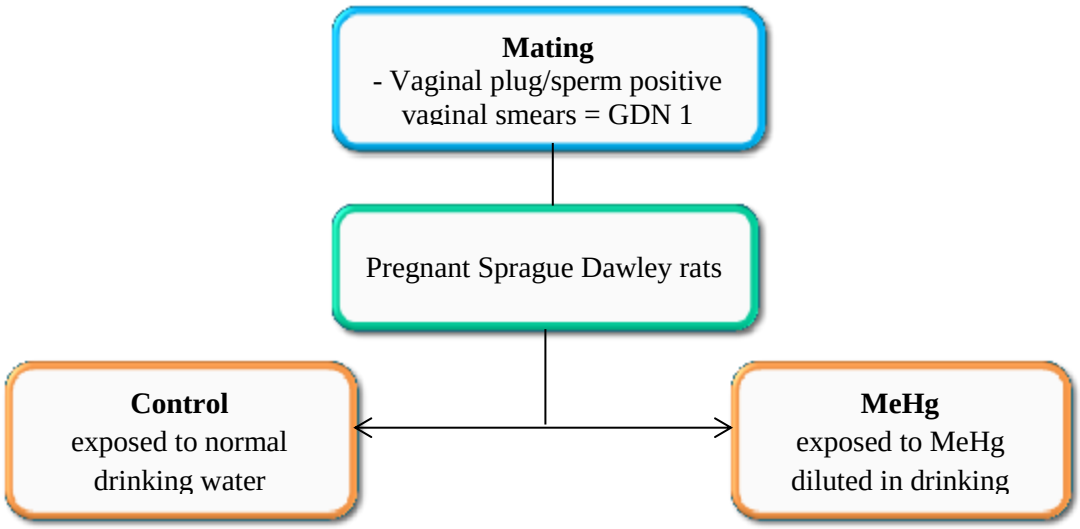
- investigate whether *SC* stem-bark extract can ameliorate the neurotoxic effects of MeHg in a 6-OHDA parkinsonian rat model

1.4 Study design

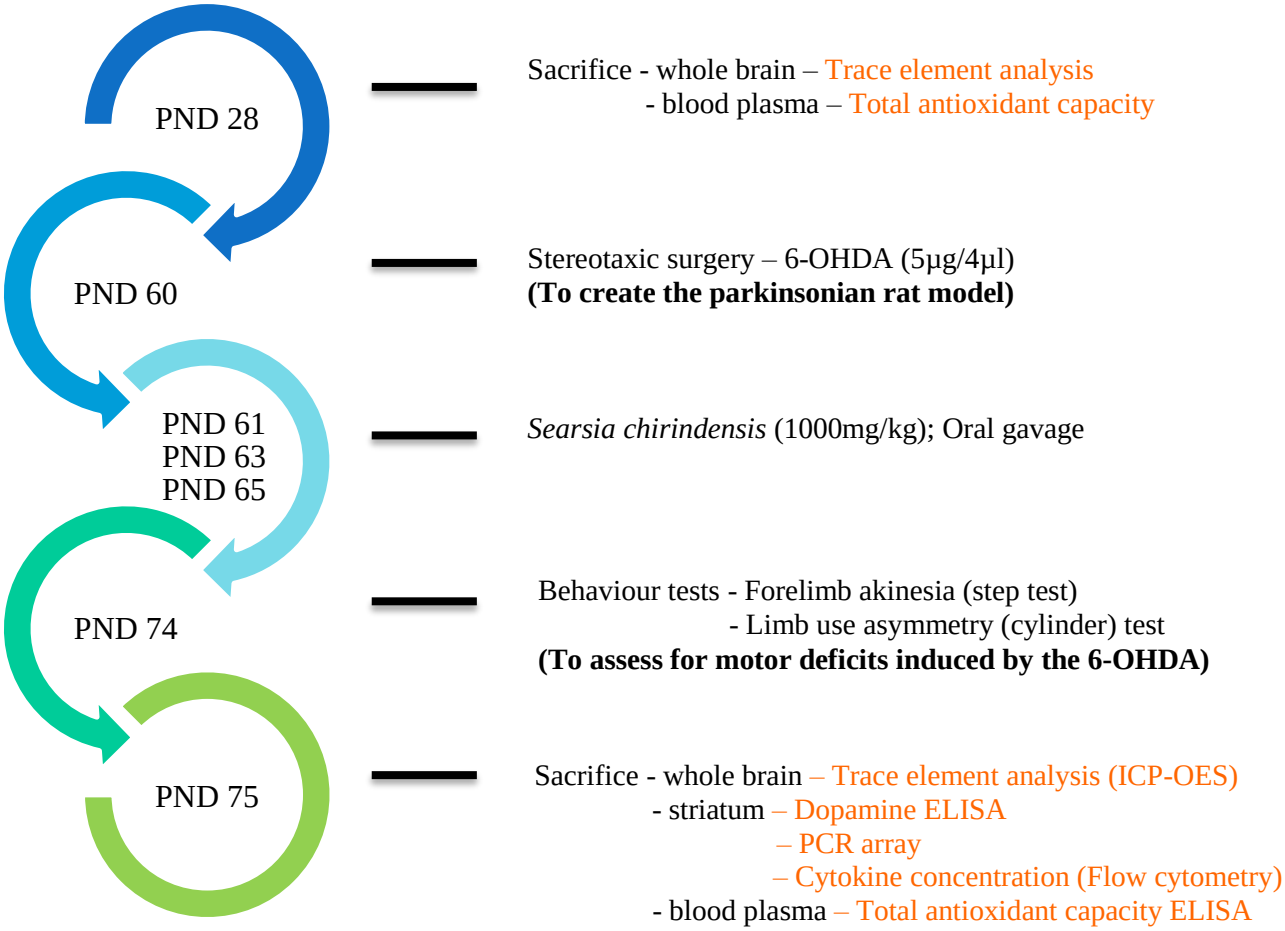
1.4.1 Plant phytochemistry and screening



1.4.2 Prenatal handling



1.4.3 Postnatal handling



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Chapter 1 reviewed the literature regarding MeHg pollution in South Africa, it's mechanisms of action and how these mechanisms may lead to neurodegeneration. Chapter 2 will investigate the effect of prenatal exposure to MeHg on the neurodevelopment of the offspring at adolescence and the consequences of a subsequent neurotoxic insult later in life.

Chapter 2

Article 1

The effects of prenatal methylmercury exposure on trace element and antioxidant levels in rat offspring following 6-hydroxydopamine-induced neuronal insult

The current article was published in the journal **Metabolic Brain Disease** (2014, Volume 29, pages 459-469)**.** The article has been presented in manuscript format according to the submission requirements of the journal. In this manuscript we have included the figures with legend as part of the results section for easier reading for the benefit of the reader.

The effects of prenatal methylmercury exposure on trace element and antioxidant levels in rats following 6-hydroxydopamine-induced neuronal insult

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Key words: methylmercury, prenatal, neurodevelopment, trace elements, antioxidants

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Abstract

Methylmercury (MeHg) is a metal toxin found commonly in the environment. Studies have shown severe neurotoxic effects of MeHg poisoning especially during pregnancy where it crosses the foetoplacental and the blood brain barrier of the foetus leading to neurodevelopmental deficits in the offspring. These deficits may predispose offspring to neurodegenerative diseases later in life. In this study we investigated the effects of prenatal methylmercury exposure (2.5mg/L in drinking water from GND 1- GND 21) on the trace element status in the brain of adolescent offspring (PND 28). Total antioxidant capacity (TAC) was measured in their blood plasma. In a separate group of animals that was also exposed prenatally to MeHg, 6-hydroydopamine (6-OHDA) was administered at PND 60 as a model of neuronal insult. Trace element and TAC levels were compared before and after 6-OHDA exposure. Prenatal MeHg treatment alone resulted in significantly higher concentrations of zinc, copper, manganese and selenium in the brain of offspring at PND 28 ($p<0.05$), when compared to controls. In contrast, brain iron levels in MeHg-exposed adolescent offspring were significantly lower than their controls ($p<0.05$). Following 6-OHDA exposure, the levels of iron, zinc, copper and manganese were increased compared to sham-lesioned offspring ($p<0.05$). Prenatal MeHg exposure further increased these trace element levels thereby promoting toxicity ($p<0.05$). Total antioxidant capacity was not significantly different in MeHg and control groups prior to lesion. However, following 6-OHDA administration, MeHg-exposed animals had a significantly lower TAC than that of controls ($p<0.05$). Brain TAC levels were higher in adult male rats than in female rats during adolescence however male rats that had been exposed to MeHg in utero failed to show this increase at PND 74. Prenatal MeHg exposure results in trace element dyshomeostasis in the brain of offspring and reduces total antioxidant capacity. This may reflect a mechanism by which methylmercury exerts its neurotoxicity and/or predispose offspring to further neurological insults during adulthood.

1. Introduction

Excess metals have long been proposed to be neurotoxic and in the young, may induce neurodevelopmental defects (Crichton et al., 2008, Giménez-Llort et al., 2001, Ferraro et al., 2009). This proposal stems from the ability of the brain to concentrate metal ions leading to its abnormal accumulation in various brain regions (Bush, 2000). The compartmentalization of ions has been shown to be harmful to the central nervous system when under inefficient homeostatic control (Bush, 2000).

Methylmercury (MeHg) is an environmental pollutant which in higher than normal concentrations, is hypothesized to be detrimental to brain structure and function (Franco et al., 2009a, Ferraro et al., 2009). For instance, prenatal MeHg intoxication has been associated with neurodevelopmental disorders such as mental retardation, as well as motor and cognitive dysfunction (Daré et al., 2003, Giménez-Llort et al., 2001, Ferraro et al., 2009, Johansson et al., 2007). The mechanism by which MeHg mediates these toxic effects remains unclear. One suggested explanation refers to the ability of MeHg to impair the antioxidant potential of the brain (Nascimento et al., 2008, Franco et al., 2009a, Farina et al., 2011a). Franco et al (2009) showed that MeHg reduces the activity of the glutathione peroxidase enzyme (GPx) in both an *in vitro* and *in vivo* model. MeHg binds readily to glutathione due to its affinity for thiol (-SH) groups. MeHg promotes the formation of free radicals such as reactive oxygen species (ROS) which also impair the glutathione antioxidant system and promote cell death by apoptosis (Farina et al., 2011a, Nascimento et al., 2008).

In addition, there is some evidence that mercury may disrupt the trace element balance in the brain (Feng et al., 2004, Zhang et al., 2007, Muto et al., 1991). Muto et al (1991) showed an increase in zinc and copper levels in the brain following MeHg treatment. This occurs because MeHg has a stronger binding affinity to sulfhydryl groups than other trace elements thereby displacing them from their active sites leading to their accumulation (Aliaga et al., 2010, Limke et al., 2004, Zhang et al., 2007). Previous studies have shown that developmental exposure to organic mercury compounds such as mercury (II) chloride ($HgCl_2$), results in higher copper and zinc levels as well as reduced iron levels in the brain of rat offspring (Feng et al., 2004, Zhang et al., 2007). These alterations are suggested to promote neurotoxicity and subsequently the development of neurodegenerative diseases.

One of the challenges in our current understanding of metal-induced toxicity is the latency in the manifestation of metal-related diseases. A possible explanation for this delay may be due to what is referred to as silent toxicity – a phenomenon described as “a biochemical or morphological injury which remains clinically unapparent unless unmasked by experimental or natural processes” (Giordano and

Costa, 2012). Under circumstances of silent toxicity, a subsequent neurotoxic injury is therefore required to trigger the onset of disease. Interestingly other trace elements such as selenium have been shown to have a protective effect against MeHg toxicity (Newland et al., 2006, Ralston et al., 2008, Ralston et al., 2007).

The aims of the present study were therefore to investigate the effects of prenatal MeHg exposure on the concentrations of a variety of trace elements in the brain, as well as the total antioxidant capacity of the brain in adolescent offspring. We also assessed the impact of prenatal MeHg exposure on the consequences of a subsequent neurotoxin later in life with respect to the levels of these trace elements and the antioxidant status of the brain.

2. Materials and methods

2.1 Animal handling and treatment procedure

Male and female Sprague-Dawley rats were obtained from the Biomedical Resource Centre at the University of KwaZulu-Natal and were housed under a 12hr light/dark cycle (6:00-18:00), with food and water *ad libitum*. All experiments were conducted with the approval of the University of KwaZulu-Natal Animal Ethics Research Committee (Ethical Clearance number: 090/12/Animal).

Synchronization: Female rats were housed in pairs in order to synchronize their oestrus cycles. The rat oestrus cycle is usually between four to five days long and is divided into 4 phases, namely: - pro-oestrus, oestrus, met-oestrus and di-oestrus (Hubscher et al., 2005, Westwood, 2008). During oestrus, the oestrogen concentration is high making it the ideal phase for pregnancy. Vaginal smears were taken daily to check for synchronization of cycles. Briefly, saline (100µl) was used to flush the vagina of female rats using a micropipette. Vaginal cells were collected, smeared on a glass slide and allowed to air-dry. Once dry, slides were prepared for staining using the Shorr stain method (Shorr, 1941, Hartman, 1944), fixed and viewed under a light microscope.

Breeding: Mating took place during pro-oestrus in anticipation of the oestrus phase. Male and female rats were housed in a 1:1 ratio and allowed to mate overnight. Females were checked for the presence of vaginal plugs the following morning and this was deemed positive for pregnancy and therefore gestational

day 1 (GND 1). In the absence of a plug, vaginal smears were performed and sperm-positive smears were regarded as GND 1.

MeHg treatment: Pregnant females were divided into two groups: - a control group which received untreated drinking water and an experimental group which was exposed to methylmercury chloride (2.5mg/L, Sigma, St. Louis MO, U.S.A.) in drinking water from GND 1 to GND 21 after which MeHg-contaminated water was replaced with normal drinking water. MeHg purity was approximately 99.5% (Sigma Aldrich Certificate of Analysis). Water intake and body weight were measured daily for each animal. A water control bottle was placed in an empty cage to control for water loss by spillage. MeHg exposure amounted to ±0.25mg/kg/day based on body weight and daily water intake. This dose was chosen as an intermediate to doses in previous publications (Coccini et al., 2000, Gralewicz et al., 2009, Guo et al., 2013) to mimic a low, chronic dose of exposure. MeHg pollution of water resources in South Africa range from below the detection limit, <0.02ng/L to ± 2.66 ng/L (Williams et al., 2010, Williams et al., 2011) depending on the site and duration of exposure. Sites closer to anthropogenic sources had higher aqueous MeHg concentration as well as high sediment MeHg levels and high concentrations in fish and other invertebrates (Williams et al., 2010, Williams et al., 2011). These contribute to the elevated levels in the aquatic food chain where recent studies have shown that the MeHg levels in fish are approaching the US EPA guidelines (300 ng/g ww). Brain mercury levels were not measured in this study but are expected to be in the range 2-4µg/ml based on a study with a similar dose (Ishitobi et al., 2010).

Postnatal handling: On postnatal day 21 (PND 21) pups were weaned and placed in a separate cage from the dam. The female offspring were separated into 2 groups: - 1) Offspring which were exposed to MeHg *in utero* (MeHg, n=7) and 2) Offspring which were not exposed to MeHg (Control, n=7). Male offspring were allowed to mature until PND 60 when 6-OHDA lesion took place (see below). Female offspring were sacrificed on PND 28 by decapitation. Blood plasma was collected for total antioxidant capacity (TAC) analysis while whole brain tissue was collected for trace element quantification. Whole brain was collected under sterile conditions using plasticware to prevent leaching of metals from dissecting equipment. The tissue was blotted on filter paper, weighed and stored at -20°C until further analysis.

Behavioural tests: Behaviour was assessed to identify motor dysfunction. This occurred both pre-lesion (PND 58) as well as post-lesion (PND 74) for comparative analysis of the neurotoxin effect to be made. The forelimb akinesia (step) test and the limb-use asymmetry (cylinder) test were conducted. Groups were randomly assigned such that the experimenter was blind to the type of treatments.

- The forelimb akinesia (step) test

This test examines movement initiation (Mabandla and Russell, 2010). The animal was held by its torso such that the hindquarters and forelimb not being tested were elevated by the experimenter resulting in the weight of the animal being supported by the forelimb being tested. The animal was then propelled forward on a non-smooth surface and the adjusting step made by the forelimb was measured using a ruler attached adjacently. This was done 3 times per limb and an average was calculated for each limb.

- The limb-use asymmetry (cylinder) test

This test examines forelimb use during explorative behaviour (Meredith and Kang, 2006, Mabandla and Russell, 2010). The animal was placed in a plexiglass cylinder (20 cm diameter and 30 cm height) for 5 minutes and its behaviour was videotaped and subsequently assessed. The animal was tested for wall exploration, contact with the wall as well as landing after wall contact, for both forelimbs (Mabandla and Russell, 2010). Animals were assessed for percentage limb-use of the impaired (contralateral) limb by using the following equation:-

$$\%\ limb\ use\ of\ impaired = \left(\frac{impaired + \frac{1}{2}\ both}{impaired + unimpaired + both} \right) \times 100$$

Where, impaired refers to the limb contralateral to the neurotoxin-injected (lesioned) hemisphere and unimpaired refers to the limb ipsilateral to the lesioned hemisphere. Both, refers to the use of both the impaired and unimpaired limbs during exploratory activity (Tillerson et al., 2001).

6-Hydroxydopamine (6-OHDA) lesion: On post-natal day 60 (PND 60), the neurotoxin 6-OHDA was injected unilaterally into the medial forebrain bundle as a model of neuronal insult (Deumens et al., 2002, Blandini et al., 2008). Male offspring (Control and MeHg groups, n=7) were first anaesthetized with sodium pentobarbital (50mg/kg i.p., Sigma, St. Louis MO, U.S.A.). After, the rat was placed in the stereotaxic frame (David Kopf Instruments, Tujunga CA, U.S.A.). The skull was exposed by making a midline incision with a scalpel. A small burr hole was drilled at the following co-ordinates: 4.7mm lateral to midline and 1.6mm caudal to bregma (Mabandla and Russell, 2010). At these co-ordinates, a Hamilton needle was slowly inserted into the brain tissue 8.4mm below the skull, to inject a fresh solution of 6-OHDA (5µg/4µl dissolved in 0.2% ascorbic acid; Sigma, St. Louis MO, U.S.A.). The 6-OHDA solution was injected into the right medial forebrain bundle at a rate of 0.5ml/min. The needle was kept in its

position for a further 3 minutes after 6-OHDA infusion and thereafter the needle was gradually removed. Sham-lesioned animals were injected with saline instead of 6-OHDA. The hole was covered with sterilized oxidized cellulose and the wound sutured thereafter. During recovery, the animals were warmed using heating pads to prevent hypothermia. They were returned to their home cages after full recovery from the surgical procedure (±2hours post-lesion).

Tissue collection: On postnatal day 75 (PND 75), animals were decapitated and blood plasma and whole brain were collected (as described for PND 28) for measurement of total antioxidant capacity (TAC) and trace elements respectively.

2.2 Biochemical analysis

2.2.1 Trace element analysis

Trace element levels were measured by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) using a method adapted from Levy et al., 2001. Briefly, whole brain tissue (1g) was homogenized in 2N hydrochloric acid (HCl) (7ml) using a Misonix Sonicator XL2000-010 (Newtown CT, USA) until a smooth homogenate was obtained. Samples were then treated with 70% perchloric acid (1ml) and incubated at 50°C for 24-36 hours in a water bath. Following incubation, samples were centrifuged at 3500rpm for 1 hour and thereafter filter-syringed through a 0.45µm pore size filter. Samples and standards were then analysed on the Perkin Elmer Optima 5300 DV Optimal Emission Spectrometer (Waltham MA, USA).

2.2.2 Total Antioxidant Capacity (TAC)

The TAC is a measure of the collective capacity of biomolecules from a sample to exert antioxidant activity. Whole blood was centrifuged at 3500rpm for 10 minutes using a Hermle Labortechnik GmbH centrifuge (Wehingen, Germany). Plasma was collected and analysed for TAC using the OxiSelect™ Total Antioxidant Capacity (TAC) Assay kit (Cell Biolabs Inc., San Diego CA, USA) according to the manufacturer's instructions.

Statistical Analysis

All data was analysed using the software programme GraphPad Prism (Version 5) and was tested for normality (Kolmogorov-Smirnov test for normality). For non-parametric data, the Kruskal Wallis test was used for comparison of more than 2 groups. The Wilcoxin matched paired test and the Mann-Whitney U test were used for comparison between 2 individual groups. For parametric data, the One-way ANOVA was performed with Tukey's Multiple Comparison test. Results were considered significant when a p-value < 0.05 was obtained.

3. Results

3.1 Water intake during pregnancy

Pregnant rats exposed to MeHg (2.5mg/L) showed no significant difference in the daily water intake compared to that of the controls (control 29.43±2.9 ml/day vs. MeHg 29.81±2.26 ml/day). Average water loss by spillage amounted to 4.8ml/day and this was corrected for in the result.

3.2 Brain weight of juvenile offspring

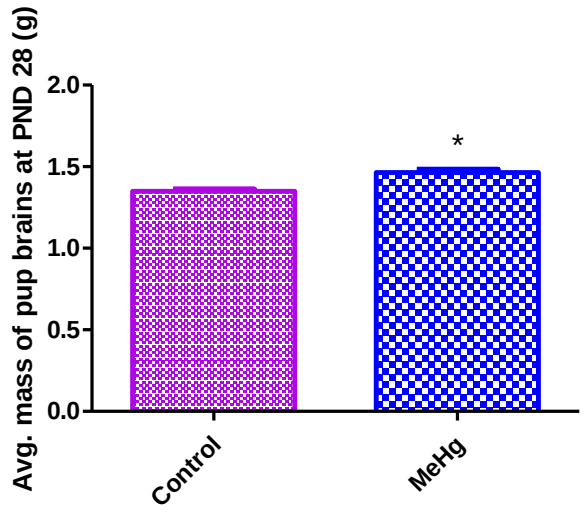


Figure 1: Graph comparing average brain mass (g) of rats exposed to MeHg or untreated drinking water at PND 28 (n=7 per group).

* $p<0.05$; significantly different from control (Mann-Whitney U test)

Offspring of MeHg-treated rats had a significantly greater brain mass when compared to non-exposed pups (Figure 1; $p<0.05$) at PND 28.

3.3 Behavioural analysis

a) Step test

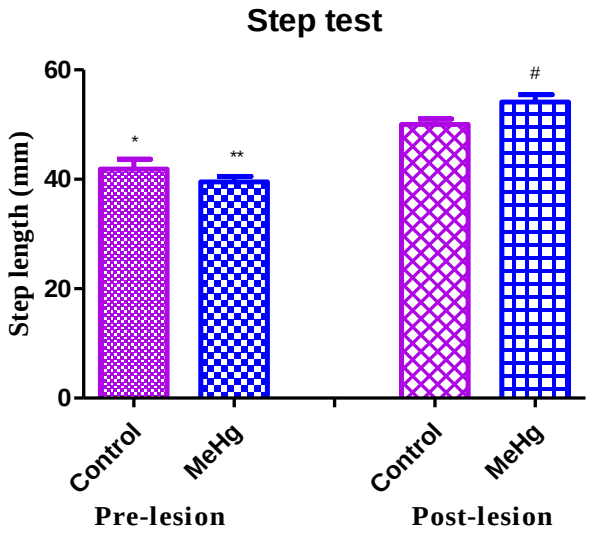


Figure 2**:** Graph showing step length of impaired limb of either MeHg-exposed rats or control rats that were exposed to untreated drinking water, before (pre-lesion) and after receiving a unilateral 6-OHDA injection (post-lesion) into their medial forebrain bundle (n=7).

*$p<0.05$; significantly different from control post-lesion group (Kruskal-Wallis followed by Wilcoxin paired test)

** $p<0.05$; significantly different from MeHg post-lesion group (Kruskal-Wallis followed by Wilcoxin paired test)

# $p<0.05$; significantly different from control post-lesion group (Kruskal-Wallis followed by Mann Whitney U test)

There was both a 6-OHDA and MeHg effect on the step length following lesion. Prenatal exposure to MeHg per se did not affect step length (Figure 2: control vs. MeHg pre-lesion), while 6-OHDA injection resulted in a significant increase in step length (Figure 2: * control pre-lesion vs. control post-lesion, **

MeHg pre-lesion vs. MeHg post-lesion; $p<0.0005$). This effect of 6-OHDA was exacerbated in animals pre-exposed to MeHg (Figure 2: $^{\#}$ control post-lesion vs. MeHg post-lesion; $p<0.05$).

b) Cylinder test

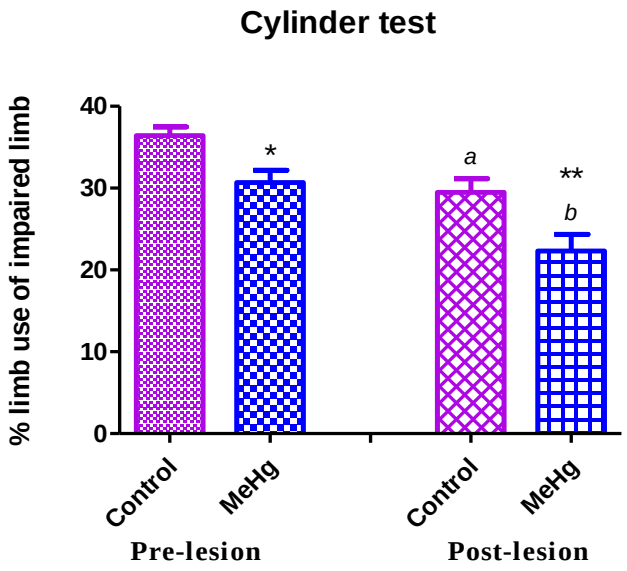


Figure 3: Graph showing percentage limb-use of impaired limb of either MeHg-exposed rats or control rats that were exposed to untreated drinking water, before (pre-lesion) and after receiving a unilateral 6-OHDA injection (post-lesion) into their medial forebrain bundle (n=7).

* $p<0.05$; significantly different from control pre-lesion group (Kruskal-Wallis followed by Mann Whitney U test)

** $p<0.05$; significantly different from control post-lesion group (Kruskal-Wallis followed by Mann Whitney U test)

^{a} $p<0.05$; significantly different from control pre-lesion group (Kruskal-Wallis followed by Wilcoxin paired test)

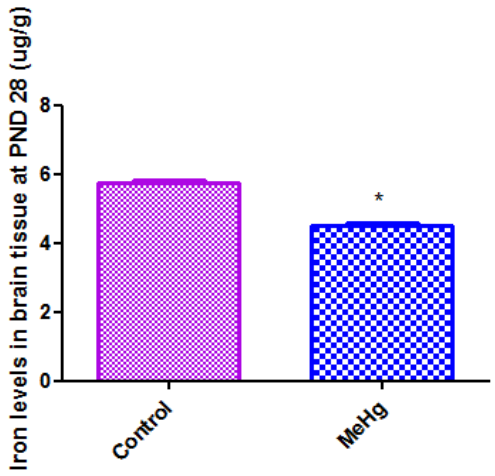
^{b} $p<0.05$; significantly different from MeHg pre-lesion group (Kruskal-Wallis followed by Wilcoxin paired test)

Offspring exposed to MeHg showed decreased locomotor activity compared to control offspring in both the impaired (Figure 3, * control pre-lesion vs. MeHg pre-lesion; $p<0.05$) and unimpaired limbs (Data not shown). 6-OHDA treatment had an effect in decreasing the percentage limb-use in control (Figure 3, ^{a} control pre- vs. post-lesion; $p<0.05$) and MeHg offspring (Figure 3, ^{b} MeHg pre- vs. post-lesion;

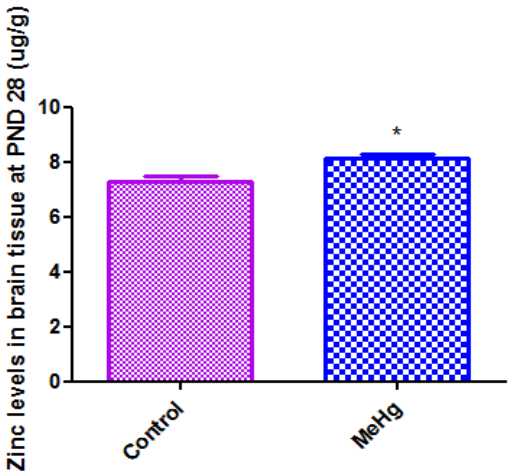
p<0.05). We also observed a significantly lower percentage limb use in the MeHg offspring post-lesion compared to that of controls (Figure 3, ** control post-lesion vs. MeHg post-lesion p<0.05).

3.4 Trace element analysis

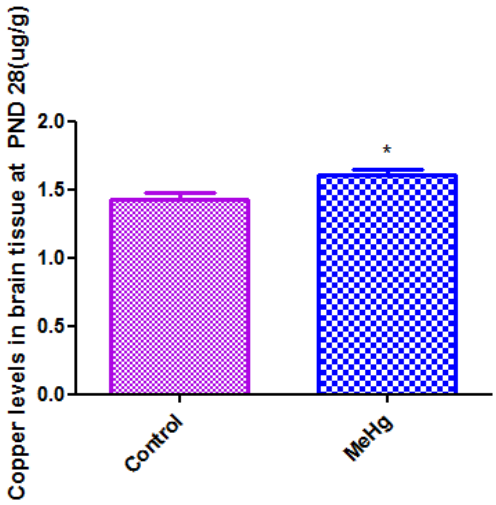
a)



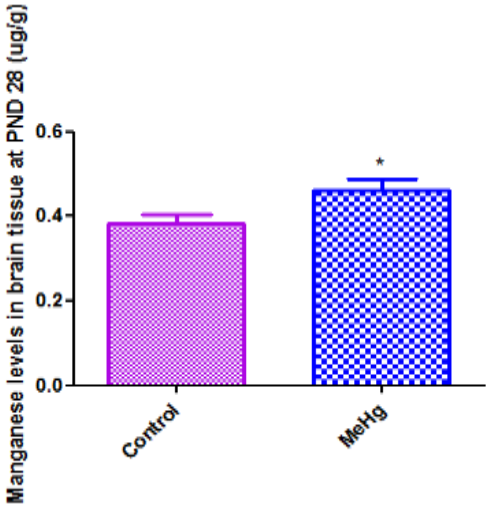
b)



c)



d)



e)

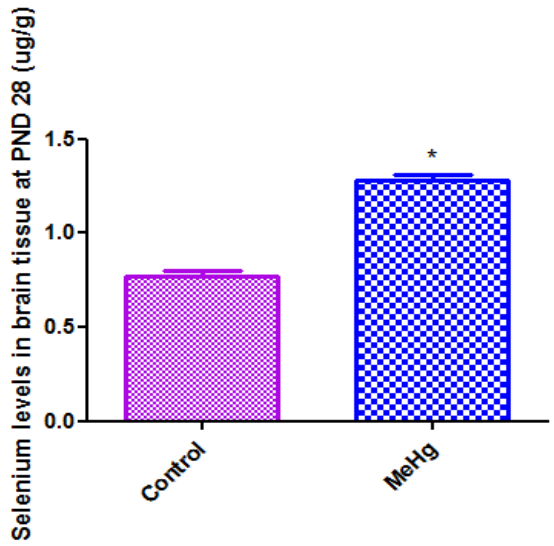


Figure 4: Graph showing trace element levels in either MeHg-exposed rats or control rats that were exposed to untreated drinking water at PND 28 (n=7 per group). Trace elements measured were a) iron b) zinc c) copper d) manganese e) selenium

* $p<0.05$; significantly different from control (Mann-Whitney U test)

Concentrations of zinc, copper, manganese, selenium and iron were quantified in brain tissue at PND 28 as well as at PND 75. Prenatal MeHg treatment resulted in decreased iron levels in the brain of offspring at PND 28 (Figure 4a; *$p<0.05$). This was accompanied by an increase in zinc, copper, manganese and selenium concentrations with MeHg exposure (Figure 4b, 4c, 4d, 4e respectively; *$p<0.05$).

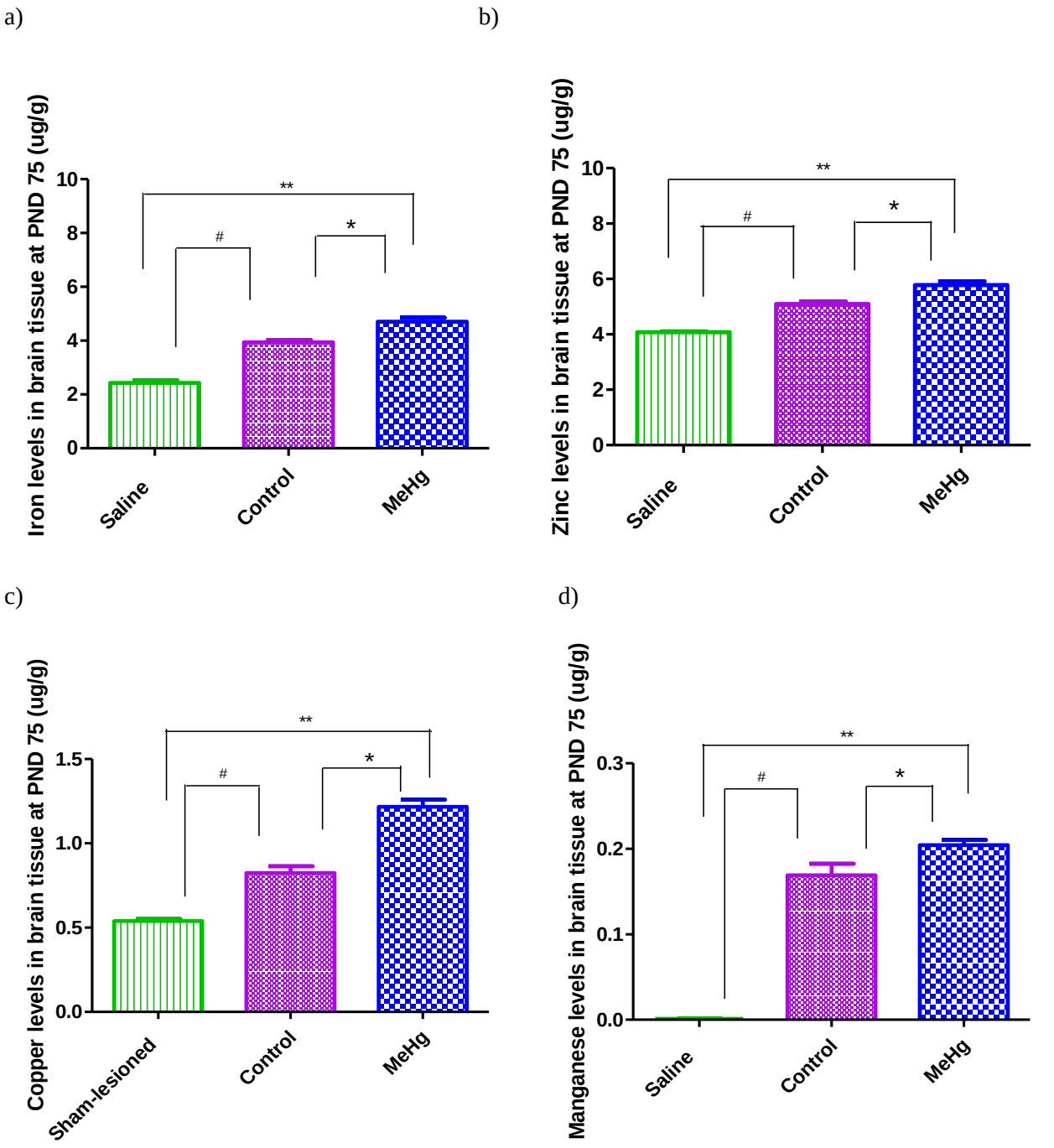


Figure 5: Graph showing trace element levels in either MeHg-exposed rats (n=8) or control rats that were exposed to untreated drinking water (n=8) after receiving a unilateral 6-OHDA injection or sham-lesion (n=5). Trace elements measured were a) iron b) zinc c) copper d) manganese

[#] $p<0.05$; significantly different from saline (Mann-Whitney U test)

*$p<0.05$; significantly different from control (Mann-Whitney U test)

** $p<0.05$; MeHg significantly different from control (Mann-Whitney U test)

Following 6-OHDA neurotoxicity, iron, zinc, copper and manganese concentrations were elevated compared to sham-lesioned animals (Figure 5a, 5b, 5c, 5d respectively; # saline vs. control, $p<0.05$). Exposure to MeHg showed a similar increase in all trace element levels (Figure 5a, 5b, 5c, 5d; ** saline vs. MeHg, $p<0.05$). Iron, zinc, copper and manganese concentrations were further increased when exposed to prenatal MeHg exposure and lesioned with 6-OHDA (Figure 5a, 5b, 5c, 5d respectively; * control vs. MeHg, $p<0.05$). Selenium levels were very low and close to the detection limit post-lesion.

3.5 Total Antioxidant Capacity (TAC)

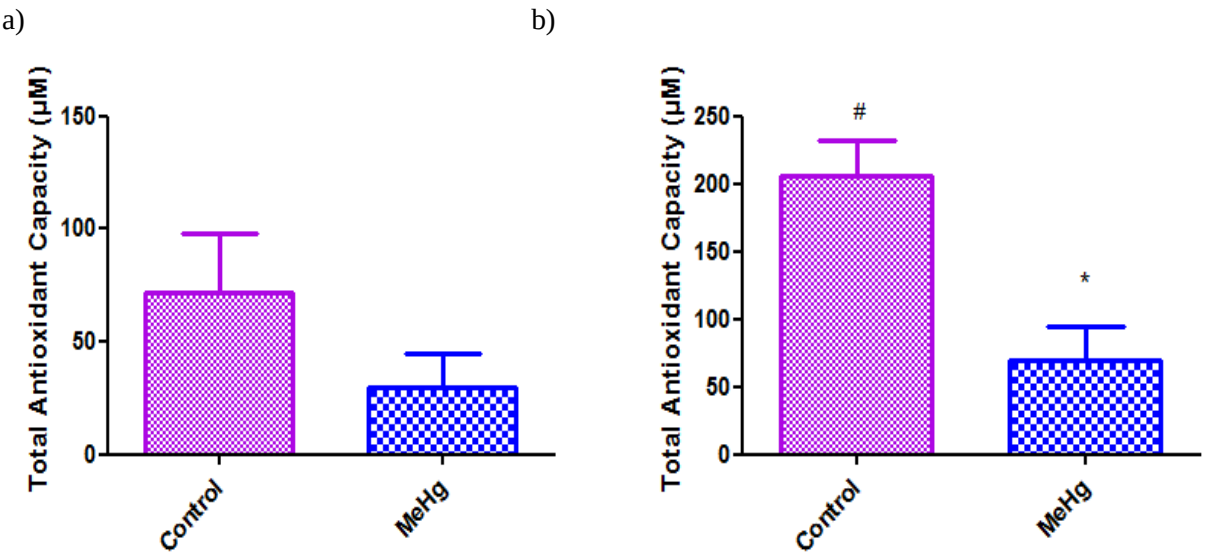


Figure 6: Graph showing total antioxidant capacity (µM) in MeHg-exposed rats or control rats that were exposed to untreated drinking water (n=7 per group) and b) after 6-OHDA lesion at PND 75 (n=7 per group).

* $p<0.05$; significantly different from control PND 75 (Mann-Whitney U test)

# $p<0.05$; significantly different from control PND 28 (Mann-Whitney U test)

Blood plasma was analysed for Total Antioxidant Capacity (TAC). Comparison between MeHg-exposed animals and controls showed no significant differences at PND 28 however, there was a tendency for the MeHg group to have a lower antioxidant capacity (Figure 6a). At PND 75, TAC was significantly lower in MeHg exposed animals compared to controls (Figure 6b, $p<0.05$). In control animals TAC was higher in older rats (Figures 6a and 6b); # control PND 28 vs. control PND 75, $p<0.05$) but there was no age-related change in total antioxidant capacity in animals that were exposed to MeHg. Although offspring were not gender-matched at PND 28 compared to PND 75, the female rats at PND 28 were pre-pubescent

and therefore there were no hormonal influences on the results. Studies have shown that the onset of puberty occurs at approximately 30 days of age and later before which there is no activity in the reproductive tract (Goldman et al., 2007, Westwood, 2008). Gender-specific differences in brain development has been suggested to occur due to the effect of these hormones which exhibit during the peri-adolescent period (PND 28-42) (Spear, 2000, Neufang et al., 2009). Thus, the gender difference would not have an impact on our results.

4. Discussion

It has been established that prenatal methylmercury (MeHg) exposure is neurotoxic to the developing foetus (Gralewicz et al., 2009, Newland et al., 2006, Carratu et al., 2008, Ferraro et al., 2009). Previous studies have shown that offspring which have been exposed to methylmercury *in utero* have neurological deficits such as motor and cognitive dysfunction (Carratu et al., 2008, Gralewicz et al., 2009). Mechanisms of methylmercury toxicity vary from the generation of reactive oxygen species to the impairment of the glutathione antioxidant system to glutamate dyshomeostasis (Aschner et al., 2007, Nascimento et al., 2008, Farina et al., 2011a). Many studies have emphasized the importance of trace element homeostasis in maintaining brain function (Bush, 2000, Feng et al., 2004, Levenson, 2005, Valko et al., 2005). The perinatal phase is especially vulnerable to dyshomeostasis and may result in neurological dysfunction (Lozoff et al., 2006b, Cordova et al., 2013). In this study, we investigated the effect of prenatal MeHg exposure on the trace element status of offspring during adolescence (PND 28). We also examined the Total Antioxidant Capacity (TAC) at this critical period.

MeHg treatment did not alter water intake during pregnancy however, offspring of dams exposed to MeHg had a greater brain mass than that of control offspring. This is in contrast to previous studies which showed no difference in offspring brain mass following prenatal MeHg exposure (Newland and Reile, 1999). This may be explained by contrasting doses of MeHg as well as different dosing regimens, i.e. in our study MeHg exposure occurred for the duration of the pregnancy only (GND 1-21) in contrast to exposure from pre-breeding to PND 16 in the above-mentioned study. Furthermore, the brain of offspring was collected at PND 28 in our study differing from Newland and Reile (1999) and Feng et al (2004) where offspring brain were collected at PND 21 and PND 20 respectively. Another study showed that mercury treatment (as $HgCl_2$ exposed from GND 0 to PND 20) in adult males resulted in increased cerebral brain mass similar to our results (Feng et al., 2004). We hypothesize that the observed increase in brain mass of MeHg-treated offspring may be due to cerebral oedema. Yamamoto et al (2012) showed that MeHg treatment increased expression of aquaporin 4 in marmoset model of MeHg toxicity.

Aquaporin 4 is the main aquaporin in the mammalian brain and is found in the end-feet of astrocytes making up the blood brain barrier (Pasantes-Morales and Cruz-Rangel, 2010, Yamamoto et al., 2012). It is responsible for regulating water balance in the brain and increased expression may result in cerebral oedema (Pasantes-Morales and Cruz-Rangel, 2010, Yamamoto et al., 2012). Aquaporin 4 is up-regulated in astrocytes and this may impair astrocyte function and contribute to MeHg toxicity (Yamamoto et al., 2012). Astrocytes are responsible for the support and nutrition of neurons (Sidoryk-Wegrzynowicz et al., 2011). They are responsible for the production and release of critical growth factors, can act as free radical scavengers and are involved in the modulation of glutathione levels (Sidoryk-Wegrzynowicz et al., 2011). MeHg binds readily to astrocytic glutamate transporters preventing glutamate uptake and thereby promoting glutamate excitotoxicity (Farina et al., 2011a, Nascimento et al., 2008). MeHg can also act within the astrocytes by inhibiting glutathione synthesis as well as binding directly to glutathione impairing antioxidant defences (Nascimento et al., 2008). Therefore, MeHg readily affects astrocyte function by impairing neuronal support and promoting oxidative stress which can lead to neuronal dysfunction.

The importance of trace elements in brain development is well established (Shanker, 2008, Dauncey, 2009). Our data showed major differences in levels of iron, copper, zinc, manganese and selenium in brains of MeHg-exposed offspring compared to controls. Results showed that iron levels were significantly decreased at PND 28 with prenatal MeHg exposure. Iron is critical during prenatal and early postnatal period due to the massive uptake needed for formation of neural circuits (Lozoff et al., 2006a). Thus, iron deficiency can lead to abnormal brain development. Iron deficiency has been strongly associated with cognitive and motor deficits due to its primary effect on the hippocampus and striatum (Lozoff et al., 2006a). Iron deficiency can also result in alterations of other essential elements (Oladiji, 2003). Oladiji *et al* (2003) showed increased concentrations of copper and zinc with iron deficiency. This result may explain the increased levels of both copper and zinc in our study both before and after 6-OHDA-induced lesion. Elevated zinc, copper and iron levels have been strongly associated with neurodegenerative diseases (Ide-Ektessabi and Rabionet, 2005, Kozlowski et al., 2009). Our study showed significantly higher manganese levels with MeHg treatment. Manganese toxicity has also been associated with iron deficiency (Cordova et al., 2013). Thus, the observed increases in copper, zinc and manganese levels may be mediated in part by the decrease in iron levels. Extremely high manganese levels leads to the neurodegenerative disorder manganism and some studies suggest that this may predispose to the development of other neurodegenerative diseases (Milatovic et al., 2009, Weiss, 2011). Copper, zinc and manganese are essential elements in normal brain development (Feng et al., 2004, Dauncey, 2009, Milatovic et al., 2009). However, alterations in their homeostasis can lead to excessive

accumulation which may be toxic leading to abnormal neuronal functioning (Cordova et al., 2013, Feng et al., 2004). Copper is a co-factor for many enzymes and is therefore essential for proper enzymatic function (Gaetke and Chow, 2003). Excess copper can be toxic by promoting the formation of free radicals thereby promoting oxidative stress (Valko et al., 2005). Copper binds readily to glutathione and forms a Cu(I)–$[GSH]_2$ complex which may react with oxygen molecules to promote superoxide radical formation (Aliaga et al., 2010). Aliaga et al (2010) showed that mercury ions can exacerbate this process leading to further oxidative damage. Zinc has been shown to be both neurotoxic and neuroprotective in the brain (Valko et al., 2005). Zinc deficiency and excess zinc readily induces apoptosis however evidence also exists that zinc has antioxidant potential (Chen and Liao, 2003). This emphasizes the importance of zinc homeostasis for proper neuronal function. Manganism occurs with excess manganese levels leading to rapid accumulation especially in the basal ganglia (Cordova et al., 2013). Manganism is characterized by motor deficits with symptoms similar to that of Parkinson's disease. Therefore, alterations in the homeostasis of these elements may contribute to neurotoxicity in offspring of MeHg-exposed animals. Franco et al (2009) showed that treatment with MeHg results in the inhibition of selenoproteins such as glutathione peroxidase (GPx) in a mouse model of neurotoxicity. Inhibition of Gpx prevents its antioxidant function promoting oxidative damage. It has been suggested that exposure to MeHg results in sequestration of selenium thereby causing selenium deficiency (Ralston et al., 2007). Supplementation of selenium in the diet has been shown to combat this selenium deficiency. Studies have shown that selenium is protective against MeHg toxicity (Meinerz et al., 2011, Ralston et al., 2007). When compared to control offspring, our data showed significantly higher selenium levels in MeHg-exposed animals. This is in contrast to previous studies where selenium levels in offspring were relatively unaffected by gestational mercury exposure (Feng et al., 2004, Newland et al., 2006). This difference may be explained by the different mechanisms of MeHg toxicity. We propose that MeHg impairs the neurocircuitry during development affecting the proper mechanisms for selenium homeostasis leading to excess selenium levels from dietary sources.

There were also imbalances in trace element concentrations after animals were subjected to a subsequent neurotoxic insult. 6-Hydroxydopamine treatment led to increases in iron, zinc, copper and manganese concentrations compared to sham-lesioned animals. Gestational exposure to MeHg resulted in higher brain iron levels than controls after 6-OHDA-induced neurotoxicity. Iron toxicity has been linked to neurodegenerative diseases such as Parkinson's disease (He et al., 1996, Graham et al., 2000). Post-mortem analysis of Parkinson's disease patients showed elevated iron levels in brain tissue (He et al., 1996, Graham et al., 2000). Iron promotes the formation of reactive oxygen species (ROS) via the Fenton reaction. Excessive accumulation of ROS may lead to oxidative stress and neuronal cell death.

Manganese levels were extremely low in sham-lesioned animals and close to the detection limit. Unpublished data from our lab showed a progressive decline in manganese levels with age as measured on postnatal day 28, postnatal day 60 and postnatal day 75. This reflects the decrease in manganese requirement in adulthood as compared to the developmental stage. Copper, zinc and manganese levels were also significantly higher following 6-OHDA exposure with the MeHg group having higher levels than control animals. Copper and zinc are redox metals and therefore imbalances in redox cycling promote the generation of ROS and oxidative stress which leads to apoptosis (Barnham and Bush, 2008, Crichton et al., 2008). Both copper and zinc have been associated with Alzheimer's disease while manganese toxicity results in manganism (Crichton et al., 2008, Kozlowski et al., 2009, Milatovic et al., 2009). Selenium levels were not detectable following 6-OHDA neurotoxicity in both control and MeHg groups. The may occur due to 6-OHDA-induced oxidative stress which up-regulates selenium-dependent enzymes such as glutathione peroxidase thereby resulting in selenium deficiency (Schweizer et al., 2004). Thus, tissue homeostasis of trace elements are essential for proper brain functioning. Alterations in their levels may contribute to MeHg neurotoxicity which could have further implications should the offspring be exposed to a subsequent neurotoxic insult.

One of the major mechanisms of MeHg toxicity is the disruption of antioxidant defences (Nascimento et al., 2008, Farina et al., 2011b). MeHg has been shown to impair the glutathione antioxidant system by binding readily to glutathione leading to glutathione depletion (Farina et al., 2011b, Kaur et al., 2011). MeHg also disrupts the antioxidant enzymes glutathione peroxidase (Franco et al., 2009a, Farina et al., 2011a). In our study, we examined the effect of developmental MeHg toxicity on the total antioxidant capacity of offspring. Results showed that MeHg exposed offspring had a tendency to have a lower antioxidant capacity than that of control animals however these results were not statistically significant. Since the Total Antioxidant Capacity (TAC) kit is not a direct measurement of antioxidant levels, damage to the antioxidant enzymes might not have been detected with this method at PND 28. Alternatively, the absence of change in the TAC could be due to compensatory mechanisms since MeHg was given prenatally and as MeHg is metabolized and cleared from the body, the antioxidant levels of the offspring may have normalized by PND 28. Our results also showed that TAC levels were higher in adult control animals compared to control animals at PND 28 but there was no difference in animals that were exposed to MeHg at the different ages. Sullivan and Newton (1988) showed that serum antioxidant levels were higher in adult rats than neonates. Our data similarly showed higher plasma TAC levels in adult offspring compared to adolescents. Animals exposed to MeHg did not show any difference in plasma TAC levels. This suggests that MeHg interferes with the development of the antioxidant system rendering the offspring more susceptible to future insult. This hypothesis is supported by the 6-OHDA result which

showed that following exposure to 6-OHDA, animals that were exposed to MeHg prenatally had a significantly lower antioxidant capacity than that of controls. MeHg toxicity has been strongly associated with the development of oxidative stress and studies have shown decreased glutathione concentrations (Kaur et al., 2006, Ni et al., 2010). This may account for the reduction in total antioxidant capacity. Although prenatal exposure to MeHg did not show a significant change in the total antioxidant capacity at PND 28, when exposed to a subsequent insult via 6-OHDA, TAC was severely reduced.

In conclusion, our data showed that developmental MeHg toxicity can disrupt the homeostasis of essential trace elements leading to deficiency or excessive accumulation. This promotes toxicity leading to neuronal dysfunction. When exposed to a subsequent neuronal insult, MeHg toxicity was exacerbated and TAC was reduced. This may have implications for the offspring in adulthood by increasing their susceptibility to neurotoxic insults. Thus, our study provides a correlation for prenatal MeHg in promoting foetal basis of adult diseases by trace element dyshomeostasis and impaired antioxidant capacity.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Chapter 2 showed that prenatal MeHg exposure impairs the antioxidant system in the brain of offspring rendering them more vulnerable to further neurotoxic insults and thereby promoting cognitive deficits. There is a need for more effective treatment strategies due to the negative side effects of current methods. Plant extracts have been highly effective in the treatment of neurological conditions and may have neuroprotective properties. Chapter 3 investigates whether *Searsia chirindensis* stem-bark extract can reduce the neurocognitive deficits associated with MeHg in a parkinsonian rat model, and to assess the possible mechanisms of action.

Chapter 3

Article 2

Searsia chirindensis stem-bark extract exacerbates 6-hydroxydopamine neurotoxicity in control rats but prevents motor deficits in offspring prenatally exposed to methylmercury

The current article has been submitted to the journal **Neurotoxicology and Teratology** *(Under Review)* according to the submission requirements of the journal. In this manuscript, we have included the figures with legend as part of the results section for easier reading for the benefit of the reader.

Searsia chirindensis stem-bark extract exacerbates 6-hydroxydopamine neurotoxicity in control rats but prevents motor deficits in offspring prenatally exposed to methylmercury

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Key words: methylmercury, *Searsia chirindensis*, trace elements, antioxidants, dopamine, gene expression

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Abstract

Methylmercury (MeHg) poisoning has received much focus due to its neurotoxic effect during foetal development. Common treatments involve the use of antioxidants and metal chelators however these are not always effective therefore there is a need for newer methods. Plant extracts have been highly effective in treating various neurological conditions and offer promise as novel neuroprotective agents. In this study, we investigated the effect of *Searsia chirindensis* (*SC*) stem-bark extract following prenatal MeHg exposure and 6-OHDA toxicity. Behaviour tests were conducted to evaluate 6-OHDA neurotoxicity with neurochemical analysis of dopamine and cytokine concentrations. Trace element levels and total antioxidant capacity were also measured and gene expression profiling was performed to assess mechanisms of toxicity. Results showed that *SC* extract (1000 mg/kg, oral gavage) prevented motor deficits in MeHg-exposed offspring as substantiated by higher dopamine levels compared to controls. Contrastingly, treatment with SC in control offspring aggravated 6-OHDA-induced neurobehavioural deficits with increased copper levels and up-regulation of the *nqo1* and *fth1* genes. This suggests that the increase in copper levels may have exacerbated the neurotoxic effects of 6-OHDA via oxidative stress thereby increasing the motor deficits. Treatment with *SC* did not affect the total antioxidant capacity (TAC) in blood plasma or cytokine concentrations. In conclusion, our study suggests that *SC* stem-bark extract may protect against 6-OHDA toxicity in MeHg-exposed offspring but exacerbates the effects of 6-OHDA in controls. This indicates that SC exerts a neuroprotective effect in MeHg-exposed offspring and therefore may be an effective treatment in preventing the development of neurodegenerative diseases.

1. Introduction

Prenatal methylmercury (MeHg) exposure has been well documented as neurotoxic, leading to neurological deficits in the offspring (Debes et al., 2016, Lam et al., 2013, Grandjean et al., 2012, Ferraro et al., 2009). We have previously shown that developmental exposure to MeHg exacerbates the neurotoxic effect of 6-hydroxydopamine (6-OHDA) by increasing trace element dyshomeostasis in the brain of offspring (Moosa et al., 2014). Since trace element imbalances result in metal-induced oxidative stress, prenatal exposure to MeHg may increase susceptibility of the offspring to further neuronal damage in later life (Kraft et al., 2016, Zheng and Monnot, 2012, Monnet-Tschudi et al., 2006, Kozlowski et al., 2009). Early-life neurotoxicity has been suggested to be a major risk factor in the development of neurodegenerative diseases (Antonelli et al., 2016, Bellinger et al., 2016, Giordano and Costa, 2012). MeHg treatment has been shown to up-regulate genes and alter proteins similar to those induced by MPP^+, a commonly used model for Parkinson's disease (PD) (Shao et al., 2015). Prenatal MeHg treatment was also shown to delay the expression of genes responsible for neurodevelopment in both the cerebrum and cerebellum (Radonjic et al., 2013). This suggests that prenatal MeHg exposure may lead to the development of neurodegenerative diseases such as PD. The mechanisms involved in both MeHg neurotoxicity and PD show similarities such as oxidative stress and neuroinflammation (Kirkpatrick et al., 2015, Takahashi et al., 2015, Collins et al., 2012, Pradhan and Andreasson, 2013, Ezcurra et al., 2010). Therefore, in this study we investigate the link between prenatal MeHg exposure and PD by studying markers of oxidative stress, neuroinflammation and PD. We also evaluate the mechanisms of toxicity by gene expression profiling.

The mechanisms of MeHg neurotoxicity have been well studied, however not much attention is given to the treatment of this disorder. Common remedies involve using selenium compounds (e.g. diphenyl diselenide, ebselen), antioxidant compounds (e.g. pyrroloquinoline quinone, N-acetyl cysteine) as well as metal chelators (Joshi et al., 2014, Carvalho et al., 2011, Yin et al., 2011, Zhang et al., 2009). However, some of these therapies have negative side effects and may be ineffective in the treatment of prenatal toxicity (Ye et al., 2016, Crisponi et al., 2015). The use of plant extracts has become increasingly popular for the treatment of many common ailments and medical conditions (Del Rio et al., 2013, Itokawa et al., 2008, Ojewole, 2007). Plant extracts and their phytochemical constituents have been shown to be highly effective in the treatment of various neurological conditions (Solayman et al., 2016, Beppe et al., 2014, Sapkota et al., 2010, Aruoma et al., 2003). *Searsia chirindensis* (*SC*) (Baker F.) (Anacardiaceae), commonly known as 'Red Currant' is a semi-deciduous tree used regularly in South Africa for the treatment of mental illnesses (Madikizela et al., 2013, Ojewole, 2007). It is an effective anticonvulsant,

and can attenuate the development of febrile seizures in young rats by reducing the seizure-induced increase of the pro-inflammatory interleukin 1β (Qulu et al., 2016). Plant extracts have also been effective in the treatment of MeHg toxicity (Lucena et al., 2013, Farina et al., 2005). Therefore, we hypothesize that *SC* may have potential to alleviate MeHg neurotoxicity and/or reduce neurodegeneration. Hence, the aim of this study was to evaluate the effects of *SC* stem-bark extract following prenatal MeHg exposure in a 6-OHDA model of neurotoxicity.

2. Materials and Methods

Female and male Sprague-Dawley rats were obtained from the Biomedical Resource Centre at the University of KwaZulu-Natal. They were housed under a 12 hr. light/dark cycle and were maintained on standard food pellets and water *ad libitum*. All experiments were conducted with the approval of the University of KwaZulu-Natal Animal Ethics Research Committee (Ethical Clearance number: 037/14/animal).

2.1 Animal handling and breeding

Female rats (180-200g) were mated with a male rat in a 2:1 ratio. Females were checked for the presence of vaginal plugs every morning and if present, this was deemed as positive for pregnancy and therefore gestational day 1 (GND 1). In the absence of a plug, vaginal smears were taken to check for the presence of sperm thereby confirming pregnancy.

2.2 MeHg treatment

Pregnant females were then administered drinking water according to the following treatment protocol:- the control group (C) received untreated drinking water while the experimental group (M) was exposed to 2.5mg/L methylmercury chloride (99.5% purity) (Sigma, St. Louis MO, U.S.A.) in drinking water for the duration of the pregnancy (GND 1 to GND 21). MeHg-containing water was replaced with normal drinking water following birth of the offspring. Water intake and body weight were measured daily for each animal. A water control bottle was placed in an empty cage to control for water loss by spillage. MeHg exposure amounted to ±0.25 mg/kg/day based on body weight and daily water intake. This dose was used previously as a low, chronic dose and was shown to have neurotoxic effects.

2.3 Postnatal handling

Following birth, pups remained with their dams until PND 21 after which they were culled to male offspring only. The male offspring were then separated into 2 groups: - a) Offspring which were exposed to MeHg *in utero* (MeHg) b) Offspring which were not exposed to MeHg (Control).

2.4 Behavioural analysis

Behavioural tests were performed to test motor activity. The forelimb akinesia (step) test and the limb-use asymmetry (cylinder) test were performed prior to 6-OHDA lesion (PND 58) as well as post-lesion (PND 74) for comparative analysis. Groups were randomly assigned such that the experimenter was blind to the type of treatments.

2.4.1 The forelimb akinesia (step) test

The step test was performed to test motor initiation as described previously (Mabandla and Russell, 2010). Briefly, the animal was held suspended in mid-air such that body weight was supported on the forelimb being tested. The animal was then propelled forward on a non-smooth surface and the adjusting step made by the forelimb was measured using a ruler attached adjacently. Each forelimb was tested 3 times each and an average step length was calculated for each limb.

2.4.2 The limb-use asymmetry (cylinder) test

The cylinder test assesses the percentage usage of the forelimbs during explorative behaviour (Mabandla and Russell, 2010, Meredith and Kang, 2006). Each animal was placed in a clear, plexiglass cylinder (20 cm diameter and 30 cm height) for 5 minutes and its behaviour was videotaped and subsequently assessed for wall exploration, contact with the wall as well as landing after wall contact, for both forelimbs and the percentage limb-use was calculated (Mabandla and Russell, 2010).

2.5 The 6-Hydroxydopamine (6-OHDA) parkinsonian rat model

A parkinsonian rat model was created by injection of 6-Hydroxydopamine chloride (Sigma, St. Louis MO, U.S.A.) into the medial forebrain bundle at PND 60 (Blandini et al., 2008). Animals were firstly anaesthetized with sodium pentobarbital (50mg/kg i.p., Sigma, St. Louis MO, U.S.A.) and thereafter placed in the stereotaxic frame (David Kopf Instruments, Tujunga CA, U.S.A.). The skull was exposed, and a small burr hole was drilled at the following co-ordinates: 4.7mm lateral to midline and 1.6mm

caudal to bregma. At these co-ordinates, a Hamilton needle was slowly inserted into the brain tissue 8.4mm below skull. The 6-OHDA solution (5µg/4µl dissolved in 0.2% ascorbic acid) was then injected into the left medial forebrain bundle at a rate of 0.5ml/min. The needle was kept in its position for a further 3 minutes after 6-OHDA infusion; thereafter the needle was removed and the wound was sutured. The animals were allowed to recover from the surgical procedure and were returned to their home cages thereafter.

2.6 *Searsia chirindensis (SC)*

Extraction: SC stem-bark was harvested from the University of KwaZulu Natal, Howard College campus after positive identification by a qualified botanist as *Searsia* (Baker F.) (Anacardiaceae) with original voucher number 4594000, asersion no. 1228/ward herbarium. The bark was allowed to air-dry at room temperature after which it was milled into a powder. The powder (500 g) was immersed completely in methanol (1L) and allowed to soak for 48 hours. Following this, the solution was filtered on filter paper and thereafter evaporated at 180 rpm and 50°C on a Heidolph rotary evaporator. The mixture was thereafter freeze-dried at -80°C for approximately 24-48 hours until a powder extract was formed (Yield = 5.5%).

2.6.1 Treatment

Animals were treated with *SC* crude extract (1000 mg/kg) dissolved in saline by oral gavage. Treatment occurred once daily on PND 61, PND 63 and PND 65.

2.7 Tissue collection

Animals were sacrificed by decapitation on PND 67 and PND 75. Striatal tissue was collected on PND 67 for cytokine quantification. On PND 75, blood plasma was collected for measurement of total antioxidant capacity (TAC) analysis while whole brain tissue was collected for trace element quantification. Whole brain was collected under sterile conditions using plasticware to prevent leaching of metals from dissecting equipment. The tissue was blotted on filter paper, weighed and stored at -20°C until further analysis. Striatal tissue was also dissected for dopamine quantification as well as PCR gene expression. The blood plasma and striatal tissue were stored at -80°C in a biofreezer until biochemical tests were performed.

2.8 Neurochemistry

2.8.1 Trace element analysis

Trace element levels were measured by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) using a method adapted from Levy et al., 2001. Briefly, whole brain tissue (1g) was homogenized in 2N hydrochloric acid (HCl) (7ml) using a Misonix Sonicator XL2000-010 (Newtown CT, USA) until a smooth homogenate was obtained. Each sample was then incubated with 70% perchloric acid (1ml) at 50°C in a water bath for 24-36 hours. Following incubation, each sample was centrifuged on a Hermle Labortechnik GmbH centrifuge (Wehingen, Germany) at 3500 rpm for 1 hour and thereafter filter-syringed through a 0.45 µm pore size filter. Samples and standards were then analysed on the Perkin Elmer Optima 5300 DV Optimal Emission Spectrometer (Waltham MA, USA).

2.8.2 Total Antioxidant Capacity (TAC)

The TAC is a measure of the collective capacity of biomolecules from a sample to exert antioxidant activity. Whole blood was centrifuged at 3500 rpm for 10 minutes on a Hermle Labortechnik GmbH centrifuge (Wehingen, Germany). Plasma was collected and analysed for TAC using the OxiSelect™ Total Antioxidant Capacity (TAC) Assay kit (Cell Biolabs Inc., San Diego CA, USA) according to the manufacturer's instructions.

2.8.3 Dopamine quantification

Striatal tissue was homogenized in EDTA (0.1M)-HCl (0.1N) buffer using a Misonix Sonicator XL2000-010 (USA). Buffer (700 µl per sample) was added to the tissue and homogenized until a smooth homogenate was obtained. The homogenate was thereafter centrifuged at 13500 rpm at 4°C for 15 minutes. The supernatant was collected and used for dopamine quantification using a Dopamine ELISA kit according to manufacturer's instructions.

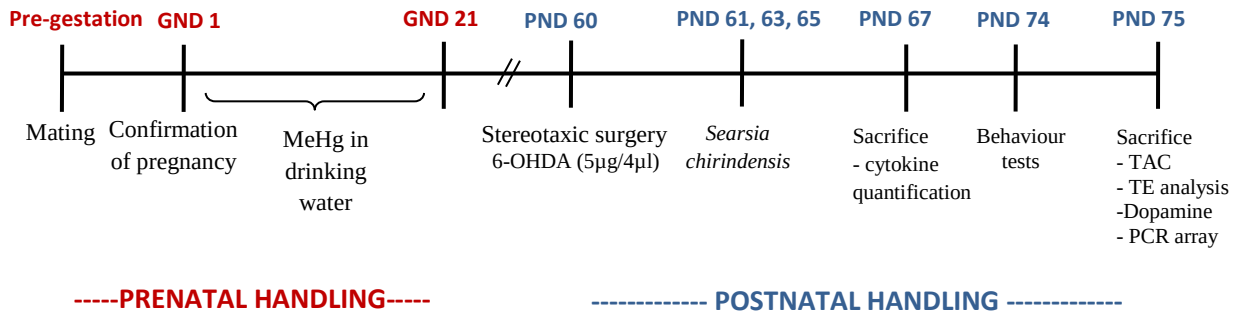
2.8.4 RT^2 Profiler PCR array

The RT^2 Profiler PCR array was performed to screen for gene expression changes in the Stress and Toxicity pathway. Prior to isolation of RNA, striatal tissue was chopped and then briefly homogenized on ice with a Misonix Sonicator XL2000-010 (USA). Thereafter total RNA was extracted using the RNeasy Microarray Tissue kit (Qiagen, USA) according to manufacturer's instructions. The purity of isolated RNA was then assessed by UV spectrophotometry using the Nanodrop 1000 (Thermo Scientific, USA). Samples with an A_{260}:A_{280} ratio between 1.8 and 2.0 was considered pure and the relative concentration per sample was calculated using the A_{260} value. Following this, RNA samples were converted to cDNA

and amplified using the RT^2 Profiler PCR array kit according to manufacturer's instructions. Results are represented as a scatter graph showing fold-change differences, with direct comparison of normalized expression between groups. A cut-off of a 4-fold change was defined as a significant result, with all genes beyond this limit highlighted in the graph.

2.8.5 Cytokine quantification

Cytokine levels were measured by flow cytometry. Striatal tissue was prepared into single-cell suspension using the BD Medimachine according to manufacturer's instructions for brain tissue. Samples were then fixed and permeabilized using the Perfix-nc kit (Beckman Coulter, USA). Briefly, Fixative Reagent (5 µl) was added to each sample (50 µl) in a Falcon tube, vortexed and incubated for 15 minutes at room temperature. Following fixation, 300 µl of Permeabilizing Reagent was added and the sample was vortexed. Thereafter the samples were stained for interleukin 6 (anti-rat CD126-IL-6Rα; Biolegend, San Diego CA) and tumour necrosis factor alpha (anti-rat TNFα; Biolegend, San Diego CA) and incubated for 30 minutes in the dark at room temperature. Following incubation, samples were re-suspended in 1X Final Reagent (3 ml), vortexed and then analysed on a BD FACSCanto flow cytometer. Each sample represents three independent experiments. Results were analyzed using Flowjo software and represented as bar graphs.



Timeline representing study design

2.9 Statistical Analysis

All data was analysed using the software programme GraphPad Prism (Version 5) and was tested for normality (Kolmogorov-Smirnov test for normality). For non-parametric data, the Kruskal Wallis test was used for comparison of more than 2 groups. The Wilcoxin matched paired test and the Mann-Whitney U test were used for comparison between 2 individual groups. For parametric data, the One-way ANOVA was performed followed by Tukey's Multiple Comparison post-hoc test. Results were considered

significant when a p-value < 0.05 was obtained. PCR results were assessed using the Qiagen data analysis software and results for flow cytometry were analyzed using Flowjo software.

3. Results

3.1 Behavioural analysis

Table 1: Behavioural results at PND 74 following treatment with *SC.*

GROUP	A: Step test		B: Cylinder test		
	Step length (mm)		% limb use of impaired limb		Reference
	Pre-lesion	Post-lesion	Pre-lesion	Post-lesion	
Control (C)	41.86 ± 8.35	50.05 ± 4.39 [a]	36.42 ± 1.05	29.48 ±1.66 [a]	(Moosa et al., 2014)
MeHg (M)	39.52 ± 4.56	54.14 ± 6.12 [a, b]	30.67 ± 1.51	22.31 ± 2.04 [a, b]	(Moosa et al., 2014)
Control Searsia (CS)	39.29 ± 7.33	60.14 ± 6.62 [a, c]	34.51 ± 1.00	23.84 ± 1.26 [a, c]	-
MeHg Searsia (MS)	41.71 ± 7.60	47.95 ± 10.1 [a, d]	31.96 ± 1.25	24.06 ± 0.82 [a]	-

Data presented as mean ± SEM (n= 7).
Alphabets represent significant differences between groups.
[a] pre. vs. post-lesion for each group respectively; p<0.05
[b] C vs. M; p<0.05 (For each test respectively)
[c] C vs. CS; p<0.05(For each test respectively)
[d] M vs. MS; p<0.05 (Step test)

3.1.1 Step test

Following 6-OHDA lesion, the step length was increased in all groups (Table 1A; [a] pre-lesion vs. post-lesion, p<0.05). MeHg-treated offspring had a bigger step length than control offspring (Table 1A; [b] Control post-lesion vs. M post-lesion, p<0.05). Administration of SC increased the step length in control animals (Table 1A, [c] C post-lesion vs. CS post-lesion) but decreased the step size in MeHg-treated offspring (Table 1A, [d] M post-lesion vs. MS post-lesion); p<0.05).

3.1.2 Cylinder test

Administration of 6-OHDA resulted in a decrease in the percentage limb use in control as well as MeHg-exposed offspring (Table 1B; [a] C pre-lesion vs. post-lesion, [a] M pre-lesion vs. post-lesion, p<0.05). MeHg exposure decreased percentage limb use as compared to controls (Table 1B; [b] C post-lesion vs. M post-lesion, p<0.05). Treatment with SC showed a significantly decreased limb use compared to control offspring alone (Table 1; [c] C post-lesion vs. CS post-lesion, p<0.05). Treatment with SC did not affect limb use in MeHg-exposed offspring.

3.2 Trace element levels

Table 2: Trace element levels at PND 75 following treatment with *SC*.

TRACE ELEMENTS	GROUPS				
	A	**B**	**C**	**D**	**E**
	Control (C)	**MeHg (M)**	**Saline (s)**	**Control *Searsia***	**MeHg *Searsia***
IRON (µg/g)	3.89 ± 0.29 [a]	4.66 ± 0.45 [b]	2.43 ± 0.22	4.13 ± 0.34	5.14 ± 0.45
ZINC (µg/g)	5.046 ± 0.30 [a]	5.821 ± 0.41 [b]	4.076 ± 0.07	5.506 ± 0.45	5.751 ± 0.39
COPPER (µg/g)	0.859 ± 0.11 [a]	1.217 ± 0.14 [b]	0.539 ± 0.03	1.056 ± 0.12 [c]	1.192 ± 0.10
MANGANESE (µg/g)	0.181 ± 0.02 [a]	0.205 ± 0.02 [b]	0.001 ± 0.01	0.181 ± 0.03	0.196 ± 0.02
	Reference: (Moosa et al., 2014)				

Data presented as mean ± SEM (n= 5).
Alphabets represent significant differences between groups for each metal respectively.
Columns A and B represent data published by our lab previously which is included for comparative purposes with *Searsia chirindensis*.
[a] s vs. C; $p<0.05$
[b] C vs. M; $p<0.05$
[c] C vs. CS; $p<0.05$

Trace element levels were quantified after different treatments. Our previous study showed that trace element levels were increased with 6-OHDA administration (Table 2, [a] s vs. C, $p<0.05$). Data is represented for comparative purposes. We also showed that prenatal MeHg exposure enhanced the elevation of these trace metals (Table 2, [b] C vs. M, $p<0.05$). Treatment with SC did not have an effect on iron, zinc and manganese levels (Table 2) however copper levels were significantly increased in control animals compared to non-treated offspring (Table 2, [c] C vs. CS, $p<0.05$). Contrastingly SC did not have an effect on copper levels of MeHg-exposed offspring.

3.3 Total Antioxidant Capacity (TAC)

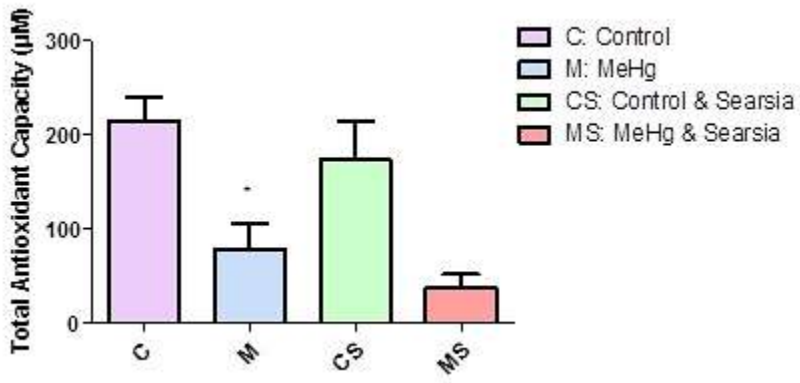


Figure 1: Graph showing TAC in blood plasma following prenatal MeHg exposure and treatment with *SC* stem-bark extract in a parkinsonian rat model. Data presented as mean ± SEM (n=5).

Prenatal MeHg exposure has been shown to decrease the TAC in adult offspring (Moosa et al., 2014). Treatment with SC did not have an effect on the TAC levels in both control and MeHg-exposed offspring (Figure 1).

3.4 Dopamine quantification

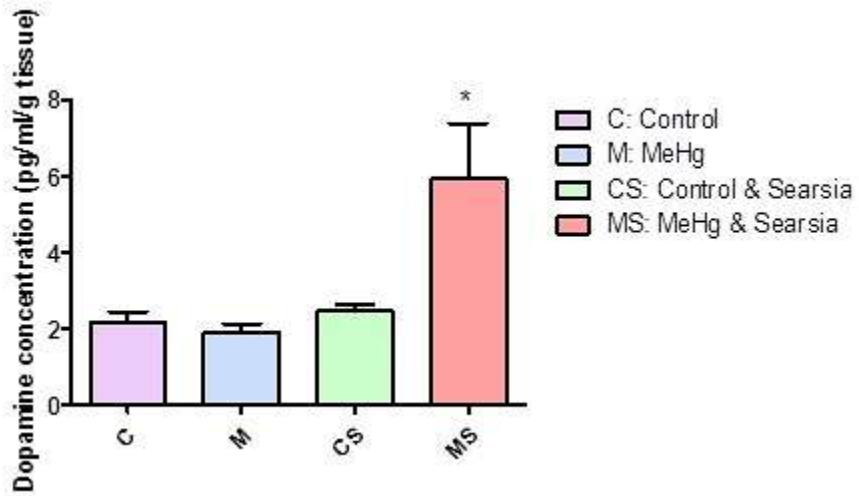
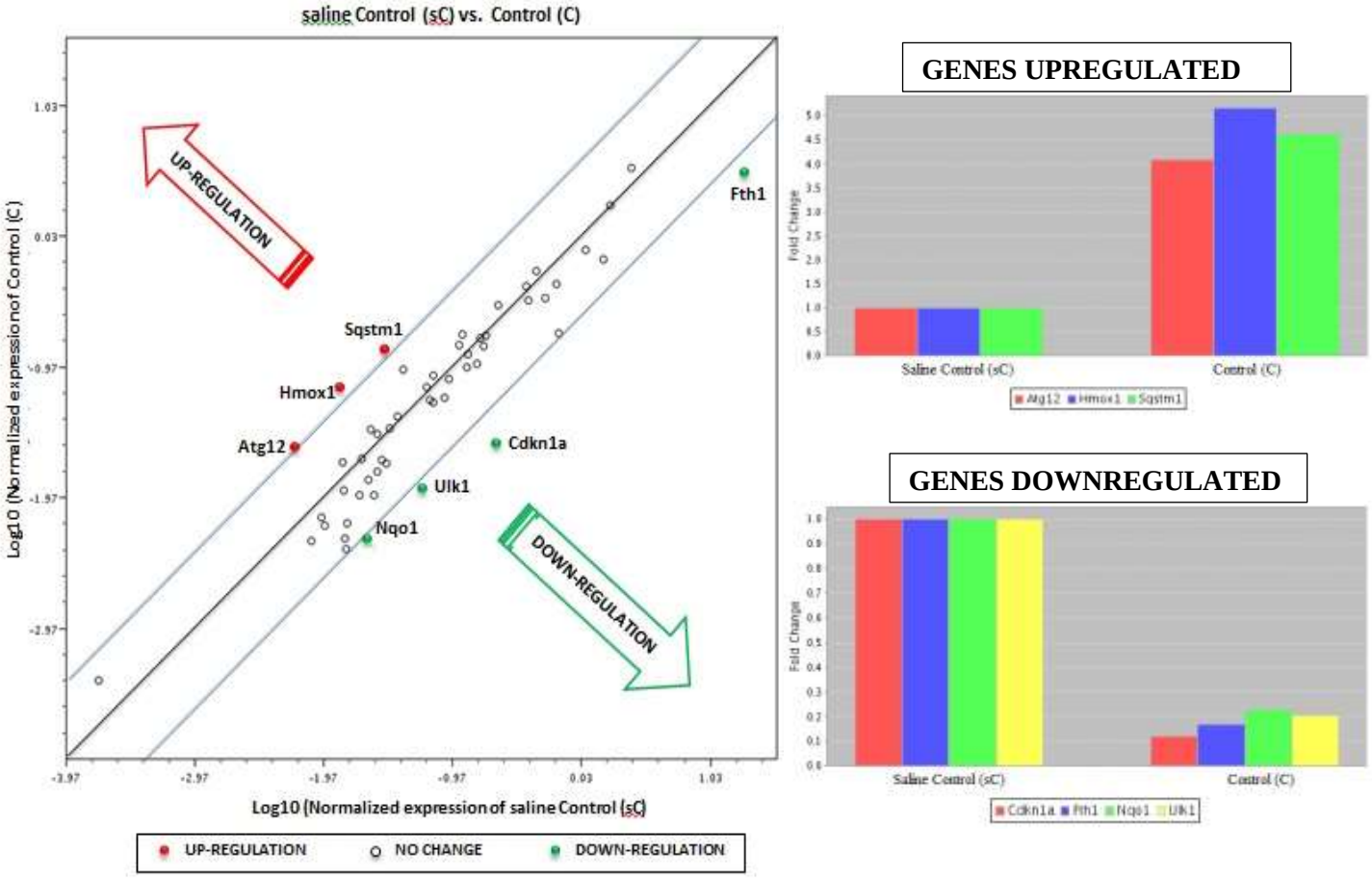


Figure 2: Graph showing striatal dopamine concentration following prenatal MeHg exposure and treatment with *SC* stem-bark extract in a parkinsonian rat model. Data presented as mean ± SEM (n=4). * M vs. MS, $p<0.05$.

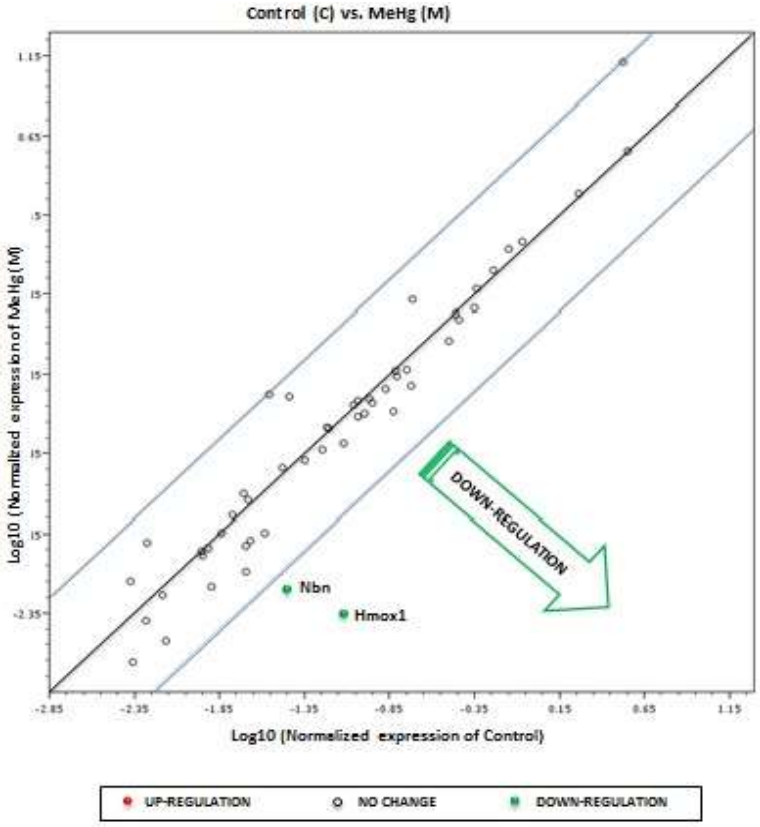
There were no significant differences in dopamine concentration after MeHg exposure however when treated with *SC*, dopamine concentration was increased (Figure 2, * M vs. MS, $p<0.05$).

3.5 Gene expression

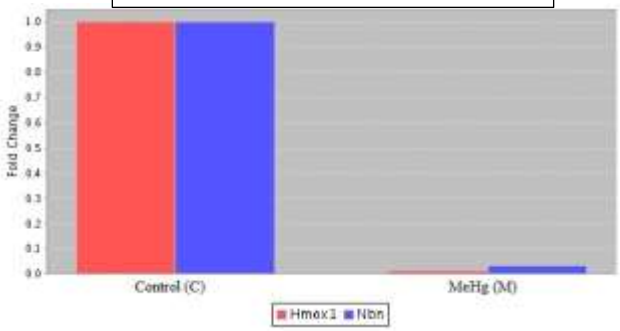
a.



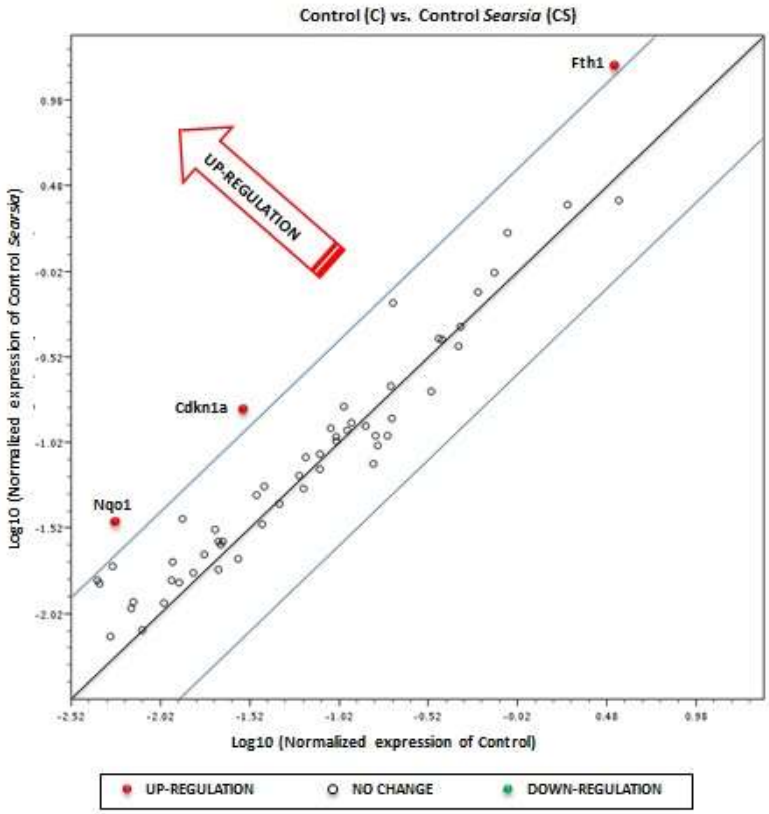
b.



GENES DOWNREGULATED



c.



GENES UPREGULATED

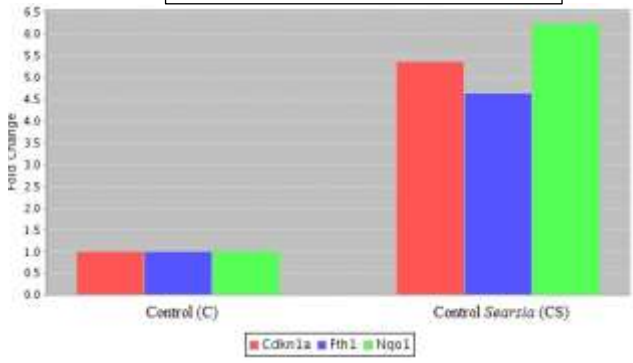


Figure 3: Scatter plot showing fold-change differences comparing normalized expression between a) saline vs. Control b) Control vs. MeHg and c) Control vs. Control *Searsia*. A cut-off of a 4-fold change was defined as a significant result (as delineated by blue lines) and all genes beyond this limit are displayed as significantly different. Abbreviations: ***hmox1:*** Heme oxygenase (decycling) 1; ***fth1:*** Ferritin, heavy polypeptide 1; ***nqo1:*** NAD(P)H dehydrogenase quinone 1; ***sqstm1:*** Sequestosome 1; ***atg12:*** ATG12 autophagy related 12 homolog; ***ulk:*** Unc-51 like kinase 1; ***cdkn1a:*** Cyclin-dependent kinase inhibitor 1A; ***nbn1:*** Nibrin.

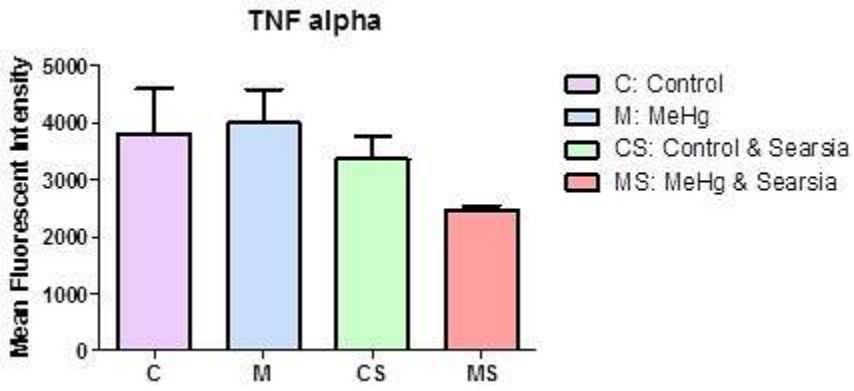
A screening of genes involved in stress and toxicity was performed to evaluate the mechanisms of toxicity in our study. Results showed that 6-OHDA had a major effect on genes involved in the oxidative stress pathway (Figure 3a: sC vs. C). There was 4-fold upregulation of the *hmox1* gene while the *fth* and *nqo1* genes were down-regulated. Autophagy genes *sqstm1* and *atg12* were also up-regulated with a down-regulation in the *ulk1* gene (Figure 3a: sC vs. C).

We also evaluated the effect of MeHg on gene expression compared to the control. Results showed that prenatal exposure to MeHg down-regulated the expression of the *hmox1* and *nbn1* genes (Figure 3b: C vs. M).

Treatment with *SC* in control animals showed an up-regulation of the *fth, nqo1* and *cdkn1a* genes (Figure 3c: C vs. M) however *SC* did not have an effect on gene expression when pre-exposed to MeHg (results not displayed).

3.6 Cytokine quantification

a.



b.

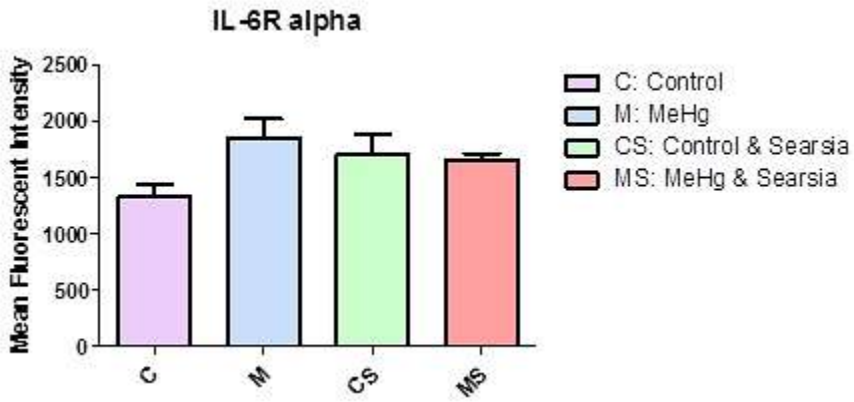


Figure 4: Graph showing cytokine levels for a) tumour necrosis factor alpha (TNFα) and b) interleukin 6 receptor (IL-6Rα) in striatal cells, following prenatal MeHg exposure and treatment with *SC* stem-bark extract in a parkinsonian rat model.

Flow cytometry was used to measure the levels of TNFα and IL-6 in striatal cells. Our results showed no significant differences in both TNFα and IL-6 levels amongst the different groups (Figure 4a and b, respectively).

4. Discussion

Treatments for methylmercury poisoning generally involve the modulation of glutathione levels, using selenium compounds as antioxidants, as well as using metal chelators (Branco et al., 2012, Kaur et al., 2006, Ralston et al., 2007). However, some of these treatments are not long-lasting and may have adverse effects. Traditional therapies have long been used among indigenous people to combat mental disorders. In this study we investigated the effect of *SC* stem-bark extract in offspring that were prenatally exposed to methylmercury in a parkinsonian rat model.

Behavioural tests were conducted to confirm 6-OHDA toxicity. Behavioural results showed that *SC* administration decreased motor deficits caused by MeHg in the step test only and not the cylinder test, thereby indicating a positive effect on motor initiation. Contrastingly, *SC* exacerbated 6-OHDA toxicity in control offspring in both the step and cylinder tests. Therefore, *SC* may be effective in alleviating the neurotoxic effect of prenatal MeHg exposure following a subsequent neurotoxic effect by 6-OHDA but exaggerates toxicity in the absence of MeHg. This is supported by the dopamine results which show an increase in dopamine concentration in MeHg groups treated with *SC*. Several studies have indicated that plant-derived phytochemicals such as polyphenols may have both pro- and antioxidant properties dependant on certain conditions (Yordi et al., 2012, Lee et al., 2005, Sakano et al., 2005). It has been shown that antioxidant compounds may exert a pro-oxidant effect in the presence of metal ions such as Cu^{2+}, thereby promoting the formation of the superoxide radical via the Fenton reaction leading to oxidative damage (Sakano et al., 2005). The pro-oxidant nature of a plant extract may be dependent on the specific phytochemical compounds as well as the amount of ROS present (Babich et al., 2011). Lee et al., (2005) showed greater ROS generation in the presence of phenolic acids than flavonoids. A recent study in our lab has shown that *SC* comprises primarily phenolic compounds (Moosa and Mabandla, 2017). In this study, our findings indicate a dual-effect of *SC*, with a pro-oxidant effect seen in control offspring while MeHg exposure leads to an antioxidant effect thereby preventing motor deficits. This finding is supported by results of trace element analysis. Our results also showed that treatment with *SC* stem-bark extract did not affect iron, zinc and manganese levels in both control and MeHg-exposed offspring, however copper levels were significantly higher after treatment with *SC* in control animals. Copper levels were not altered in MeHg-exposed animals following *SC* administration. The increased copper levels may in part explain the motor deficits seen in control offspring treated with *SC*. Administration of *SC* elevated copper levels thereby exerting a pro-oxidant effect in control offspring. This may promote the development of oxidative stress which could exacerbate the effect of 6-OHDA, thereby increasing the motor deficits seen in our behavioural test (Kozlowski et al., 2009).

Animals exposed to 6-OHDA showed significant changes in gene expression. In the control group the *nqo1* and *fth* genes were downregulated while *hmox1* was upregulated. NQO1 is responsible for the conversion of quinones to hydroquinones thus circumventing the formation of semiquinones (Siegel et al., 2004, Ross et al., 2000). Semiquinones promote the formation of the superoxide radical and in turn other reactive oxygen species leading to oxidative stress (Siegel et al., 2004, Ross et al., 2000). Therefore, decreased expression of NQO1 promotes oxidative stress-mediated neurodegeneration. The *fth* gene which encodes the heavy chain of Ferritin (FTH1) is the principal iron storage protein in cells (Baraibar et al., 2012). The FTH1 sub-unit is responsible for converting iron from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state, consequently reducing the formation of the hydroxyl radical during the Haber-Weiss reaction (Friedman et al., 2011). Therefore, up-regulation of *fth* implies an antioxidant mechanism by diminishing the levels of oxidative stress (Eid et al., 2016). Upon treatment with 6-OHDA we found down-regulation of the *fth* gene thereby reducing antioxidant activity and promoting neurodegeneration (Friedman et al., 2011). Iron overload is a well-known contributor to oxidative stress and has been implicated in the development of many neurodegenerative diseases including Parkinson's disease (Muhoberac and Vidal, 2013, Kozlowski et al., 2009, Rhodes and Ritz, 2008). Moreover, the increase expression of Heme Oxygenase 1 (HMOX1) supports these results. HMOX1 is an iron regulatory protein with increased expression in response to oxidative stress and iron overload (Ayuso et al., 2014). Recent studies have also implicated HMOX1 as a therapeutic target of neuroinflammation (Schipper, 2015, Ambegaokar and Kolson, 2014, Syapin, 2008). Results also suggest that animals exposed to 6-OHDA present with increased autophagy-mediated neurodegeneration, characterized by the up-regulation of *atg12* and *sqstm1* genes and decrease expression of ULK1. Autophagosome formation is promoted by the presence of ATG12 as well as the inhibition of ULK while Sequestosome levels increase with the formation of misfolded proteins (Frake et al., 2015). These results support the neurotoxic effect of 6-OHDA as an appropriate model of Parkinson's disease.

Gene expression profiling also showed that treatment with SC in control animals reduced 6-OHDA toxicity by the up-regulation of the *fth* and *nqo* genes. Increased levels of FTH1 indicate enhanced iron storage which may be a direct response to excessive iron concentrations (Friedman et al., 2011). As discussed previously, both FTH1 and NQO1 express antioxidant activity providing a protective mechanism (Friedman et al., 2011, Siegel et al., 2004, Ross et al., 2000). This suggests that SC stimulated an up-regulation in *fth1* and *nqo1* as a protective mechanism against 6-OHDA toxicity. This result is supported by other studies which showed that neuroprotective agents induced an upregulation in NQO1 in response to neurotoxicity (Jia et al., 2008). *SC* treatment also induced an up-regulation in the *cdkn1a* gene

indicating a possible inflammatory effect. This could in part explain the negative effects of SC on behavioural function in control animals. Contrastingly, *SC* did not have any effect on gene expression when prenatally exposed to MeHg. This is supported by the other neurochemical tests which showed that *SC* did not affect trace element levels, TAC or cytokine levels in offspring pre-exposed to MeHg.

In conclusion, our study evaluated the effect of *Searsia chirindensis* stem-bark extract in a model of prenatal MeHg toxicity as well as a subsequent neurological insult to the adult offspring. We assessed behavioural tests, examined trace element levels in brain, striatal dopamine levels and also examined plasma total antioxidant capacity (TAC). Our results showed that *SC* may ameliorate the neurotoxic effects of MeHg after 6-OHDA exposure but not in control offspring. Rather, *SC* exacerbates 6-OHDA toxicity in control offspring. *SC* increased copper levels in the brain control offspring and did not alter other trace elements. Total antioxidant capacity was not affected by *SC*. The mechanisms behind these effects are unknown and warrant further study. Current studies in our laboratory involve the isolation and characterisation of the compounds within the extract. This may provide insight into the mechanisms of *SC* effects.

5. Acknowledgements

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6. Conflict of interest

The authors declare that there are no conflicts of interest.

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Chapter 3 studied the effect of *Searsia chirindensis* (SC) stem-bark extract on neurocognitive deficits and its possible mechanisms of action. Contrary to expectation, we found that SC exacerbated neurotoxicity in control offspring but prevented behavioural deficits in MeHg-exposed animals. This suggests that SC has a negative impact when administered in control animals. Therefore in Chapter 4, we investigate the effect of SC on normal body parameters to assess for toxicity. We also quantify the relative levels of phytochemicals to identify possible reasons for the contradictory action of SC in different paradigms.

Chapter 4

Article 3

The effect of *Searsia chirindensis* stem-bark extract on renal and liver function in a rat model of neurotoxicity

The current article has been accepted for publication in the journal **Biomedicine and Pharmacotherapy** (2017, volume 86, pages 368-372). The article has been presented in manuscript format according to the submission requirements of the journal. In this manuscript we have included the figures with legend as part of the results section for easier reading for the benefit of the reader.

The effect of *Searsia chirindensis* stem-bark extract on renal and liver function in a rat model of neurotoxicity

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Highlights

- *Searsia chirindensis (SC)* does not alter liver and kidney function.
- *SC* comprises primarily phenolic acids and triterpenoids.
- These compounds may account for the strong free radical scavenging activity shown.
- Therefore, *SC* does not alter homeostasis of other bodily systems in our model.

Abstract

Searsia chirindensis (*SC*) (Baker F.) (Anacardiaceae) has shown potential for the treatment of many neurological diseases however its effect on other bodily systems in neurotoxic models is not well-documented. In this study we investigated the effects of *SC* on blood glucose homeostasis, as well as its effect on liver and kidney function by assessing lipid peroxidation using the TBARS assay and measuring plasma and urinary electrolyte concentrations in a 6-hydroxydopamine parkinsonian model. The relative levels of phytochemicals were also quantified, along with testing free radical scavenging activity via the DPPH assay. Our results showed that *SC* decreases blood glucose levels but did not alter the liver and kidney function as reflected by the absence of electrolyte imbalances and lipid peroxidation damage. We also found that *SC* comprises primarily phenolic acids (945.73±154.01 mg GA/g *SC*) and triterpenoids (1997.21±404.04 mg OA/g *SC*) with minimal flavonoid content (12.98±2.75 mg Q/g *SC*) and exhibited strong free radical scavenging activity (≈ 80%). In conclusion, these results suggest that *SC* does not alter the renal and liver function at least in the different parameters studied.

Key words: *Searsia chirindensis*; flavonoids; phenolic acids; triterpenoids; free radical scavenging; electrolytes

1. Introduction

Conventional treatments for many neurological and neurodegenerative disorders include the use of synthetic drugs as well as invasive procedures (Connolly and Lang, 2014, Walter and Vitek, 2004). These treatments are often very expensive, not readily available to socio-economically challenged communities and generally develop side-effects with prolonged use making them ineffective as long-lasting treatments (Rascol et al., 2006, Keränen et al., 2003). There is a growing industry in plant pharmacology and the use of plant extracts in the field of medicine (Martin et al., 2011, Itokawa et al., 2008). Plant extracts have been shown to be a rich source of many molecules including flavonoids, steroids, phenolic acids as well as triterpenes (Halliwell et al., 2005). These molecules are known to have significant effects in the biological system providing a rich source of nutritional supplementation and may assist in the prevention and treatment of many disorders.

The validation for novel pharmacotherapy from medicinal plants for neurotoxic diseases has progressed significantly in the past decade. This is reflected in the large number of herbal preparations for which neuroprotective potential has been evaluated in a variety of experimental models (Taïwe and Kuete, 2014). Plants of the *Searsia* (Formally known as Rhus) family in particular have shown neuroprotective effects by reducing apoptosis as well as up-regulating neurotrophic factors in dopaminergic cell lines (Sapkota et al., 2010, Sapkota et al., 2011, Kim et al., 2011). *Searsia chirindensis (SC)* is a semi-deciduous tree found throughout southern Africa particularly in Zimbabwe, Mozambique and in the Western Cape and KwaZulu-Natal provinces of South Africa (Ojewole, 2008, Moffett, 2007). It is used commonly by traditional healers for the treatment of mental disorders, heart diseases and rheumatism (Stafford et al., 2008, Madikizela et al., 2013). *SC* also exhibited anticonvulsant properties, reducing seizure severity and decreasing interleukin 1 beta (Ilβ) concentration (Ojewole, 2008, Qulu et al., 2016). This suggests that *SC* may have anti-inflammatory effects and therefore may be effective in the treatment of other inflammation-based neurological conditions such as Parkinson's disease. However, in this process there is a paucity of data of the collateral effect of this treatment in the function of other systems

apart from the brain. Hence the present study aims to assess the effect of SC on homeostasis in a 6-hydroxydopamine (6-OHDA) model of Parkinson's disease.

2. Methods

2.1 Drugs

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated.

2.2 Plant material and preparation of extract

Searsia (Rhus) chirindensis (*SC*) stem-bark was harvested from the University of KwaZulu Natal, Howard College campus after positive identification by a qualified Taxonomist/Curator botanist as Searsia (Baker F.) (Anacardiaceae) with original voucher number 4594000, asersion no. 1228/ward herbarium. The bark was air-dried until completely dry and thereafter milled into a powder. The powder (500g) was immersed completely in methanol (1L) and allowed to soak for 48 hours. Following this, the solution was filtered using filter paper and evaporated on a rotary evaporator at 180 rpm and 50°C. The mixture was freeze-dried at -80°C until a powder extract was formed (Yield = 5.5%).

2.3 *In vivo*

Male Sprague-Dawley rats at postnatal day (PND) 60 were obtained from the Biomedical Resource Unit at the University of KwaZulu-Natal following ethical clearance from the Animal Ethics Sub-Committee (Ethical Clearance number: 049/13/animal).

2.3.1 The 6-Hydroxydopamine (6-OHDA) neurotoxic model

6-Hydroxydopamine chloride (Sigma, St. Louis MO, U.S.A.) was injected into the medial forebrain bundle at postnatal day (PND) 60 as a model of neurotoxic insult (Blandini et al., 2008). The animals were first anaesthetized with sodium pentobarbital (50mg/kg i.p., Sigma, St. Louis MO, U.S.A.) and

thereafter placed in the stereotaxic frame (David Kopf Instruments, Tujunga CA, U.S.A.). A small burr hole was drilled into the skull at the following co-ordinates: 4.7mm lateral to midline and 1.6mm caudal to bregma and a Hamilton needle was slowly inserted into the brain tissue 8.4 mm below skull to inject a fresh solution of 6-OHDA (5µg/4µl dissolved in 0.2% ascorbic acid) into the left medial forebrain bundle at a rate of 0.5 ml/min (Mabandla and Russell, 2010). After 6-OHDA infusion, the needle was kept in its position for a further 3 minutes before being removed. The hole was covered with sterilized oxidized cellulose and the wound sutured thereafter. During recovery, the animals were warmed using heating pads to prevent hypothermia. They were returned to their home cages after full recovery from the surgical procedure (± 2 hours post-lesion).

2.3.2 *SC* treatment

Animals were treated with *SC* crude extract (1000 mg/kg) dissolved in saline by oral gavage. This dose was chosen based on a preliminary study done in our lab. Treatment occurred once daily on PND 61, PND 63 and PND 65. Blood glucose concentration was also measured to assess potential hypoglycemia. The tail-prick method was used and glucose concentration was measured using a glucometer up to PND 74.

To investigate the effects of *SC* on renal function, animals were placed in metabolic cages overnight and urine samples were collected the following morning at 08h00 on PND 74. Urinary and serum electrolyte concentrations were analyzed using an electrolyte analyzer (Beckman Coulter Synchron, CX 3 Delta Clinical System, USA).

2.3.3 Tissue collection

Animals were sacrificed by decapitation on PND 75. Blood plasma was collected for electrolyte analysis while liver and kidney tissue were collected for the determination of lipid peroxidation.

2.3.4 Thiobarbituric Acid Reacting Substances (TBARS) Assay

The TBARS assay was conducted to determine whether *SC* had an effect on liver and kidney function. Liver/kidney tissue (50 mg) was homogenized in 450 µl of 0.2% phosphoric acid. The homogenate was then centrifuged at 10 000 rpm for 10 minutes and the supernatant was collected and transferred to glass tubes. A solution of 2% phosphoric acid (500 µl) was then added to each tube followed by 7% phosphoric acid (200 µl). This was followed by the addition of the TBA/BHT solution (400 µl) and 100 µl 1M HCl. The tubes were then heated in a water bath at 100°C for 15 minutes. After cooling at room temperature, 1.5 ml of butanol was added to all samples, vortexed and the top phase was transferred to a 96 well microplate. Each sample was measured in triplicate and the absorbance was read at 532 nm and 600 nm on the Spectrostar Nano spectrophotometer (BMG LABTECH, Germany). Concentration was determined according to the following formula:

$$\text{Concentration} = \frac{absorbance}{absorptivity\ coefficeint} = \frac{A_{532nm} - A_{600nm}}{156mm^{-1}} \quad \text{(convert to \%)}$$

2.4 Phytochemistry

2.4.1 Total Flavonoid content

The total flavonoid content of *SC* extracts was measured by the aluminium chloride colorimetric method as described previously (Liu et al., 2014c). Briefly, the extracts were dissolved in ethanol at concentrations of 0, 5, 10, 50, 100, 500 and 1000 µM. Each sample (500 µl) was mixed with 10% aluminium chloride (100 µl) and 1M potassium acetate (100 µl). Thereafter 2.3 ml distilled water was added and the mixture was incubated at room temperature for 30 minutes after which absorbance was measured in triplicate at 415 nm (BMG Labtech, Ortenburg, Germany). Total flavonoid content was expressed as a measure of quercetin (mg Q/g *SC*) which was used as a standard. Results reflect an average of three independent experiments.

2.4.2 Total Phenolic content

The Folin-Ciocalteu method was used to determine the total phenolic content of *SC* extracts as described previously, adapted for use by a plate reader (Chew et al., 2009). Each sample (300 µl) was dissolved in deionised water according to the concentrations above and was added to a 24 well plate. Thereafter Folin-Ciocalteu reagent (1.5 ml, 10% v/v) was added to the sample in the dark. Sodium carbonate solution (1.2 ml, 7.5% w/v) was then added to the mixture. The mixture was then thoroughly mixed and incubated in the dark for 30 minutes. Following incubation, the absorbance was measured in triplicate at 765 nm on the SPECTROstar Nano plate reader (BMG Labtech, Ortenburg, Germany). Gallic acid was used as a standard and total phenolic content was expressed as mg gallic acid per g of plant extract (mg GA/g *SC*). Results reflect an average of three independent experiments.

2.4.3 Total Triterpenoid content

The amount of total triterpenoids was quantified using the method described by Chang *et al* (2012) (Chang et al., 2012) previously. *SC* extracts were dissolved in methanol (100 µl) and together with vanillin-glacial acetic acid solution (5% w/v, 150 µl), were incubated at 60ºC for 45 minutes in a water bath. Immediately thereafter, the solutions were immersed in ice-cold water and cooled to room temperature. Following this, a final volume of glacial acetic acid (2.25 ml) was added to the mixture and the absorbance of the solution was measured in triplicate at 548 nm using the Spectrostar Nano spectrophotometer. A known triterpene, oleanolic acid (OA) was used as a reference standard and results were expressed as milligram oleanolic acid equivalents (mg OA/g *SC*). Results reflect an average of three independent experiments.

2.4.4 The 2.2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

We also performed the DPPH assay to assess the *SC* extract for potential antioxidant activity. DPPH solution (0.1 mM, MeOH) was incubated with the plant extracts for 30 minutes in the dark, at room temperature. Following incubation, the absorbance was measured in triplicate at 517 nm, using methanol

as a control. The percentage free radical scavenging activity was calculated as follows: $\%\ scavenging\ activity = \frac{ODcontrol - ODsample}{ODcontrol} \times 100$

Results reflect an average of three independent experiments.

2.5 Statistical analysis

A two-factor ANOVA (treatment × time) was performed to analyse glucose results followed by the Bonferroni post-hoc test. A one-way analysis of variance ANOVA was performed for all other analyses. The Tukey's multiple comparison post-hoc tests was performed for the phytochemistry results while t-tests were done for the TBARS and electrolyte data. Differences at $P < 0.05$ were considered statistically significant. The results were presented as mean values ± SEM (standard error of means).

3. Results

3.1 Blood glucose levels

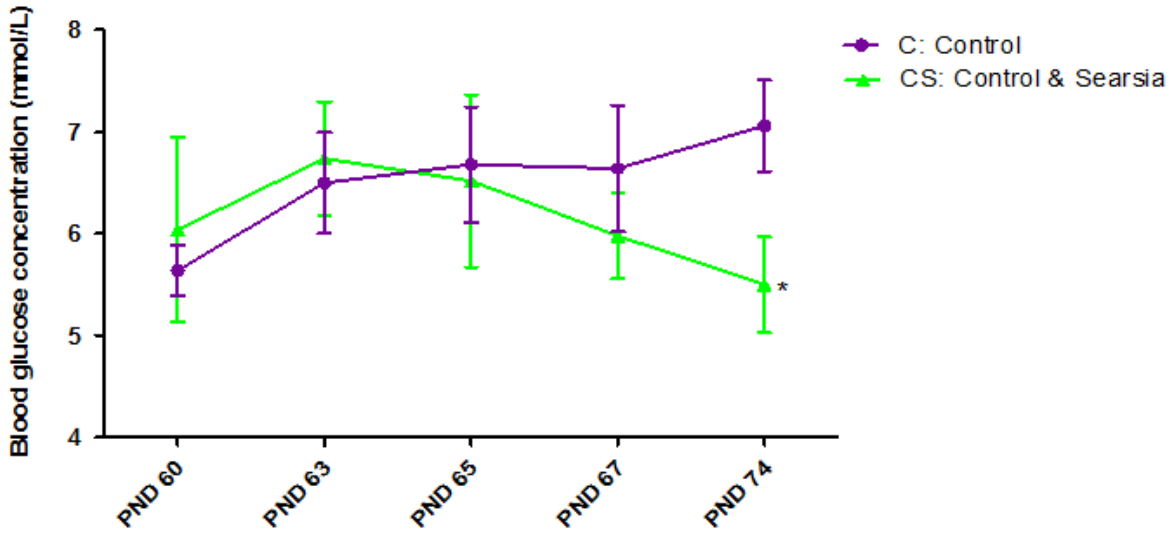


Figure 1: Blood glucose concentration following treatment with *SC* stem-bark extract. Data presented as mean ± SEM. (n=5). * (C vs. CS, $p<0.05$). *SC* = *Searsia chirindensis*

3.2 Electrolyte analysis

Table 1: Plasma and urinary electrolyte levels after SC treatment.

	Creatinine (U/mol/L)		**Urea (mmol/L)**		**Sodium (mmol/L)**		**Potassium (mmol/L)**	
	Plasma	Urine	Plasma	Urine	Plasma	Urine	Plasma	Urine
Control	33.75±1.93	6.44±0.71	5.9±0.15	165.76±6.37	132.5±6.91	152.33±11.13	8.1±1.45	311.22±35.63
Control + *SC*	32.5 ± 2.87	5.97± 0.76	5.1 ±1.65	144.6±13.29	138±0.82	182±26.89	8.63±0.53	356.35±29.14

Data presented as mean ± SEM. (n= 5). *SC* = *Searsia chirindensis.*

Plasma and urinary electrolyte levels were measured for creatinine, urea, Na^+, and K^+. There were no significant differences in electrolyte concentrations in both plasma and urine for all groups (Table 1).

3.3 TBARS assay

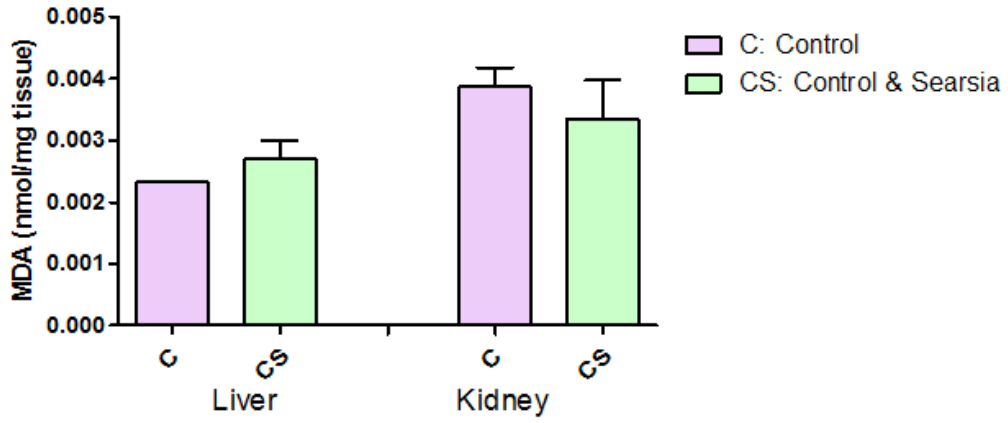
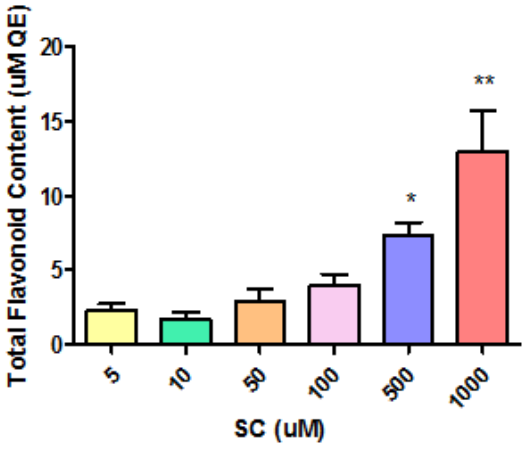


Figure 2: MDA concentration in liver and kidney tissue after treatment with *SC* stem-bark extract. Data presented as mean ± SEM. (n=3). *SC* = *Searsia chirindensis.*

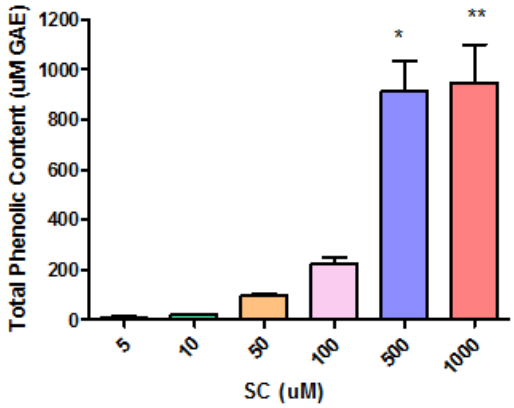
Lipid peroxidation was assessed in liver and kidney tissue by measuring malondialdehyde (MDA) levels. MDA is produced as a normal by-product of lipid peroxidation. Our results showed that there were no significant changes in MDA levels in *SC*-treated animals in both liver and kidney tissue (Figure 2).

3.4 Phytochemistry

a)



b)



c)

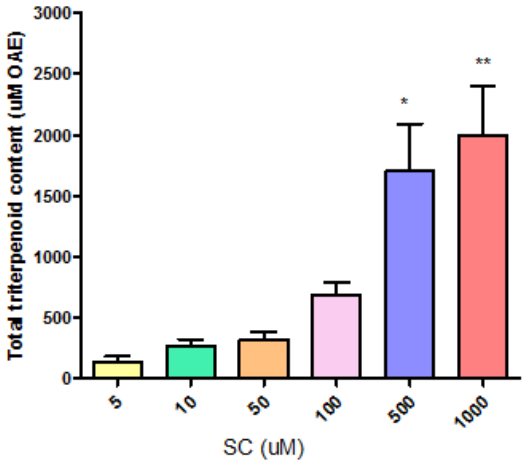


Figure 3: Phytochemical profile of *SC.* a) total flavonoid content b) total phenolic content and c) total triterpenoid content. Data presented as mean ± SEM. (n=3). * (5 vs. 500, $p<0.05$) and ** (5 vs. 1000, $p<0.05$). *SC* = *Searsia chirindensis.*

We tested *SC* for total flavonoid content. Overall our results showed that *SC* had low levels of flavonoids with only 12.98±2.75 mg Q/g *SC* at 1000 µM dose (Figure 3a). The amount of phenolics and triterpenoids in *SC* was also quantified. Results showed that at a low concentration of *SC* (5 µM), the phenolic content was 11.28±0.72 mg GA/g *SC* which increased majorly to 912.72±121.07 mg GA/g *SC* and 945.73±154.01 mg GA/g *SC* at 500 µM and 1000 µM doses respectively (Figure 3b: * (5 vs. 500, $p<0.05$) and ** (5 vs. 1000, $p<0.05$). The total triterpenoid content showed a similar increase from 138.43±44.39 mg OA/g *SC* at 5 µM to 1703.69±385.48 mg OA/g *SC* and 1997.21±404.04 mg OA/g *SC*, respectively at 500 µM and 1000 µM doses (Figure 3c: * (5 vs. 500, $p<0.05$) and ** (5 vs. 1000, $p<0.05$).

3.5 DPPH scavenging activity

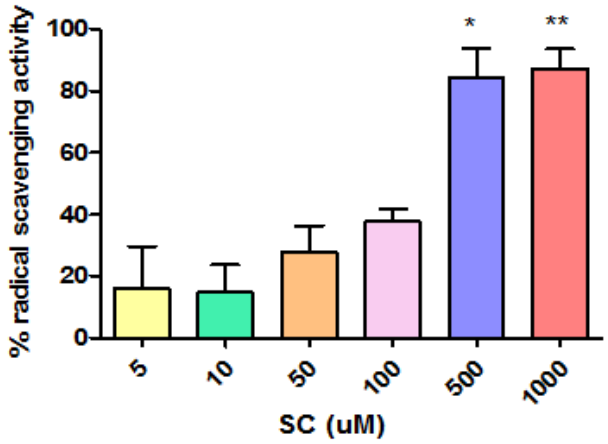


Figure 4: Radical scavenging activity of *SC* stem-bark extract. Data presented as mean ± SEM. (n=3). * (5 vs. 500, $p<0.05$) and ** (5 vs. 1000, $p<0.05$). *SC* = *Searsia chirindensis.*

SC was tested for potential free radical scavenging activity. Results showed that 500µM and 1000µM doses of *SC* showed approximately 80% radical scavenging activity (87.22 ± 6.39) (Figure 4: * 5 vs. 500, $p<0.05$; ** 5 vs. 1000, $p<0.05$).

4. Discussion

Conventional treatments for many neurological and neurodegenerative disorders have been ineffective as long-lasting treatments due to side effects as well as socio-economic issues (Rascol et al., 2006, Keränen et al., 2003). This has promoted the use of medicinal plants as a therapeutic intervention in the treatment of neurological conditions. However, many such plant extracts have not been tested for concurrent effects on systems outside of the central nervous system. In this study we investigate the effects of *SC* stem-bark extract on liver and kidney function in a 6-hydroxydopamine model of Parkinson's disease.

Blood glucose homeostasis was assessed following *SC* treatment to screen for hypoglycaemic effects. Our results showed no significant changes in blood glucose levels during the treatment period. However, *SC* reduced blood glucose levels in animals nine days post-treatment. Since *SC* has been shown to act as a hypoglycaemic agent in diabetic studies (Ojewole, 2007) these effects suggest that sustained treatment may cause adverse effects in normal individuals over a long-term period. However in our study, the decreased blood glucose did not reflect hypoglycaemia as glucose levels falls within the normal range of 4-6mmol/l in adult rats. Moreover, previous studies have indicated that severe hypoglycaemia leads to hypokalemia (Kang, 2015, Christensen et al., 2009), which leads to an electrolyte imbalance. As *SC* did not affect the electrolytes levels as established by no difference between the groups in the urine and plasma, this confirms the non-severe state of hypoglycaemia.

We also assessed the level of urea and creatinine to evaluate glomerular filtration. Our findings showed that *SC* did not alter urea and creatinine in blood plasma and urine. Urea and creatinine levels can be used as markers of glomerular filtration while sodium and potassium ion balance is essential for maintaining osmotic homeostasis and neuromuscular excitability (Dzoyem et al., 2014). Therefore, our results indicate that *SC* did not have an effect on glomerular filtration and therefore did not alter normal homeostasis.

We also investigated the effects of *SC* on MDA levels in liver and kidney tissue. Results of the TBARS assay showed that *SC* did not cause any lipid peroxidation in the liver and kidney (Figure 2). These results

are supported by the DPPH assay which indicates high free radical scavenging activity by *SC* (Figure 4). We also found that *SC* stem-bark extract consists of a large amount of phenolic acids and triterpenoids with minimal flavonoid content (Figure 3). These phytochemicals increased in a dose dependent manner, with an initial gradual increase, which is then greatly amplified at the 500μM dose and then reaches a plateau with no further increase. Phenolic acids such as caffeic acid and dihydrocaffeic acid have been shown to possess strong antioxidant activity and function by acting as free radical scavengers (Han et al., 2007). Previous studies have shown that *Rhus verniciflua Stokes* (RVS) bark extract increases neurotrophic factor levels, decreases inflammation and reduces cognitive deficits in mice (Cho et al., 2013, Sapkota et al., 2010). *Searsia dendata* leaf extract and *SC* stem-bark extract were both shown to exhibit anticonvulsant effects (Pedersen et al., 2010, Ojewole, 2008, Qulu et al., 2016). These effects may be mediated via the phytochemical constituents in the plant. Triterpenoids were also found abundantly in *SC* stem bark extract (Figure 3c). Triterpenes such as asiatic acid, oleanolic acid and lupeol are known to have anti-inflammatory properties which may reflect the mechanism of action of *SC* (Xu et al., 2012, Martin et al., 2012, Badshah et al., 2016). Besides those effects, triterpenes have been shown to possess hepatoprotective activity (Liu et al., 2014a, Liu et al., 2012) and has also been shown to protect against renal damage (Sherif, 2015, Vyas and Argal, 2012). Moreover, plants rich in phenolic acids have also been shown to protect against hepatotoxicity and nephrotoxicity in animal models (Amat et al., 2010, Bag and Mumtaz, 2013, Domitrović et al., 2014). Together these results show that *SC* stem-bark extract contains triterpenoids and phenolic acids which may be responsible for at least in part the strong free radical scavenging activity displayed.

SC during its action in neurotoxic disease did not alter homeostasis, at least in the different parameters related to the homeostasis functions studied. However further studies are still needed to explore its activity in other system homeostasis.

5. Acknowledgements

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Chapter 5

Synthesis of research findings

The following chapter summarizes the findings of this thesis, provides a concluding statement and suggests recommendations for future research.

Environmental pollutants are known to trigger many health-related deficiencies and disease conditions (Hong et al., 2012, Franco et al., 2009b, Migliore and Coppedè, 2009, Järup, 2003). Methylmercury in particular, is a metal toxin transmitted to humans primarily from occupational and anthropogenic sources, typically close to water sources where they accumulate within the aquatic food chain (Walters et al., 2011, Masekoameng et al., 2010, Papu-Zamxaka et al., 2010b). Prenatal MeHg toxicity is particularly hazardous as it undergoes biomagnification leading to massive accumulation in the brain where it disrupts neurodevelopment resulting in impaired cognitive function in the offspring at adulthood (Debes et al., 2016, Jacobson et al., 2015, Ferraro et al., 2009, Bisen-Hersh et al., 2014). Methylmercury neurotoxicity has been suggested to be an element of silent neurotoxicity, whereby early life events may contribute to the development of adult-onset disorders when unmasked by a trigger (Kraft et al., 2016, Giordano and Costa, 2012, Weiss et al., 2002). In this study, we investigate whether developmental MeHg exposure increased susceptibility to the development of Parkinson's disease. We also propose the use of a plant extract, *Searsia chirindensis (SC)* as a potential neuroprotectant to reduce neurobehavioural deficits. *SC* has been shown to act an anticonvulsant and therefore it could possibly block inflammatory-mediated neurodegeneration (Qulu et al., 2016, Ojewole, 2008). Plant extracts such as *SC* are used commonly within South Africa by traditional healers for the treatment of various ailments including mental disturbances (Ojewole, 2008). With the implementation of the Traditional Health Practitioners Act, 2007 by the South African government, traditional medicine has become a recognized form of medicine leading to a surge in the use of plant extracts (Moagi, 2009). However, since these plant extracts are administered in their crude form as concoctions, the beneficial properties of the plant are not target specific. This could lead to unexpected secondary effects which may be detrimental to health.

Overall the results of our study support the concept of developmental MeHg as a silent neurotoxicant. We found that exposure to MeHg during the prenatal period led to an imbalance in overall trace element levels at early adolescence with no apparent symptoms. We then induced a model of Parkinson's disease at adulthood which led to motor deficits accompanied by impaired antioxidant mechanisms. This includes up-regulation of genes which induce autophagy and oxidative stress as well as decreased total antioxidant capacity. Furthermore, MeHg impaired Nibrin function, which promotes neurodegeneration thereby exacerbating the motor deficits caused by 6-OHDA. Treatment with *SC* stem-bark extract was shown to reduce the motor deficits in MeHg-exposed offspring as exhibited by an increased dopamine concentration. Contrastingly, *SC* in the absence of MeHg was shown to increase copper levels as well as increased expression of CDKN1A which promotes 6-OHDA-mediated motor dysfunction. Surprisingly, there was also a slight upregulation in antioxidant genes suggesting that *SC* possessed antioxidant activity however this was not reflected by our behavioural results. These conflicting results indicate that the

composition of the crude extract is varied and may be responsible for the differential effects with MeHg. This suggests that *SC* crude extract may have a toxic effect. To evaluate this, we investigated the effect of *SC* on glucose levels as well as on liver and kidney function. Our results show no adverse effect of *SC* on other bodily systems implying that the differential effects may be specific to the central nervous system.

Overall our study has shown for the first time that developmental MeHg exposure may have effects on the brain during the adolescent period with no clinical symptoms displayed. We also showed that upon a further neurotoxic insult such as 6-OHDA, MeHg effects become apparent and exacerbate motor deficits. Our study also investigated a novel plant extract, *Searsia chirindensis* as a potential neuroprotectant. Our findings indicate that *SC* may protect from 6-OHDA-induced neurodegeneration when pre-exposed to MeHg however the mechanisms surrounding this result is inconclusive since *SC* exhibits contradictory effects in the absence of MeHg. We propose that *SC* may have both pro- and antioxidant properties where the pro-oxidant nature is induced by the elevated copper levels (Babich et al., 2011, Sakano et al., 2005). The antioxidant effects of SC are supported by the up-regulation of antioxidant genes in the striatum as well as the absence of oxidative damage observed in liver and kidney. Therefore, further research is needed to clarify the mechanisms of action of *SC*. Future recommendations include investigation of the effect of *SC* on dopamine receptor function dopamine metabolite concentration. Although our study did not show an effect of *SC* on cytokine levels, further inquiries on inflammatory processes are recommended. Therefore, analysis of glutamate concentration and NMDA receptor density is suggested. Another treatment paradigm may also be considered by administration of *SC* pre-lesion, thereby providing for the treatment of asymptomatic MeHg effects which may then prevent the exacerbation of neurodegeneration.

We conclude that early diagnosis of MeHg neurotoxicity in their asymptomatic phase may be a promising strategy to reduce neurocognitive defects and prevent neurodegeneration. A novel treatment strategy by the use of plant extracts such as SC may be favorable however further investigation into the effects of SC must be done to fully elucidate its function.

Appendices

Appendix A

Protocol for trace element analysis

Brain tissue was prepared for trace element analysis in the following manner:-

- HCl (2N) was added to whole brain tissue in the ratio 1g tissue: 5ml HCl
- Brain tissue was then homogenized using the Misonix Sonicator XL2000-010 (Newtown CT, USA) until a smooth homogenate was obtained
- A further 2ml HCl was added to the homogenated followed by 1ml Perchloric acid (70%)
- Samples were then incubated in a water bath at 50°C for approximately 24-48 hours for digestion of tissue
- After digestion samples were spun on a centrifuge at 3500rpm for 1hour
- The supernatant was thereafter removed and filtered with a syringe through a .45micron pore.
- Samples were then read on a Perkin Elmer Optima 5300 DV Optimal Emission Spectrometer using prepared standards for iron, zinc, copper, manganese and selenium. Samples were read at the following wavelengths:-

  Iron - 259 nm
  Zinc - 213 nm
  Copper - 324 nm
  Manganese - 257 nm
  Selenium - 196 nm

- Concentrations for standards were as follows:-

  Zinc, Copper, Manganese, Selenium } 1, 2, 5, 10, 20 µg/ml

  Iron – 1, 5, 10, 20, 50 µg/ml

Appendix B

Protocol for Total Antioxidant Capacity (TAC)

Product Manual

OxiSelect™ Total Antioxidant Capacity (TAC) Assay Kit

Catalog Number

STA-360 200 assays

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

CELL BIOLABS, INC.
Creating Solutions for Life Science Research

Appendix C

Protocol for Dopamine ELISA

IBL INTERNATIONAL

Instructions for Use

Dopamine ELISA

Manual and automated enzyme immunoassay for the *in-vitro diagnostic* quantitative determination of dopamine in human plasma and urine. Further the Test can be used for research of tissue homogenates and cell culture supernatants.

REF **RE59161**

Σ **96**

2-8°C

EU: IVD CE U.S.: *For in-vitro diagnostic use only.*

I B L I N T E R N A T I O N A L G M B H
Flughafenstrasse 52a
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Appendix D

Protocol for RNA isolation (RNeasy Microarray Tissue kit)

December 2014

RNeasy® Microarray Tissue Handbook

For purification of total RNA from all types of tissue for microarray analysis

Sample & Assay Technologies

Appendix E

Protocol for RT^2 Profiler PCR array kit

Appendix F

Protocol for Perfix-nc kit

PerFix-nc Kit
(no centrifuge assay Kit)

REF B31167
75 tests

ENGLISH

For Intra- & Extra- Cellular Staining Preparation

IVD CE

	PerFix-nc Buffer 1 B31164	PerFix-nc Buffer 2 B31165	PerFix-nc Buffer 3 B31166
	Fixative Reagent	Permeabilizing Reagent	Final 10 X Solution
Formulation	Liquid	Liquid	Liquid
Volume	1.8 mL	22.5 mL	26.4 mL
Number of vials	1 vial	1 vial	1 vial
Volume per test	5 or 25 µL	300 µL	320 or 350 µL

USE

PerFix-nc Kit is used to prepare biological samples for analysis by flow cytometry. PerFix-nc kit lyses red blood cells and induces permeability in the cytoplasmic membrane of leukocytes simultaneously for the permeabilization of intracellular and extracellular antigenic determinants by means of fluorescein probes.

PRINCIPLE

The PerFix-nc Kit (no centrifuge assay Kit), for Intra- & extra- Cellular Staining Preparation, consists of two ready-to-use reagents, and one reagent requiring a 10-fold dilution before use. Its purpose is to induce permeability in the cytoplasmic membrane of leucocytes for the demonstration of intracellular antigenic determinants by means of cyes or fluorochrome-conjugated antibodies. PerFix-nc Kit can be used to prepare biological samples for analysis by flow cytometry. It has been developed to enhance the signal to noise ratio of cellular staining and to simplify the workload necessary for the sample preparation. Accurate detection of both intracellular and extracellular epitopes is obtained, while:

- There are no washing steps through the procedure. A final wash is also optional.
- Several surface markers can be added together with the intracellular markers and incubated simultaneously during the permeabilization step.

STORAGE AND STABILITY

PerFix-nc Buffers are stored at 18 – 25°C.
Stability of closed vial: see expiration date on vial. Do not freeze.
Stability of opened vial: the reagent is stable for 90 days

EVIDENCE OF DETERIORATION

In case of packaging deterioration or if data obtained show some performance alteration, please contact your local distributor or use the following e-mail address:
immunotechsupp@beckmancoulter.com

PRECAUTIONS

1. Do not use reagents beyond the expiration date shown on the label.
2. Do not expose reagents to strong light during incubation.
3. Avoid microbial contamination of the reagents, or incorrect results may occur.
4. PerFix-nc Buffer 1 (Fixative Reagent) and Buffer 3 (Final 10 X Solution) contain formaldehyde. Formaldehyde is toxic and allergenic and is considered as a carcinogenic agent. Handle with care in well ventilated areas. Never pipet by mouth, avoid all contacts with skin, mucous membranes, eyes and clothing (wear protective gloves, glasses and gown).
5. PerFix-nc Buffer 2 (Permeabilizing Reagent) contains ProClin 300. ProClin 300 is a potentially irritating component. Handle with care in well ventilated areas. Never pipet by mouth, avoid all contacts with skin, mucous membranes, eyes and clothing (wear protective gloves, glasses and gown).
6. Biologic hazard: All biologic samples must be considered as potentially infectious and must be handled with care (in particular the wearing of protective gloves, gowns and goggles).
7. Blood tubes and disposable material used for handling should be disposed off with proper precautions.
8. Never pipet by mouth and avoid contacts of samples with skin, mucous membranes and eyes.
9. Use good laboratory practices when handling the PerFix-nc buffers and all other reagents.

GHS STATEMENT CLASSIFICATION

PerFix-nc Buffer 1, Fixative Reagent
DANGER

H302 Harmful if swallowed.
H315 May be harmful in contact with skin
H314 Causes severe skin burns and eye damage.
H317 May cause an allergic skin reaction.
H341 Suspected of causing genetic defects.
H350 May cause cancer.
H370 Causes damage to organs.
P201 Obtain special instructions before use.
P280 Wear protective gloves, protective clothing and eye/face protection.
P303+P361+P353
IF ON SKIN (or hair): Rinse skin with water.
P305+P351+P338
IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P308+P311
If exposed or concerned: Call a doctor/physician.
P308+P313
If exposed or concerned: Get medical advice/attention.
P362+P364
Take off contaminated clothing and wash it before use.

Methanol 1 - 2%
Formaldehyde 2 - 5%
PerFix-nc Buffer 2,Permeabilizing Reagent
WARNING

H317 May cause an allergic skin reaction.
P280 Wear protective gloves, protective clothing and eye/face protection.
P333+P313
If skin irritation or rash occurs: Get medical advice/attention.
P362+P364
Take off contaminated clothing and wash it before use.

PerFix-nc Buffer 3, Final 10X Solution
DANGER

H302 Harmful if swallowed.
H315 May be harmful in contact with skin
H314 Causes severe skin burns and eye damage.
H317 May cause an allergic skin reaction.
H341 Suspected of causing genetic defects.
H350 May cause cancer.
H370 Causes damage to organs.
P201 Obtain special instructions before use.
P280 Wear protective gloves, protective clothing and eye/face protection.
P303+P361+P353
IF ON SKIN (or hair): Rinse skin with water.
P305+P351+P338
IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P308+P311
If exposed or concerned: Call a doctor/physician.

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