

Potential neuroprotective effects of fermented rooibos herbal tea in a rat model of ischemic brain injury



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By

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A thesis submitted in fulfillment of the requirements for the Degree of Magister Scientiae (MSc) in the Department of Medical Biosciences, Faculty of Natural Sciences, University of the Western Cape.

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May 2015

DECLARATION

I, Olusiji Alex Akinrinmade, hereby declare that the dissertation “**Potential neuroprotective effects of fermented rooibos herbal tea in a rat model of ischemic brain injury**” submitted by me for the Masters degree in Medical biosciences at the University of the Western Cape, South Africa is my own original work and has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name: Olusiji Alex Akinrinmade

Date signed:



DEDICATION

I dedicate this dissertation to the Glory of God almighty, the One who saw the completion of my master's programme before I started, who went ahead of me to South Africa and perfected all of my ways. He bestows life to my soul, clear wisdom to my mind and skills to my hand. I am forever thankful O' Lord.

To the glory of God, I also dedicate this dissertation to my mother, Mrs. E.A Akinrinmade, her motherly love, support, prayers and the grace of God upon her life are the many reasons why I am continually thankful to God.

And also to my late father, Mr. L.O Akinrinmade, your uttermost desire was that I and my siblings be educated. Your priority was our education; hence you provided all the needed resources and love. You always said "we will be great if we read our books" and now I see how the sermon of education you preached to us daily has brought me thus far. I remain thankful to you. We might also learn from you how a loving heart and the desire to live can serve as a neuroprotectant against stroke, as you lived more than a decade after having your first and second episode of stroke with its attendant neurologic impairment. I pray that God almighty grant you an eternal rest.

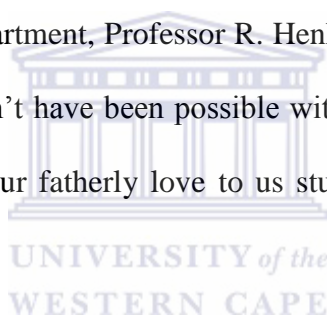
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ABSTRACT

Stroke is the third leading cause of death in South Africa, killing about 240 people a day and leaving survivors with residual disabilities. There is no clinically approved neuroprotective agent for stroke at the moment but the consumption of plant polyphenols has been suggested to offer neuroprotection against stroke and other neurodegenerative diseases. In this study, we investigated the effects of long term consumption of fermented rooibos herbal tea (FRHT) on ischemia reperfusion brain injury (I-RBI) in rats. Male adult Wistar rats were fed FRHT *ad libitum* for 7 weeks prior to the induction of ischemic injury by the transient bilateral occlusion of the common carotid arteries (BCCAO) for 20 minutes followed by 24 hours, 4 and 7 days of reperfusion respectively. Rats were then evaluated for neurologic deficits before sacrifice and brains harvested for assessment of brain oedema, blood-brain-barrier (BBB) integrity through Evans blue extravasation (EBE), immunohistochemical studies of apoptosis and lipid peroxidation. Oxygen radical antioxidant capacity and ferric reducing antioxidant power assays were also conducted to assess total antioxidant capacity after ischemia-reperfusion injury. Notably, the long term consumption of fermented rooibos herbal tea prevented brain oedema by reducing cerebral swelling induced by I-RBI. We also observed that fermented rooibos herbal tea offered neuroprotection against damage to the BBB and delayed neuronal death associated with BCCAO as fewer apoptotic cells were identified 7 days post BCCAO reperfusion. Significantly reduced levels of lipid peroxidation and increased levels of total antioxidant capacity were also observed in brain specimens of rats treated with FRHT. Rats treated with FRHT also showed improved neurologic outcomes when compared with the untreated animals. Our results show that FRHT has potent antioxidant and anti-inflammatory properties which can provide neuroprotective effects against neuronal cell loss, cerebral swelling, BBB disruption, lipid peroxidation and neurologic deficits following I-RBI. The use of FRHT is therefore highly recommended for patients with conditions that predispose them to stroke.

KEYWORDS

Stroke

Cerebral ischemia

Bilateral common carotid artery occlusion

Ischemia-reperfusion injury

Fermented rooibos herbal tea

Neuroprotection

Antioxidants

Hippocampus

Apoptosis

Brain oedema

Blood brain barrier integrity

Lipid peroxidation

Oxygen radical antioxidant power

Ferric reducing antioxidant power

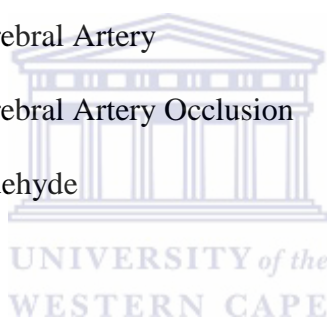


ABBREVIATIONS, NOTATIONS AND SYMBOLS

°C	Degree Celsius
µl	Microlitres
2-AAF	2-acetylaminofluorene
AAPH	2,2'-azobis-2-methyl-propanimidamide, dihydrochloride
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AFB1	Aflatoxin B1
ACA	Anterior Cerebral Artery
ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
AQP4	Aquaporin 4
AST	Aspartate Aminotransferase
ATP	Adenosine Triphosphate
ATPase	Adenosine triphosphatase
AVM	Arteriovenous Malformations
BA	Basilar Artery
BCCAO	Bilateral Common Carotid Artery Occlusion
BBB	Blood Brain Barrier
Bcl-2	B-cell lymphoma 2
CA1	Cornu Ammonis 1
Ca ²⁺	Calcium Ions
CCA	Common Carotid Artery
CCL	Carbon tetrachloride
CCM	Cerebral Cavernous Malformations
CD	Conjugated dienes

CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CT	Computerized Tomography
Cu	Copper
CV	Cresyl Violet
CVA	Cerebrovascular Accident
CVD	Cardiovascular disease
CVM	Cerebral Vascular Malformations
DAB	Diaminobenzidine
DNA	Deoxyribonucleic acid
DND	Delayed Neuronal Death
DSS	Dextran Sodium Sulfate
EB	Evans Blue
EBE	Evans Blue Extravasation
ECA	External Carotid Artery
FE ²⁺	Ferrous Ion
Fl	Fluorine
FRAP	Ferric Reducing Antioxidant Power
FRHT	Fermented Rooibos Herbal Tea
GFAP	Glial Fibrillary Acidic Protein
GSH	Glutathione
GSSG	Glutathione Disulfide
H ⁺	Hydrogen Ion
HCL	Hydrochloric Acid
HDL	High Density Lipoprotein

HFSA	Heart Foundation, South Africa
H ₂ O ₂	Hydrogen Peroxide
ICA	Internal Carotid Artery
ICH	Intracerebral Hemorrhage
IHC	Immunohistochemistry
IP	Intraperitoneal
I-RBI	Ischemia Reperfusion Brain Injury
K ⁺	Potassium ion
LDL	Low Density Lipoprotein
LPO	Lipid Peroxidation
MCA	Middle Cerebral Artery
MCAO	Middle Cerebral Artery Occlusion
MDA	Malondialdehyde
Mn	Manganese
ml	Millilitre
MRI	Magnetic Resonance Imaging
NC	Neurochemistry
NaCl	Sodium Chloride
NCDs	Non-communicable diseases
NIH	National Institutes of Health
NO	Nitric oxide
NOS	Nitric oxide synthase
O ²⁻	Superoxide anion radical
OF	Open Field
OH ⁻	Hydroxide Ion



ORAC	Oxygen Radical Antioxidant Capacity
ONOOH	Peroxynitrous Acid
PBS	Phosphate Buffered Saline
PET	Position Emission Tomography
PCA	Posterior Cerebral Artery
RNA	Ribonucleic Acid
RT	Room Temperature
rt-PA	Recombinant Tissue Plasminogen Activator
ROS	Reactive Oxygen Species
SAH	Subarachnoid Hemorrhage
SEM	Standard Error of Mean
SOD	Superoxide Dismutase
STAIR	Stroke Therapy Academic Industry Roundtable
TPTZ	Tripyridyltiazine
TAC	Total Antioxidant Capacity
TBA	Thiobarbituric Acid
TCA	Trichloroacetic Acid
TBARS	Thiobarbituric Acid Reactive Substances
TdT	Deoxynucleotidyl Transferase
TEAC	Trolox Equivalent Antioxidant Capacity
TIA	Transient Ischemic Attack
TUNEL	Terminal dUTP Nick-END Labeling
USD	United States Dollar
USA	United States of America
WHO	World Health Organization
VO	Vessel Occlusion

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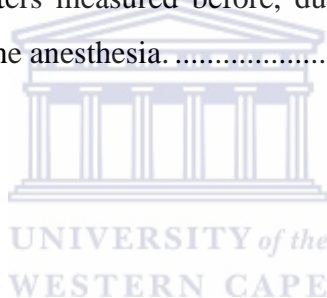
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CHAPTER ONE

INTRODUCTION

1.0 BACKGROUND

Epidemiological transition is the complex changing relationship between humans and human diseases and occurs as a country undergoes socio-economic advancement from a less-developed country to become a developed nation (Omran, 2005). During this transition, communicable diseases decline and non-communicable / lifestyle related diseases increase in prevalence (McKeown, 2009). According to World Health Organization (WHO) factsheet, 36 million people die from non-communicable diseases (NCDs) annually, with 80% of these deaths (29 million) occurring in less-developed (low or middle income) countries (WHO, 2015b). Of all NCDs related deaths, cardiovascular diseases (CVDs) account for most (or 17.3 million deaths) annually, followed by cancers (7.6 million), respiratory diseases (4.2 million), and diabetes (1.3 million) (Lim *et al.*, 2013; WHO, 2015b).

Stroke is the most common of all cardiovascular diseases (CVDs) in the epidemiologic or health transition, and there are indications that South Africa is undergoing the transition (Steyn *et al.*, 2006). Recent statistics from the Heart Foundation, South Africa (HFSA) show that about 130 heart attacks and 240 stroke episodes occur daily in South Africa, implying that 10 people will suffer a stroke and 5 people will suffer a heart attack every passing hour (Heart Foundation South Africa, 2013). The burden of stroke does not only lie in its high mortality but also in the high morbidity which leaves up to 50% of survivors with chronic disability (Wilkinson *et al.*, 1997). In developed countries, stroke is a significant economic burden as the total cost of stroke in the United States is estimated at about 38.6 billion USD / Year (Heidenreich *et al.*, 2011), while costs after hospital discharge were estimated at 2.9 billion Euros for the year 2002 in France (Feigin *et al.*, 2007).

The direct and indirect costs of death and disability from heart diseases and stroke in South Africa are estimated to exceed 8 billion Rand per year (Jozi, 2007). If there were effective prevention programmes and medication for diseases of lifestyle, such finances could otherwise be used to meet other socio-economic needs of South Africa. Most of the approved medications for the treatment of stroke [e.g. alteplase (rt-PA), reteplase, tenecteplase, anistreplase, streptokinase and urokinase] are known to restore blood flow (Duggal and Harger, 2011), but some have been found to be neurotoxic, to disrupt neurovascular matrix and increase the risk of intracerebral hemorrhage (ICH) (Wang *et al.*, 2003). Hence, no clinically effective neuroprotective drug has yet been licensed for stroke (Macrae *et al.*, 2011). The development of therapies that can limit stroke-induced brain damage and disability has been identified as a worthy research interest in the last 15 years (Macrae *et al.*, 2011).

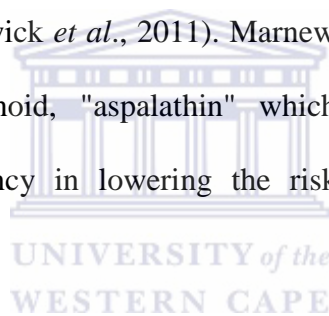
Acute ischemic stroke, the most common form of stroke, is caused by blood clotting in cerebral arteries leading to brain oxygen deprivation and cerebral infarction. The events involved in stroke include neuron death, oedema, blood-brain barrier (BBB) disruption, and hemorrhage (Sumii and Lo, 2002). The pathophysiology of cerebral ischemia is complicated by the fact that the extent of cellular damage is modulated by numerous secondary consequences of the primary ischemic impact (Hagl *et al.*, 2003). Recirculation disturbances, stress responses, peroxidative changes, or the activation of genomic responses are only a few examples of the many haemodynamic and molecular responses that determine the final outcome of stroke (Suzuki *et al.*, 1980; Kogure and Kogure, 1997). This means that the presence of systems to prevent or reduce the severity of the primary ischemic attack may reduce the extent of the secondary damage as well as the overall extent of cellular injury. For example, mitochondrial function disrupted by reactive oxygen species (ROS) during cerebral ischemia will set up a feedback cycle in which ROS-mediated oxidative damage to mitochondria will favor more ROS generation (Qi *et al.*, 2010). This subsequently triggers the release (secondary

consequences) of critical apoptotic activators and effectors of cell death (such as: cytochrome c and apoptosis-inducing factor) through exacerbation of intracellular calcium levels. This results in mitochondrial swelling, opening of the mitochondrial permeability transition pores and depolarization of mitochondrial membrane (Christophe and Nicolas, 2006). This means that the presence of a ROS scavenging system may help prevent or reduce insults to the mitochondria, thereby preventing or reducing the detrimental chain of events that may have accompanied the above-mentioned vicious cycle (Qi *et al.*, 2010).

Neuroprotection as explored by scientists aims to prevent neuronal injury or slow disease progression by halting or at least slowing the loss of neurons (Seidl and Potashkin, 2011). Of all symptoms or injuries associated with Central Nervous System (CNS) disorders, neuroprotective treatments often target oxidative stress and excitotoxicity, both of which are principal mechanisms of cell loss in a variety of CNS diseases (Boll *et al.*, 2011). The use of antioxidants to tackle oxidative stress is plausible because free radicals are known to distort such biochemical components of cells and tissues as DNA, RNA, carbohydrate moieties, unsaturated lipids, proteins and micronutrients (Lobo *et al.*, 2010). The elevated ROS levels, subsequent mutation of antioxidant enzymes and depletion of existing antioxidants (due to high levels of ROS) results in failure in protecting the neurons from oxidative damage (Qi *et al.*, 2010). In recent times, there has been increasing scientific interest in the potential health benefits of long term consumption of antioxidant-rich food substances and beverages and their possible use as neuroprotective agents when administered prior to onset of an ischemic brain injury.

Rooibos tea is a very popular beverage in South Africa and has increasingly gained more popularity among international consumers due to its well acclaimed health benefits (Mahomoodally, 2013). Recent animal studies have shown that rooibos tea has potent

antioxidant, antimutagenic, immune-modulating and chemopreventive effects (Van der Merwe *et al.*, 2006; Ichiyama *et al.*, 2007 and Marnewick *et al.*, 2011). Inanami *et al.* (1995) reported the effects of rooibos tea on older and new born rats following 2-year administration. This study found that rooibos tea protected against age-related changes in the brains of rats compared to the controls. The scientists concluded that this protection was due to the ability of rooibos tea to prevent the age-related accumulation of lipid peroxides in the brain (Inanami *et al.*, 1995). Another study showed that high intake of rooibos tea resulted in significant reductions in lipid peroxidation, Low Density Lipoprotein (LDL) cholesterol, triglycerides, and an increase in High Density Lipoprotein (HDL) cholesterol levels compared with the control group. The researchers concluded that rooibos tea lowered the risk factors for cardiovascular and degenerative diseases (Marnewick *et al.*, 2011). Marnewick *et al.* (2011) also showed that Rooibos tea contains the flavonoid, "aspalathin" which could account for its strong neuroprotective effects and potency in lowering the risk factors for cardiovascular and degenerative diseases.



Taken together, these data suggest that rooibos tea possesses antioxidant properties that could reduce neurodegeneration in the brain. Its protective effects against ischemic brain injury have not been previously studied. Previous studies have shown that 40% of patients with traumatic brain injury and stroke experience deteriorating conditions after hospitalization (Narayan, 2002). Thus a study of the protective effects of antioxidants against neuronal damage following an ischemic injury is plausible. Lestage *et al.* (2002) reported that a "good" *in vivo* animal model of stroke must reproduce the etiology, anatomical, functional and metabolic consequences of human pathology involving mechanisms in both ischemic and reperfusion pathophysiology. This informed our use of a rat model of ischemic-reperfusion injury in this study.

1.1 THE SPECIFIC OBJECTIVES OF THE RESEARCH

The general objective of our research was to determine the neuroprotective properties of rooibos tea to complement existing modalities used for the management of ischemic brain injury. The specific objectives included:

- i. Investigation of the anti-inflammatory potential of fermented rooibos herbal tea against brain oedema and relative brain weight in a rat model of cerebral ischemia after 20 minutes of bilateral common carotid artery occlusion.
- ii. Investigation of the possible protection of fermented rooibos herbal tea on the integrity of the blood-brain-barrier in a rat model of cerebral ischemia after 20 minutes of bilateral common carotid artery occlusion (BCCAO).
- iii. Investigation of the neuroprotective potential of fermented rooibos herbal tea against oxidative stress-induced apoptosis in rat brains following ischemic brain injury.
- iv. Investigation of the anti-oxidant and neuroprotective potential of fermented rooibos herbal tea in modulating lipid peroxidation in rat brains after ischemic brain injury.
- v. Investigation of the neuroprotective potential of fermented rooibos herbal tea in enhancing the antioxidant capacity of the brain to withstand oxidative stress induced by 20 minutes bilateral common carotid artery occlusion.
- vi. Evaluation of neurobehavioral outcomes in treated rats at day 1, 4, 7 after 20 minutes of bilateral common carotid artery occlusion reperfusion injury.

1.2 HYPOTHESIS OF STUDY

We hypothesize that regular consumption of fermented rooibos herbal tea could protect the brain against the severity of an ischemic brain injury. This hypothesis is based on the knowledge that the antioxidants in rooibos tea (especially its flavonoid contents) have been previously reported to confer some beneficial properties.

1.3 SIGNIFICANCE OF STUDY

About 6.3 million people live with high blood pressure in South Africa and are more prone to such life-threatening diseases as stroke (Heart Foundation South Africa, 2013). According to the South African Heart and Stroke Foundation, stroke is the third leading cause of death in South Africa and is a leading cause of adult disability. It affects about 240 South Africans per day, a quarter of which do not survive (Steyn, 2007). Most stroke survivors end up with long-term residual disabilities (Heart Foundation South Africa, 2013). The pathogenesis of stroke shows that its debilitating effects are often associated with ischemia and multifactorial cell death. Profound ischemia results in necrosis, and less severe ischemia triggers a series of perturbations that may lead to apoptosis in the stroke penumbra, including cortical spreading depressions, excitotoxicity and oxidative stress (Maas and Furie, 2009).

It is therefore plausible that measures aimed at preventing the occurrence of ischemic stroke and its attendant neurological manifestations be encouraged especially by susceptible individuals. Amelioration of oxidative stress and free radical production could provide a potential source of protection. In this study, the effects of prolonged consumption of fermented rooibos tea in modulating neurological outcome after an ischemic brain injury were investigated.

Rooibos tea has been adjudged the preferred tea in South Africa (Fukasawa *et al.*, 2009) and is well known for preventing lipid oxidation while its antioxidant and free-radical scavenging properties are also well documented (Marnewick *et al.*, 2011). In spite of the growing population of rooibos tea consumers and a plethora of information about its health benefits, very little scientific reports are available in literature regarding its effects on the nervous system.

This study aims to evaluate the potential neuroprotective effects of fermented rooibos herbal tea especially when consumed for long periods prior to an acute ischemic event.

CHAPTER TWO

LITERATURE REVIEW

2.0 INTRODUCTION

Globally, cerebrovascular diseases remain a leading cause of death (Truelsen *et al.*, 2000). It has been estimated that 5.5 million people (equivalent to 9.6 % of all global deaths in 2001) died from cerebrovascular diseases (Truelsen *et al.*, 2000). With about two-third of stroke-related deaths occurring in people living in low- and middle-income countries (WHO, 2002), prevention is possible by addressing risk factors as well as managing and counselling individuals at risk (Di Legge *et al.*, 2012).

2.1 CEREBROVASCULAR DISEASE

Cerebrovascular disease (CVD) is defined as any abnormality of the brain characterized with damage to the vascular integrity of the brain (McCance and Huether, 2014). It is described as the most common of all neurological disorders (Mangiapane and Salter, 1999), and ranks as the only neurological disorder among the top ten causes of death worldwide (WHO, 2012). Cerebrovascular accident (CVA) often called stroke is documented as the most common of all cerebrovascular disease reported in hospitals around the world (Craft *et al.*, 2013).

2.1.1 Types of Cerebrovascular Diseases

1. Cerebral aneurysms: are abnormal areas of cerebral blood vessels that bulges out like a balloon and become filled with blood. Aneurysms are mostly congenital but could also result from weakening of blood vessels by diseases or injury. A ruptured cerebral aneurysm often results in stroke, permanent neuronal damage or death (Novitzke, 2008).

2. Cerebral vascular malformations (CVMs): includes any congenital vascular anomalies of the brain that are present at birth, and are clinically evident either during childhood or

adulthood (Yakes, 2004). They may include any vascular element (e.g arteries, veins, capillaries and lymphatics) (Yakes, 2004). The most common of CVMs are arteriovenous malformations (AVMs) and cerebral cavernous malformations (CCMs) (Leblanc *et al.*, 2009). AVMs occur when there is an abnormal connection between the arteries and veins in the brain in which arteries connect directly to veins without having a true capillary bed between them (Kim *et al.*, 2011). CCMs do occur when there are enlarged capillaries with immature vessel wall components in the cerebral vasculature. They both often result in brain hemorrhage in the event of a rupture (Leblanc *et al.*, 2009; Kim *et al.*, 2011).

3. Cerebrovascular accident: often referred to as a brain attack or stroke, occurs when blood supply to any part of the brain is interrupted or blocked. It ranks as the second largest cause of death behind ischemic heart disease with 6.7 million deaths worldwide in the year 2012 (WHOa, 2015). An estimated 28% of people younger than 65 years of age experience an event of stroke during their lifetime (Perkin, 2002) but it is most common in individuals above the age of 60 years (Moghtaderi and Alavi-Naini, 2012).

2.1.2 **Major Risk Factors Associated With Stroke**

The term risk factor is defined as a trait associated with a pathological medical condition. Such factors when observed before the onset of a stroke can be related to the occurrence of stroke later in life (Lindgren, 2014). Most of the risk factors associated with stroke are shown in table 2.1. Arterial hypertension and both elevated systolic and diastolic blood pressures are independent risk factors for stroke (Perkin, 2002). Cigarette smoking has also been reported to increase the risk of stroke by 25% to 50% (Buttaro et al., 2013). Another risk factor associated with stroke is diabetes which increases the risk of ischemic stroke by 2.5 to 3.5 times (Schwartzman, 2006). Non-thematic atrial fibrillation have also been associated with an increase in the incidence of ischemic stroke (Norris and Vladimir, 2001).

Table 2.1: Risk factors for stroke

Hypertension ^a	Cigarette Smoking ^b	Diabetes ^{c,d}	Atrial fibrillation ^e
Increased arterial pressure can damage inside of cerebral arterial wall.	Increases oxidative stress from cigarette smoke and is a potential stimulus that initiates cardiovascular dysfunction.	Chronic hyperglycemia can lead to damage to the vascular endothelium.	Atrial fibrillation can cause blood flow to pool and clot in the atrial chambers.
Damaged or altered blood cell-endothelium interaction can subsequently lead to local thrombi formation.	Cardiovascular dysfunction can progress to the development of atherothrombotic disease.	Widening of the basement membrane does compromise the integrity of adjacent vascular smooth muscle cells, pericytes and astrocytic end feet.	Blood clot in the heart can travel long distances from the heart into the cerebral vasculature.
Degenerative changes in smooth muscle cells can accelerate atherosclerotic process which predisposes vessel to stroke	Atherosclerotic plaque may narrow cerebral arteries and limit the flow of oxygen-rich blood to the brain resulting in stroke.	Diffuse swelling of the astrocytic end feet compromises the blood brain barrier (BBB) causing cytotoxic oedema and stroke.	Occlusion of a cerebral vessel by such blood clot is the cause of cardio-embolic stroke.

a = (Johansson, 1999), b = (Mazzone *et al*, 2010), c = (Ergul *et al*, 2012), d = (Kagansky *et al*, 2001), e = (Arboix and Alió, 2010)

2.1.3 **Classification of Stroke**

The brain abnormalities induced by cerebrovascular accidents are classified according to their pathophysiology and include:

1. Ischemic stroke (thrombotic or embolic)
2. Hemorrhagic stroke

2.1.3.1 **Ischemic Stroke**

2.1.3.1.1 **Thrombotic stroke**

Cerebral thrombosis is the most common type of ischemic stroke documented in clinical studies (Nagaraj *et al.*, 2011). It arises from arterial occlusion caused by thrombi (blood clots) formed along the walls of major arteries supplying the brain (figure 2.1) (Appel and Llinas, 2007). Cerebral thromboses are mostly associated with areas where inflammatory and atherosclerotic processes have caused narrowing of blood vessels. Atherosclerotic plaques are known to cause the smooth area of blood vessel walls to degenerate, forming an ulcerated area of the vessel wall which attracts platelets and fibrins to adhere to the damaged wall (Porth, 2011). This subsequently results in clot formation which gradually occludes the artery and prevents blood flow to the remaining part of the brain. More-often, portions of a thrombotic clot may break off and travel up the cerebral vessel to distant sites where occlusion could occur, producing a thromboembolic stroke (McCance and Huether, 2014). This results in death or necrosis of the parts of the brain deprived of blood and oxygen. Thrombotic strokes may be further subdivided on the basis of their clinical manifestations into: Transient ischemic attacks (TIAs), Strokes-in-evolution and Completed strokes.

In a true TIA, the neurologic deficits are of short duration and are completely clear within 24 hours, leaving no residual dysfunction (Good, 1990) as the temporary disturbance in cerebral blood flow reverses before infarction occurs (Porth, 2011). The typical development process of

thrombotic stroke causes the clinical syndrome known as stroke-in-evolution (progressive stroke), while a completed stroke is a CVA that has reached its maximum destructiveness in producing neurologic deficits (McCance and Huether, 2014).

2.1.3.1.2 Embolic stroke

An embolic stroke is caused by a moving clot which travels from a distant source (outside the cerebrovascular system) and becomes trapped in small cerebral vessels, mostly at bifurcations (Rink and Khanna, 2011). The most frequent site of embolic strokes is the middle cerebral artery distribution (Karen and Aidin, 2012). While most cerebral emboli originate in the thrombus in the left heart, they may also originate from an atherosclerotic plaque in the carotid arteries (Pooler, 2009). Long periods of bone / cardiac surgeries are known to also produce emboli in the form of blood, fat, or air (Shahpouri *et al.*, 2012). Rheumatic heart diseases, atrial fibrillation, recent myocardial infarction are various conditions that predispose to the formation of emboli. In persons who experience an embolic stroke, a second stroke usually follows at some point because the source of emboli continues to exist (Pooler, 2009).

2.1.3.2 **Hemorrhagic Stroke**

Hemorrhagic stroke or intracranial hemorrhage is defined as an acute neurological injury which occurs when weakened cerebral arteries bleed into the head (figure 2.1) (Smith and Eskey, 2011). It is the third most common cause of CVA (McCance and Huether, 2014). Hemorrhagic stroke is of two distinct types: intracerebral hemorrhage (ICH) which involves vessel bleeding directly into the brain parenchyma (ICH), and subarachnoid hemorrhage (SAH) which occurs when there is bleeding into the cerebrospinal fluid (CSF), the sulci, fissures, and cisterns (Smith and Eskey, 2011). Hypertension (56% to 81%), ruptured aneurysms, vascular malformations, head trauma and illicit drug use remain the most common cause of hemorrhagic

stroke (McCance and Huether, 2014). Other risk factors for hemorrhagic stroke include previous cerebral infarct, coronary heart disease and diabetes mellitus (Pooler, 2009).

About a quarter of most strokes which occur during sleep (Soler and Ruiz, 2010); however cerebral hemorrhage is known to occur suddenly usually when the person is active (Porth, 2011). Most people may complain of a severe headache and stiff neck (nuchal rigidity), as a result of blood entering the cerebrospinal fluid (CSF) which may subsequently be accompanied by focal neurologic signs or other symptoms such as nausea/vomiting, loss of consciousness, or seizure depending on the vessel involved (Smith and Eskey, 2011). There is usually contralateral hemiplegia, with initial flaccidity progressing to spasticity. The hemorrhage and resultant oedema exert great pressure on the brain substance, and the clinical course progresses rapidly to coma and frequently to death (Pooler, 2009).

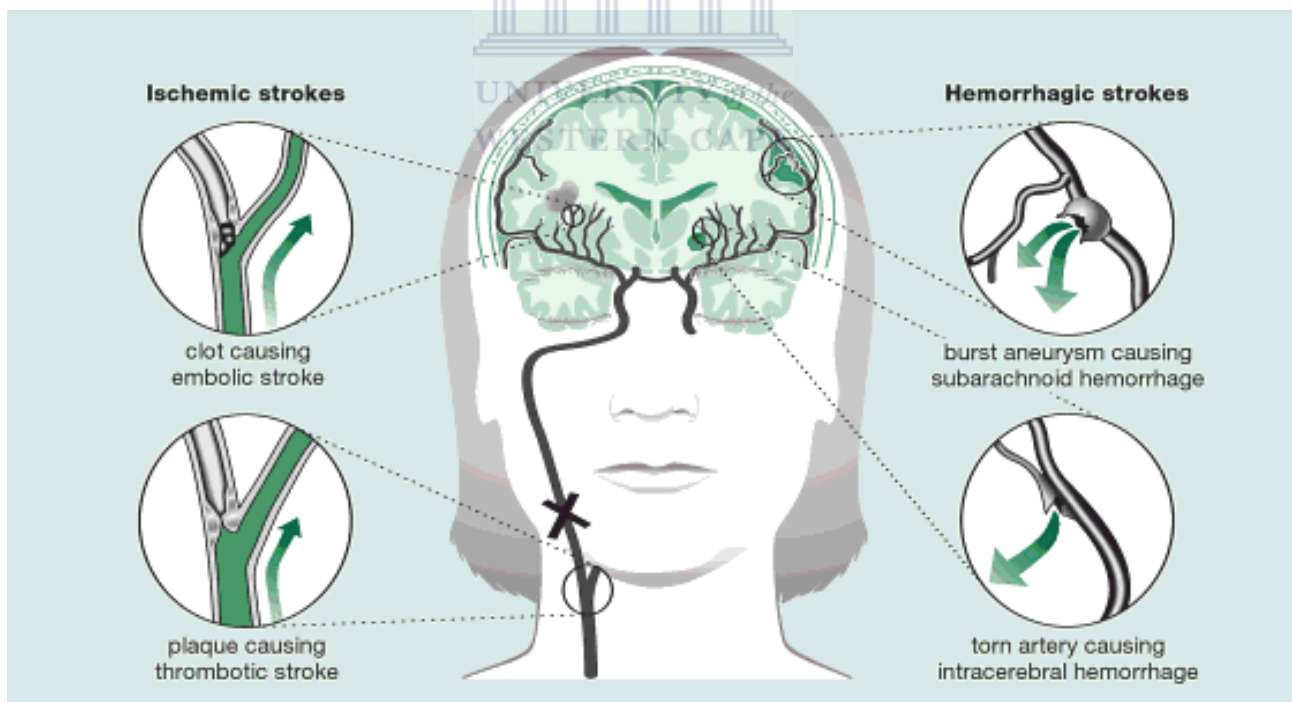


Figure 2.1: Representation of different stroke types. Ischemic stroke as depicted on the left side of the picture shows blockage of blood flow to the brain by a blot clot and plaque. The leakage of blood into the brain is known as hemorrhagic stroke and is shown on the right side of the picture (Adapted from Appel and Llinas, 2007).

2.1.4 **Pathophysiology of Ischemic Brain Injury**

Ischemia is defined as a decrease in blood flow to organs or tissues sufficient enough to alter cellular metabolic demands, and if not corrected, will cause death of the cells and tissues (Woodruff *et al.*, 2011). In the brain, the threshold of ischemic injury varies in the different regions with the white matter being more resilient than the gray matter (Troncoso *et al.*, 2010). A phenomenon due to the fact that areas of the brain with high energy consumption becomes rapidly depleted of energy during an ischemic episode and are therefore most vulnerable (Huang and Castillo, 2008). The impact of an ischemic injury on the structure and function of the brain also depends on the severity and duration of the blood flow reduction. In ischemia produced by occlusion of the middle cerebral artery, the impact is more rapid, severe and irreversible in the “ischemic core” which is the area where blood flow is lowest and less than 20% of its normal rate (Iadecola, 1999). However, the ischemic damage is less severe in the ischemic penumbra (the region around the ischemic core) because of collateral blood supply from adjacent non-ischemic territories which keep the region functionally silent, but metabolically active and potentially salvageable (Iadecola and Anrather, 2011).

The pathophysiology of cerebral ischemia involves a series of complex events best studied in animal models of stroke (Smith, 2004). These pathologic events include morphological alterations in brain cells, energy failure, loss of cell ion homeostasis, increased intracellular calcium levels, excitotoxicity, free radical-mediated toxicity, disruption of the blood-brain barrier (BBB), activation of glial cells, and infiltration of leukocytes (Woodruff *et al.*, 2011).

2.1.4.1 **Morphological Alterations in the Brain Cells after an Ischemic Brain Injury**

The cellular changes occurring after an ischemic brain injury are well defined in literature. These reports (Iadecola, 1999; Kalogeris *et al.*, 2012) describe the response of tissue cells to ischemia in experimental models of cerebral ischemia in which blood flow to the brain was

compromised to mimic clinical conditions in humans. The resultant pathologic cellular events are briefly summarized and tabulated below (Table 2.2).

2.1.4.1.1 Neurons

Deficiency in the amount of oxygen and glucose reaching neurons during cerebral ischemia presents pathomorphological changes in the nuclei and cytoplasm. During the first few hours of ischemia, disseminated eosinophilic ischemic neurons with granular chromatin condensation, dilatation of the endoplasmic reticulum and swelling of the mitochondrial inner matrix occur (Ito *et al.*, 2006). Because neurons do not store alternative source of energy (Bramlett and Dietrich, 2004), they remain more sensitive to ischemia than any other cell in the brain (Damjanov, 2012).

2.1.4.1.2 Glial cells

Glial reactions to ischemic injury are reported to be quantitative or qualitative and may involve all four cell types (astrocytes, microglial, oligodendrocytes and ependymal cells) (Damjanov, 2012). Astrocytes are star shaped glial cells derived from neural stem cells. These cells are the only glycogen-storing cells in the brain, known to be less susceptible to ischemic injury. Astrocytic glycogen breaks down during ischemic conditions to lactate as a temporary substitute for glucose (Nikonenko *et al.*, 2009). During ischemia, astrocytes are reported to swell, elongate and undergo cytoplasmic fragmentation processes, with an increase in the expression of glial fibrillary acidic protein (GFAP) in the ischemic penumbra (Iadecola, 1999 and Nikonenko *et al.*, 2009). During an ischemic injury, microglia retract their processes and assume an amoeboid morphology, typical feature found on activated microglia (Taylor and Sansing, 2013). Oligodendrocytes which make up the myelin that wrap around axons in both the grey and white matter are specifically sensitive to hypoxia and ischemia in premature

infants. Ependymal cells which line the walls of the ventricles and form the specialized choroid epithelium which secretes cerebrospinal fluid (CSF) may also be injured during ischemia.

2.1.4.1.3 Neutrophils and Macrophages

The role of inflammation in the pathogenesis of ischemic brain injury is also well documented. There are several cells within the brain tissue that are able to secrete pro-inflammatory mediators after an ischemic insult. These include endothelial cells, astrocytes, microglia and neurons. During cerebral ischemia, the brain responds by recruiting various types of inflammatory cells (neutrophils, different T cells subtypes, monocyte/macrophages, etc.) into the ischemic brain tissue. The influx of neutrophils peaks at 48-96 hours following permanent occlusion of the middle cerebral artery after which their number decrease rapidly with time. Blood-borne macrophages migrate into the inflammatory site to become the most predominant cell 5-7 days after ischemia (Jin *et al.*, 2010a).

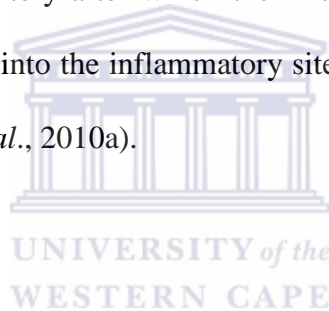


Table 2.2: Cellular changes occurring after focal cerebral ischemia in the rat brain (Iadecola, 1999)

Time after Ischemia	Neurons	Astrocytes	Microglia	Neutrophils	Macrophages
30 minutes	Shrinkage	Swelling; Degeneration of GFAP+ cells	-	-	-
1 hour	Swelling; Vacuolation; Chromatin clumping	-	-	-	-
6 hours	-	Increase in GFAP+ cells at the infarct periphery	-	Adhesion to endothelial cells; Brain infiltration begins	-
12 hours	Axonal swelling; Cytoplasmic and nuclear disintegration; Mitochondrial densities	-	Activation in the ischemic area	-	-
1 day	-	Ghost cells	-	-	Brain infiltration begins
2 – 3 days	Ghost neurons	-	Brain infiltration maximal	-	-
4 – 5 days	-	-	Activation in distant regions	-	-
7 days	-	Focused gliosis; Glial scar	-	-	-

2.1.4.2 Molecular Alterations in Brain Cells after an Ischemic Injury

2.1.4.2.1 Glutamate excitotoxicity

Glutamate is one of the most prominent and an essential neurotransmitter that regulates the functions of the brain (Kim *et al.*, 2011). It is required for the rapid synaptic transduction in the nervous system and plays important roles in normal brain function, neuronal growth and synaptic plasticity in health and disease (Lai *et al.*, 2014). While glutamate is essential, it is also very toxic at high extracellular concentration (Gillessen *et al.*, 2000), and has been reported to be involved in the early pathologic process of ischemia-induced neuronal damage during the early hours of ischemic injury (Woodruff *et al.*, 2011). The reduction or cessation of blood flow to neurons during an ischemic brain injury results in the failure of energy-dependent cellular pumps which in turn results from failure of ATP generation by hypoxic neurons. The failure of such energy-dependent processes (e.g. sodium-potassium ATPase) subsequently result in an ionic imbalance, cellular swelling through osmosis, cellular depolarization and inhibition of the re-uptake of excitatory neurotransmitters such as glutamate from the extracellular space. Under this circumstance, excess glutamate binds to its receptors hereby promoting an excessive influx of calcium ions (Ca^{2+}) into the cell, triggering a wide array of downstream phospholipases and lipases which in-turn degrade membranes and proteins essential for cellular integrity, thus ending in cell death (Cross *et al.*, 2010).

2.1.4.2.2 Oxidative stress

Oxidative stress is defined as the imbalance between pro-oxidants and antioxidants in systemic homeostasis resulting in excessive production of reactive oxygen species (ROS) which leads to tissue damage (Allen and Bayraktutan, 2009). All cells in the body such as neurons are normally exposed to a baseline level of oxidative stress from both exogenous and endogenous sources (Woodruff *et al.*, 2011) with a balance maintained by the body's antioxidant defense systems which involve enzymatic and non-enzymatic processes (Łagowska-Lenard *et al.*,

2008). ROS are highly reactive molecules which are naturally produced as a by-product of normal oxygen metabolism (Uttara *et al.*, 2009). In aerobic organisms, superoxide anion radical ($O_2^{\cdot-}$) is the primary ROS which also generates hydrogen peroxide (H_2O_2) by dismutation. Other ROS forms are generated by the reaction of oxygen radical with other tissue components; $O_2^{\cdot-}$ with nitric oxide (NO) produces peroxynitrous acid (ONOOH) that spontaneously decomposes to produce OH^{\cdot} (Allen and Bayraktutan, 2009). Despite their beneficial role in cell signaling during normal biologic process, ROS at toxic levels play a key role in brain tissue damage after cerebral ischemia where they cause injury to cell structures including lipids, membranes, proteins, and DNA (Olmez and Ozyurt, 2012). Shortly after the onset of brain ischemia, several detrimental processes accompany the loss of oxygen and glucose supply to neurons. These include the accumulation of lactic acid in neurons following energy (ATP) depletion with a resultant acidic cellular environment which promotes an increase in H^+ concentration which in turn enhance the overproduction of oxidants, inactivation of detoxification systems, and consumption of endogenous antioxidants (Allen and Bayraktutan, 2009). These changes consequently cause the disruption of the brain's antioxidative defense ability (Chen *et al.*, 2011). The overproduction of ROS in the absence of an endogenous antioxidant defense system activates several pathways involved in cell death; this includes apoptosis and inflammation (Woodruff *et al.*, 2011).

2.1.4.2.3 Inflammation

Inflammation, either acute or chronic is a defense reaction to tissue insult or injury (Kriz, 2006). Acute inflammation comprises of an early response against an injury which helps in the repair of the damaged site, while chronic inflammation results from persistent and unresolved harmful stimuli (Streit *et al.*, 2004). Experimental and clinical evidence continues to support the involvement of inflammatory processes in the post-ischemic events that accompany a primary ischemic insult (Amantea *et al.*, 2009). In animal models of focal cerebral ischemia,

time-dependent recruitment and activation of neutrophils, T cells and monocytes / macrophages have confirmed the involvement of inflammatory process in the patho-mechanism of ischemic brain injury, with inhibition of such inflammatory response seen to reduce brain damage (Jin *et al.*, 2010a). In the early hours of ischemic brain injury, increased levels of pro-inflammatory mediators (cytokines and chemokines) released or present at the site of the blood-brain-barrier (BBB) increases the expression of adhesion molecules on cerebral endothelial cells and leukocytes, and as a result facilitates the migration of peripheral inflammatory cells (circulating neutrophils and monocytes) from blood into the brain tissue (Stanimirovic and Satoh, 2000). The brain's inflammatory response is further amplified by the release of more cytokines and chemokines during the sub-acute phase (hours to days) of ischemic injury by infiltrating leukocytes which leads to disruption of the BBB, brain oedema, neuronal death, and hemorrhagic transformation (Jin *et al.*, 2010a).

2.1.4.2.4 Necrosis and Apoptosis

Necrosis and apoptosis are two most commonly described forms of neuronal cell death following cerebral ischemia in scientific literature (Pang and Geddes, 1997). As reported by Onténiente *et al.*, (2003), characterization and differentiation of these cell death pathways remains an area of intense investigation in the search for a neuroprotective drug against ischemic cell death. While, necrosis is characterized by rapid cell swelling and cell lysis which mostly results in the rupture of cell content, and predominantly occurs in area of severe ischemic insult (mostly especially in the infarct core area after focal cerebral ischemia) (Liu *et al.*, 2004), apoptosis may occur in the ischemic penumbra (areas of mild ischemic injury with longer survival periods). The key features of apoptosis are cell body shrinkage, cytoplasmic and nuclear fragmentation and internucleosomal chromatin cleavage (Pang and Geddes, 1997). In contrast to necrosis, apoptosis appears to be a coordinated process of energy-dependent programmed cell death (Elmore, 2007).

There are two inter-connected pathways that lead to apoptosis: the intrinsic pathway which is initiated by internal cellular events and is dependent on the release of apoptogenic protein - cytochrome C from disrupted mitochondria leading to downstream activation of caspase cascade, and an extrinsic pathway which is activated by specific ligands binding to death receptors on the surface of the cell (Elmore, 2007; Broughton *et al.*, 2009). Specifically, the most important regulators of the intrinsic pathway of apoptosis are the Bcl-2 family which protect neurons and other cell types against a wide variety of apoptotic insults and are labelled antiapoptotic proteins (Soane *et al.*, 2011). After cerebral ischemia, a downregulation of Bcl-2 levels and an upregulation of proapoptotic protein Bax levels result in activation of caspases which are principal triggers of apoptosis (Figure 2.2) (Phan *et al.*, 2002; Broughton *et al.*, 2009).

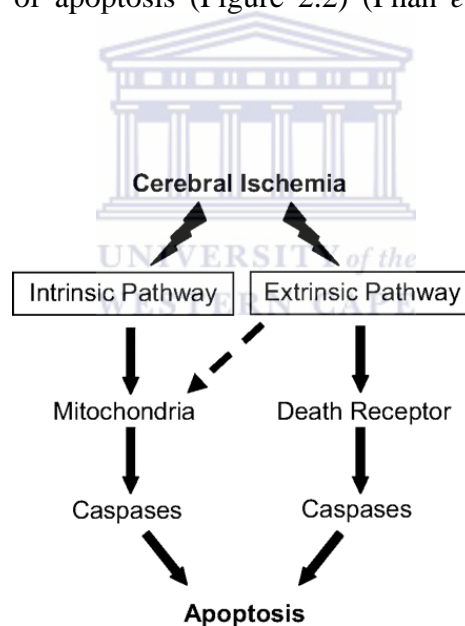


Figure 2.2: Apoptotic signaling cascade after cerebral ischemia (Adapted from Broughton *et al.*, 2009).

2.1.4.3 Ischemic reperfusion injury

The prompt restoration of blood flow to an ischemic tissue is indisputably considered the most effective therapeutic response to reducing the severity or preventing neurologic damage in patients suffering from an ischemic brain injury (Sanderson *et al.*, 2013). However, restoration

of blood flow may aggravate the initial injury caused by ischemia by triggering a multifactorial process of (molecular and biochemical) events that antagonize the beneficial role of reperfusion (Aronowski *et al.*, 1997). Yang and Betz (1994) showed that 3 hours of MCA occlusion followed by 3 hours of reperfusion exacerbated brain infarct and damage to the blood-brain-barrier when compared to 6 hours of permanent MCA occlusion.

The involvement of leukocytes in ischemia-reperfusion damage is common and extensively discussed in literature (Panetta and Clemens, 1993). Though important in tissue protection and repair, activated leukocytes may release substances that are harmful to vessel wall upon interacting with the vascular endothelium, and examples of such substances are the products of phospholipase which can damage the endothelium to allow transmigration into the ischemic brain tissue (Vasthare *et al.*, 1990). Upon transmigrating into the brain parenchyma, activated leucocytes release toxic ROS, proteases and elastases, which result in oedema, thrombosis and parenchymal cell death (Eltzschig and Collard, 2004).

2.1.5 **Diagnosis of Acute Ischemic Brain Injury**

A complete history and thorough physical and neurologic examination is important to accurately diagnose and characterize stroke into either ischemic or hemorrhagic (Pooler, 2009). In recent years, computerized tomography (CT) scans and magnetic resonance imaging (MRI) have become the most commonly used techniques in the assessment of acute cerebral vascular disease (Birenbaum *et al.*, 2011). While CT does not present much information in the first 24 hours of an ischemic insult, positron emission tomography (PET) is considered the gold standard in defining the location and size of neuronal loss and assessment of potentially salvable tissue in the penumbra (Sá de Camargo and Koroshetz, 2005). The rapid onset of irreversible brain injury necessitates the need for an urgent diagnosis and selection of an efficient treatment for acute ischemic brain injury (Kasner and Gorelick, 2004).

2.1.6 **Treatment of Acute Ischemic Brain Injury**

Although preclinical studies have greatly enhanced our knowledge of the pathomechanism underlying an acute ischemic brain injury, translating such knowledge from bench to bedside for clinical use remains a challenge in healthcare (Balkaya *et al.*, 2013). The main therapeutic goal in the acute phase of an ischemic brain injury is the restoration of blood flow either naturally or with the aid of a thrombolytic agent that dissolves blood clots (Woodruff *et al.*, 2011). Urokinase / streptokinase and recombinant tissue plasminogen activator (rt-PA, alteplase) are examples of thrombolytic drugs used clinically for the removal of blood clot in acute ischemic stroke (Bivard *et al.*, 2013). The use of intravenous rt-PA has also been found beneficial in improving functional outcomes in patients if administered during the first 3 hours of acute ischemic stroke (Wardlaw *et al.*, 2012). Clinical studies of desmoteplase and tenecteplase are also ongoing to alleviate the short time therapeutic window, risk of hemorrhage, and limited efficacy of alteplase on large clot removal (Bivard *et al.*, 2013). However, because reperfusion of an ischemic tissue may magnify tissue injury and worsen the challenge of treatment (Nour *et al.*, 2012), thrombolytic therapy for acute ischemic stroke is seldom used and has only been found useful in 2% of eligible persons (Miller *et al.*, 2011). Other vascular therapies include the prevention of microcirculatory disturbance, protection of the BBB and augmentation of collateral blood flow and cellular therapy by protection of neurons (Tanaka, 2013).

2.1.7 **Animal Models of Ischemic Brain Injury**

Animal models of ischemic brain injury have been developed to mimic or recreate the pathophysiological mechanisms of ischemic brain injury seen in humans (Belayev, 2012). An understanding of these mechanisms is believed to be critical to enhancing our knowledge of the pathological changes that occur during and after the onset of an ischemic brain injury for new

therapeutic strategies for stroke to be developed (Sicard and Fisher, 2009). The two main animal models of ischemic brain injury often used in preclinical studies include the focal ischemic and global ischemic stroke models (Belayev, 2012), both of which involve a reduction of oxygen and glucose supplies to brain tissue. Although both models are similar in many respects, their differences are discussed in the following sections.

2.1.7.1 Focal Ischemic Stroke Model

Focal ischemic stroke models usually involve the occlusion or blockage of the middle cerebral artery (MCA) (Yang *et al.*, 2006) which is the most commonly affected artery in human ischemic stroke patients (Uluç *et al.*, 2011). The technique employed (see figure 2.3) include the occlusion of the middle cerebral artery by either of the following methods; (1) suture ligation, (2) electrical cauterization, (3) intraluminal filament occlusion (4) blood clot injection and (5) Photothrombotic occlusion (Wang-Fischer and Koetzner, 2008). Depending on the research interest, each method provides a valuable tool for studying the neuroprotective potentials of agents against cellular, neurological and behavioral outcomes over a period of time (Sicard and Fisher, 2009). The major outcome is however the final infarct size and sensorimotor deficits with the latter more relevant in evaluating neuroprotective agents for clinical trials (Macrae, 2011).

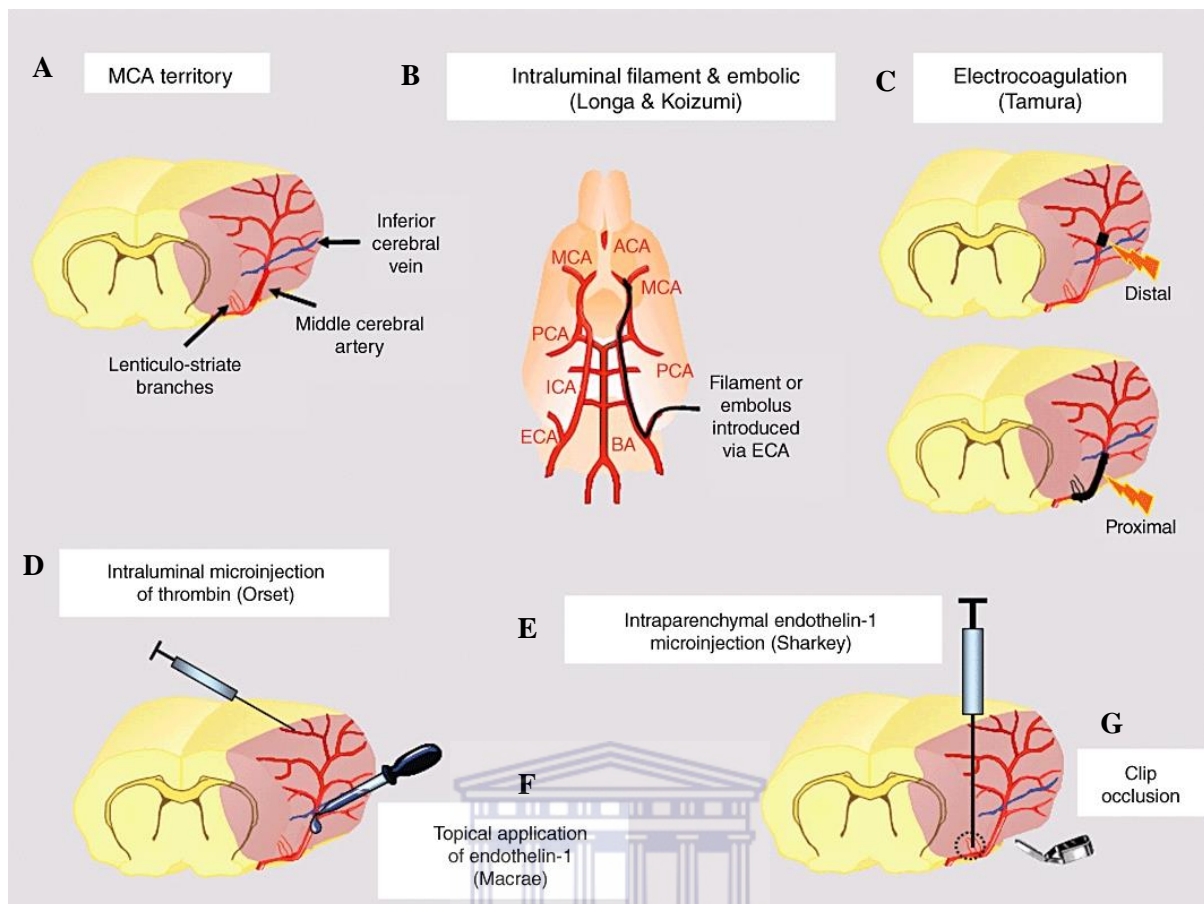


Figure 2.3: Diagrammatic illustrations of middle cerebral artery (MCA) occlusion models in rats and mice. Pink shading on MCA territory in the diagram represents part of the brain tissue supplied by the MCA (A). (B) Shows the intraluminal method of inserting an occluding filament or embolus through the external carotid artery (ECA) into the origin of the MCA. (C-G) shows animal models of ischemic stroke which uses craniotomy to directly access the MCA or its branches for electrocoagulation, (D) intraluminal injection of thrombin, (E and F) microinjection or topical application of endothelin-1 and (G) Clip occlusion of MCA (Adapted from Macrae, (2011)). (ACA: anterior cerebral artery, PCA: posterior cerebral artery, ICA: Internal cerebral artery, BA: basilar artery).

2.1.7.2 Global ischemic stroke model

Global ischemic models unlike focal ischemic models involve the occlusion of two or more blood vessels to reduce blood flow to the entire brain mimicking cerebral ischemia resulting from cardiac arrest and severe hypotension (Wang-Fischer and Koetzner, 2008). It is reported to be a very suitable model for histopathological, molecular, biochemical and physiological evaluation of neuroprotective agents (Raval *et al.*, 2009) and mainly involves 2-Vessel occlusion (VO) of both common carotid arteries (CCA) or 3-Vessel occlusion of the CCAs and

the basilar artery (Figure 2.4) (Woodruff *et al.*, 2011). The advantage of the 2-VO model in producing forebrain ischemia is that it involves a more simple surgical preparation, produces a highly reproducible ischemic damage and reperfusion can easily be accomplished with high animal survival rate making it suitable for chronic survival studies (Traystman, 2003). The 2-VO ischemia model produces selective neuronal vulnerability typical of the CA1 pyramidal neurons of the hippocampus and also delayed neuronal death after a transient ischemic episode, 3-7 days after reperfusion (Belayev, 2012).

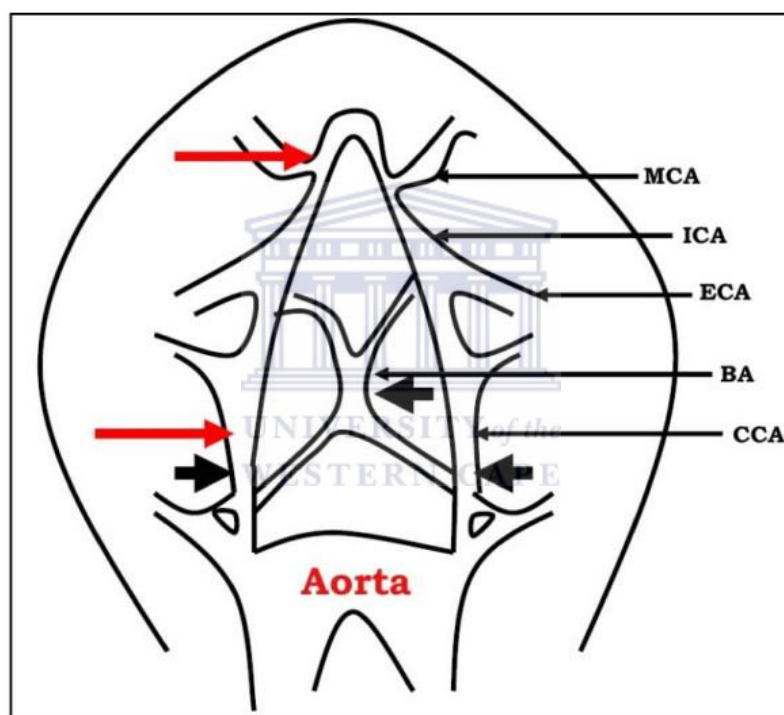


Figure 2.4: Diagrammatic illustration of occlusion points in animal models of ischemic brain injury. Black arrows and red arrows indicates global (3-VO) and focal (MCA) occlusion points respectively (CCA: Common carotid artery) (Adapted from Woodruff *et al.*, 2011).

2.2 ROOIBOS TEA

Rooibos tea or *Aspalathus linearis* is produced from the Rooibos plant which grows in the Cederberg area of the Western Cape Province of South Africa (figure 2.5 A) (Standley *et al.*, 2001). It has been consumed as a healthy beverage for more than a century in the Republic of South Africa and in Europe (Baba *et al.*, 2009). There are two types of Rooibos tea, the

fermented (red) and unfermented (green) varieties, each having a distinct flavor. The red variety is sweet and nutty, while the green variety has a malty taste. In South Africa, this tea has been said to have many functions such as increasing appetite, improving gastrointestinal motility, and controlling mental condition (Morton, 1983; Nakano *et al.*, 1997). In recent times, extracts from the fermented herb have become more popular as a beverage for everyday use (Crozier *et al.*, 2011).

2.2.1 **History of rooibos tea**

As reported by Joubert *et al.* (2008), the first recorded use of rooibos tea was during the 17th century when the leaves and stems of the rooibos plant served as beverage among the mountain-dwelling tribe of Khoi in the Clanwilliam region of the Western Cape. The Khoi tribe harvested the plant during the summer months of the year from the wild mountains, where they grew and prepared infusions of the fermented rooibos by chopping the plant stems together with their leaves, crushing them with a hammer, sweetening them in the hollows of stone reefs, before sun-drying. This process is believed to be the basis of the industrialized process for fermented rooibos that is common today (Joubert *et al.*, 2008). *Aspalathus linearis* is now cultivated commercially and there are new improvements in the methods of harvesting and drying the tea (Small, 2011). Besides being consumed as a tea, rooibos is also utilized in the production of cosmetic products, alcoholic drinks and ingredients in food preparations by the food industry (figure 2.5 B - E) (Mahomoodally, 2013). Its popularity is also reported to have increased globally among consumers due to its notable antioxidant activity (Mahomoodally, 2013).

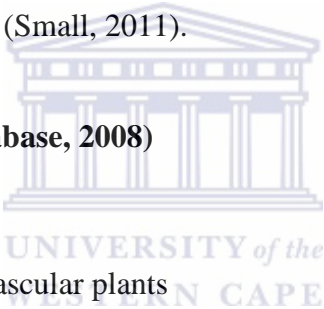


Figure 2.5: Images of rooibos plant cultivation and uses. (A): Rooibos plant growing in the Cedarberg region of South Africa (Erickson, 2003). (B-E): The use of rooibos plant does cut across the production of; (B) herbal teas, (C) cosmetics, (D) alcoholic drinks and (E & F) food ingredients and pet food (Images adapted from Rooibos Ltd, South Africa).

2.2.2 **Plant Taxonomy**

Aspalathus linearis belongs to the family of the *Fabaceae* and about 278 species in the genus *Aspalathus* are confined to South Africa (Dahlgren, 1988; McKay and Blumberg, 2007). The plant is an erect shrub of about 2 meters tall with red-brown branches and needle-like leaves which are about 1.5 to 6 centimeters long (Small, 2011). In some species the leaves bear hard, sharp, spines at their tips (Phillips, 1951) and the flowers have been seen to vary in the degree of their color complexity ranging from showy yellow, to pink and pale violet (Marloth *et al.*, 1915). Prior to the 20th Century, *Aspalathus linearis* was exclusively collected in the wild but an increasing demand has encouraged the cultivation of this plant thereby diminishing the proportion of wild rooibos available commercially (Malgas *et al.*, 2010). Approximately 60% of national harvest is exported annually (Small, 2011).

2.2.2.1 **Classification (Plant database, 2008)**



Kingdom	Plantae – Plants
Subkingdom	Tracheobionta – Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Rosidae
Order	Fabales
Family	Fabaceae – Pea family
Genus	<i>Aspalathus</i> L. – aspalathus
Species	<i>Aspalathus linearis</i> (Burm. f.) R. Dahlgren – rooibos

2.2.3 **Chemical Composition of Rooibos Tea**

There are two forms of rooibos tea that are produced commercially, the traditional fermented and the “green” unfermented rooibos tea (Marnewick, 2009). The unfermented rooibos tea has a higher antioxidant capacity when compared to the fermented rooibos, mainly because of the minimum oxidative (or fermentative) changes allowed during its production process (Joubert *et al.*, 2008). A chemical analysis of a teaspoon per cup of rooibos tea (both fermented and unfermented) was reported to contain 300 mg of protein, 7.8% Copper (Cu), 5.5-7.3% Fluorine (Fl) and 1.7-2.2% Manganese (Mn) of the recommended percentage of U.S daily values respectively (McKay and Blumberg, 2007).

Rooibos tea does not contain the stimulant caffeine, but traces of the alkaloid sparteine have been reported by Van Wyk and Verdoorn (1989). Among the phenolic compounds found in rooibos tea are tannins, which may vary in content ranging from about 3.2% to 14% and to as high as 50% depending on the two types of rooibos tea (fermented or unfermented) and the methods of extraction (dried water, methanol, etc.) (Joubert *et al.*, 2008). Several other chemical compounds are present in both fermented and unfermented rooibos and identifications of these compounds are important for proper understanding of the potential phytopharmaceutical health benefits of rooibos tea (Beelders *et al.*, 2012). Aspalathin, nothofagin, apigenin, luteolin, rutin, isoquercetin, hyperoside, quercetin, luteolin and chrysoeriol are some predominant flavonoids identified in both types of rooibos tea (McKay and Blumberg, 2007; Villaño *et al.*, 2010). Their antioxidant activity are tabulated in Table 2.3 below. Natural aspalathin has only been isolated from rooibos and remains the most abundant flavonoid and a major antioxidant in the unfermented rooibos tea and also in the water extract of fermented rooibos despite its substantial decrease during fermentation (Joubert, 1996; Bramati *et al.*, 2002). While fermented extracts of rooibos tea have been reported to demonstrate less antioxidant activity based on their reduced flavonoid content during

fermentation, several studies have shown that fermented rooibos could scavenge physiologically relevant reactive oxygen species, superoxide radical anion ($\text{O}_2^{\bullet-}$) (Yoshikawa *et al.*, 1990; Standley *et al.*, 2001; Joubert *et al.*, 2004) and hydroxyl radical ($\bullet\text{OH}$) (Yoshikawa *et al.*, 1990; Lee and Jang, 2004; Joubert *et al.*, 2005). Fermented rooibos tea also remains the most popular tea among an increasing population of consumers.



Table 2.3: Antioxidant activity of Phenolic Compounds from Rooibos and selected reference compounds (Joubert and de Beer, 2014)

Compounds	Free Radical Scavenging Assay				Lipid Peroxidation Assays		
	ABTS (IC ₅₀ , µM) ^a	ABTS (TEAC) ^b	DPPH (% Inhibition) ^c	Superoxide (% Inhibition) ^d	Microsomes (IC ₅₀ , µM) ^e	LDL (Lag Time, h) ^f	Rancimat (Lag Time, h) ^g
Aspalathin	3.33	2.62	91.74 (87.62)	81.01	50.2	6.2	2.55
Notthofagin	4.04	2.06	-	-	1388	4.3	-
Orientin	11.43	1.47	-(88.65)	72.52	137.9	2.7	-
Isoorientin	11.25	1.54	-(82.18)	63.32	480.7	3.8	-
Vitexin	> 2313	0.86	-(3.99)	10.15	> 2323	-	-
Isovitexin	1224	0.81	-	-	1689	-	-
Luteolin	10.82	-	90.85 (88.01)	57.83	185.9	-	-
Chrysoeriol	21.54	-	-(2.02)	32.93	217.7	-	-
Rutin	10.47	1.2	91.18 (66.75)	68.16	240.1	-	-
Isoquercitrin	12.89	1.23	91.99 (86.59)	66.67	111.3	9.6	4.17
Hyperoside	8.55	1.33	-	-	283.2	-	-
Quercetin	3.6	2.7	93.27 (91.11)	81.45	17.5		26.93
Procyanidin B3	-	-	-(90.16)	-	53.3	-	27.23
Caffeic acid	-	-	93.65 (-)	-	-	-	18.85
Ferulic acid	-	-	-	-	-	-	1.26
p-coumaric acid	-	-	-(58.10)	5.31	-	-	1.08
Vanilic acid	-	-	20.66* (-)	-	-	-	-

^aSnijman *et al.*, (2009), ^bKrafczyk *et al.*, (2009), ^c% inhibition at 0.25 mol compound/mol DPPH Von Gadow *et al.*, (1997) and Joubert *et al.*, (2004),

^d% inhibition of 12.5 µmol/mL, Joubert *et al.*, (2004), ^eiron-induced microsomal lipid peroxidation, Snijman *et al.*, (2009), ^fcopper-induced low –density lipoprotein oxidation at 1 nmol, Krafczyk *et al.*, (2009), ^goxidation of lard at 0.02 µg compound/100µg lard, Von Gadow *et al.*, (1997).

ABTS: 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid; DPPH: 1,1-diphenyl-2-picrylhydrazyl, IC₅₀: Concentration of sample needed to obtain 50% inhibition, LDL: Low-density lipoprotein, TEAC: Trolox equivalent antioxidant capacity.

2.2.4 **Cellular Effects of Rooibos Tea**

The protective effects of chronic intake of rooibos tea against the age-related brain changes have long been studied (Inanami *et al.*, 1995). The authors reported that the contents of the thiobarbituric acid (TBA) reactive substances (TBARS) present in the frontal cortex, occipital cortex, hippocampus and cerebellum of 24 months old rats pre-treated with rooibos tea for 21 months was insignificantly different when compared to 5 weeks old rats. Also, MRI images of the brains of the 24 months old rats pre-treated with rooibos tea were similar to those of 5-week old rats. However in aged rats without rooibos pre-treatment, the results showed significantly higher content of TBARS and a decrease in the signal intensity of the MRI imaging at the same brain regions of the 24 months old rats pre-treated with rooibos. Based on these observations, Inanami and colleagues concluded that chronic rooibos tea administration could prevent age-related accumulation of lipid peroxidases in several regions of rat brain.

The anti-inflammatory effects of unfermented rooibos tea have also been investigated in colitis-induced with dextran sodium sulfate (DSS). Treatment with rooibos tea resulted in a significant increase and decrease in serum levels of superoxide dismutase (SOD) levels and urine levels of 8-hydroxy-2'-deoxyguanosine respectively in the rooibos group compared to the controls ($P < 0.05$ for both). The study showed that unfermented rooibos tea was able to modulate serum levels of SOD which remained significantly higher in the rooibos group when compared to the controls after induction of colitis. This *in vivo* study showed that rooibos tea may prevent DNA damage and inflammation by its anti-oxidative activity (Baba *et al.*, 2009).

The antimutagenic properties of fermented and unfermented rooibos tea in preventing the transformation of a mutagenic compound into a mutagen have also been investigated with the *Salmonella typhimurium* mutagenicity assay. Briefly, aqueous extracts of fermented and unfermented rooibos tea and honeybush tea showed antimutagenic activity against 2-

acetylaminofluorene (2-AAF) and aflatoxin B(1) (AFB(1))-induced mutagenesis after using tester strains TA98 and TA100 in the presence of metabolic activation (Van der Merwe *et al.*, 2006).

Researchers at the Institute of Animal Biochemistry and Genetics (Slovak Republic) reported the possible hepatoprotective effects of rooibos tea following the treatment of rats with carbon tetrachloride (CCl₄). Rooibos tea significantly inhibited the increase of tissue malondialdehyde, triacylglycerols, cholesterol and plasma activities of aminotransferases (ALT, AST), alkaline phosphatase and bilirubin concentrations which are known markers of liver diseases. Simultaneously, the anti-fibrotic effects of rooibos tea as indicated by histological regression of steatosis and cirrhosis of the liver were reported (Ulicná *et al.*, 2003).

In humans, the effects of fermented rooibos herbal tea on biochemical and oxidative stress parameters in adults at risk of cardiovascular diseases was studied in 40 volunteers who drank six cups of fermented rooibos herbal tea daily for 6 weeks, followed by a control period. The results from the study showed that consumption of fermented rooibos herbal tea significantly increased total plasma polyphenol levels when compared to controls (from 79.8 ± 16.9 mg/L to 89.8 ± 14.1 mg/L) (Marnewick *et al.*, 2011). These findings were corroborated by further studies by same authors which showed improved lipid profiles (serum LDL-cholesterol, triacylglycerol, and HDL-cholesterol), improved redox status (total glutathione - tGSH, ratio of reduced to oxidized glutathione – GSH: GSSG) and also reduced levels of lipid peroxidation markers (conjugated dienes - CDs, thiobarbituric acid reactive substances - TBARS) when compared with controls. Lipid profiles as well as redox status are both relevant in adults at risk of developing cardiovascular diseases (Marnewick *et al.*, 2011).

CHAPTER THREE

MATERIALS AND METHOD

3.0 INTRODUCTION

This chapter summarizes how the study was carried out. It is divided into sections that explain each experimental procedure (e.g. the animal model, experimental design, protocol for collecting data, data analysis, etc.), presented in detail to allow for reproducibility of the experimental procedures.

3.1 ETHICAL CONSIDERATION

Ethical guidelines as specified by the faculty board research and ethics committee and by the senate research committee of the University of the Western Cape, Cape Town, South Africa were followed. Ethical registration and project registration numbers; 13/10/94 and ScR1Rc2013/07/18 were subsequently assigned to the research project before commencement.

3.2 MATERIALS AND DRUGS

The materials and drugs used in this study are tabulated on Table 3.1 and 3.2:

Table 3.1: Materials used in these study

Product	Supplier
Wound auto closing system	Kent Scientific (USA)
4-0 Silk Suture	Kent Scientific (USA)
Heating Pad	Docol corporation (USA)
Retractors	Kent Scientific (USA)
CODA blood pressure monitor	Kent Scientific (USA)

Table 3.2: Drugs used in this study

Product	Use	Supplier
Isoflurane	Anesthesia	Safeline Pharmaceuticals Ltd (South Africa).
Meloxicam	Analgesic	Norpharm Medical (South Africa)
Sodium Pentobarbital	Anesthesia	Norpharm Medical (South Africa)

3.3 PROCUREMENT OF ROOIBOS HERBAL TEA

The fermented rooibos (*Aspalathus linearis*) used in this study was a generous gift from Rooibos Ltd (Clanwilliam, South Africa) to the research laboratory of Prof. Thomas Moonses at the Department of Medical Biosciences, University of the Western Cape, Bellville, Cape Town, South Africa.

3.4 DAILY PREPARATION OF FERMENTED ROOIBOS TEA

A concentration of 2g / 100ml of fermented rooibos herbal tea was used throughout this study (Marnewick *et al.*, 2003; Panti *et al.*, 2011) as these concentrations have been reported to be routine for tea-making purposes (Marnewick *et al.*, 2003). Briefly, 1000 ml of freshly boiled tap water was added to 20g of fermented rooibos herbal tea leaves and stems. The infusion was allowed to stand for 5 minutes after which it was filtered using a piece of cheese cloth and Whatman's filter paper (number 4). The aqueous extract was then allowed to stand at room temperature. Each day, fermented rooibos herbal tea was prepared freshly before being fed to experimental rats *ad libitum* (Opuwari and Monsees, 2014).

3.5 ACCLIMATIZATION AND CARE OF ANIMALS

Fifty (50) healthy male wistar rats with an average weight of 250 g were procured from the University of Stellenbosch animal facility, Cape Town, South Africa and maintained at the Animal House of the Department of Medical Bioscience, University of the Western Cape,

Bellville, Cape Town, South Africa, under standard laboratory conditions of temperature ($25 \pm 2^{\circ}\text{C}$), humidity ($50 \pm 15\%$) and light period (12 h light dark cycle) for an acclimatization period of two weeks. The rats were fed freely on standard rat chow and tap water *ad libitum*. They received humane care in accordance with the Guide for the Care and Use of Laboratory Animals 8th edition (www.nap.edu).

3.6 EXPERIMENTAL DESIGN AND GROUPING

In this study, a total of 50 adult experimental rats were randomly separated into 4 main groups which consisted of a Control-sham group (15 animals), Rooibos-sham group (5 animals), Rooibos + ischemia group (15 animals) and Ischemia group (15 animals). Animals in these respective groups were used for the assessment of brain oedema, relative brain weight, blood brain barrier (BBB) Integrity, histological and immunohistochemistry (IHC) assessment of apoptosis and neurochemical studies (NS) of lipid peroxidation, oxygen radical antioxidant capacity assay (ORAC) and ferric reducing antioxidant power. All rats in the Control-sham and Ischemia groups received food and tap water *ad libitum* throughout the study while rats in the Rooibos-sham and Rooibos + ischemia groups had daily access to food and Fermented Rooibos Herbal Tea (FRHT) *ad libitum* for 7 weeks prior to BCCAO and 24 hours to 7 days post-BCCAO depending on the experimental protocol (See table 3.3).

Table 3.3: Experimental design and grouping

Experimental procedure	n = Total Number of Animals / Group			
	Control-sham (n= 15)	Rooibos-sham (n= 5)	Rooibos + Ischemia (n= 15)	Ischemia (n= 15)
Brain Oedema	5	-	5	5
BBB Integrity	5	-	5	5
IHC and NS	5	5	5	5

3.7 BODY WEIGHT MEASUREMENT

Daily body weights of rats were measured using a weighing balance (ae-adam, Keynes, United Kingdom) and weight changes relative to the initial weight were determined. In addition,

relative organ weights (organ weight-to-body weight-ratios) were calculated at the end of the study.

3.8 ROOIBOS AND WATER INTAKE

Daily measurement of fermented rooibos herbal tea and water intake was done throughout the experimental period by subtracting the volume of the remaining fluid from the volume pre-determined 24 hours prior. A visual observation was used to ascertain that no major fluid (fermented rooibos herbal tea or water) leaked from water bottles.

3.9 BILATERAL COMMON CAROTID ARTERY OCCLUSION MODEL

Acclimatized adult male Wistar rats weighing 300 – 350 g were used. Cerebral ischemia was transiently induced for 20 minutes by bilateral occlusion of the right and left common carotid arteries (CCA) according to the method described by Xi *et al.*, (2014) (See figure 3.1).

3.9.1 **Animal preparation and surgery**

On the day of surgery, rats were transferred 1 hour before surgery from the animal holding room to the operating room, for animals to acclimatize to the operating environment. A pre-operative animal weight was then recorded before induction of anesthesia by weighing each animal in a weighing scale (ae-Adam, Keynes, United Kingdom). Rats were subsequently deeply anesthetized in an induction box with 3% Isoflurane in an oxygen-nitrous oxide mixture (30:70). The degree of anesthesia was ascertained by the absence of a withdrawal reflex after performing a toe pinch on the animal (Moon *et al.*, 2012). If the animal showed no reflex, the surgical procedure was continued. Each rat was placed on a heating pad in a supine position and allowed to breathe through a facemask. Isoflurane was adjusted to 1.5 – 2 % and administered continuously in the O₂/N₂O mixture at a flow rate of 1 L/min.

Aseptic surgical procedure was followed by shaving the fur on the ventral neck area of the rat and disinfecting the surgical work surface and surrounding fur with an alcohol pad spiraling

from the centre outwardly. Pre-emptive analgesia was also given to each rat by injection of a 2 mg/kg dose of Meloxicam injection subcutaneously to prevent post-surgical pain and distress. By making a 1 – 2 cm midline neck incision, the submandibular glands were separated to allow access to the underlying muscles covering the trachea. With a self-retraining retractor, the right sternomastoid muscle and the omohyoid muscle were retracted to expose the underlying right CCA which can be easily seen pulsating. With a careful blunt dissection, the right CCA was separated from its surrounding fascia and accompanying vagus nerve (See figure 3.1).

3.9.2 **Vascular occlusion procedure**

A 10 cm 4-0 silk suture was then around the right CCA in preparation for occlusion and the same surgical procedure carried out on the left side to expose the left CCA. Once the left CCA was separated from its fascia and vagus nerve, it was occluded by tying a 4-0 silk suture around it and the time of occlusion was recorded. The right CCA was also occluded by tightening the already prepared loop within the next 30 seconds. Complete occlusion of the CCAs was confirmed by visibly monitoring the CCA for swelling around the point of occlusion and the direction of flow of blood rostral to the occlusion. Rectal temperature was monitored with a digital thermometer using a rectal probe inserted to a depth of approximately 2 cm. Core temperature was controlled during and after surgery and maintained at about 37°C by a heating pad and Infrared Lamp. The animals were maintained under Isoflurane anesthesia throughout the occlusion period until the end of ischemia (20 minutes) when reperfusion was permitted by loosening and removing the silk sutures from both CCAs. Reperfusion was confirmed before closing the incision with a wound closing system. Animals were thereafter carefully placed in a recovery cage. Post-surgery, 2ml of sterile saline was injected subcutaneously along the scruff of the neck in order to reduce dehydration. In sham animals (Control-sham and Rooibos-sham), the CCA was surgically prepared for occlusion, but was occlusion did not take place (i.e., no ischemia was induced).

3.10 PHYSIOLOGICAL PARAMETERS

As previously described by Ord *et al.* (2012) and while under anesthesia, physiological parameters were measured prior to, during and after the occlusion of the right and left common carotid arteries. Systolic and diastolic blood pressure and heart rate were measured by the CODA non-invasive tail cuff blood pressure monitor (Kent Scientific, USA) which uses the volume pressure technology. Values from each animal were determined from the mean of a minimum of 3 separate pressure and heart rate measurements. Temperature was monitored using a rectal thermometer and was maintained at about 37°C using a heating pad and infrared heating lamp.

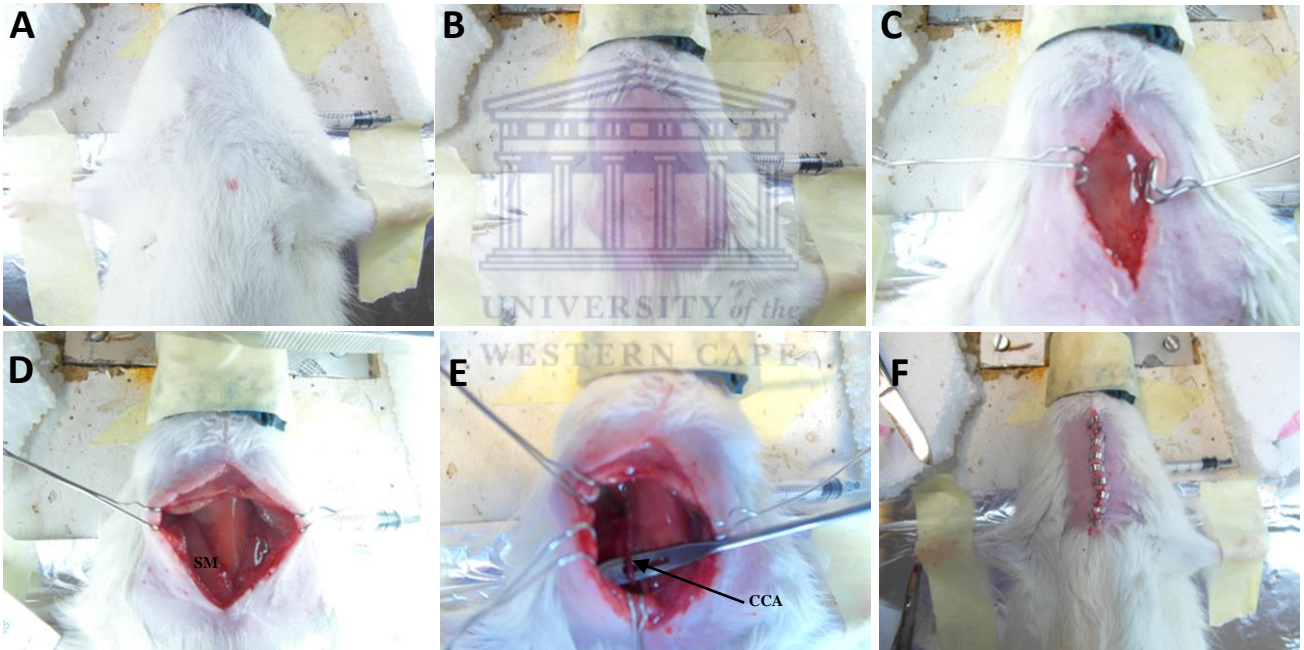


Figure 3.1: Bilateral common carotid artery occlusion (BCCAO) in rat. (A-D). (A) Each rat was placed in a supine position and the nostrils connected to an Isoflurane anesthetic system through a facemask. (B) The fur on the ventral neck area disinfected using an alcohol pad. (C-D) A 1-2 cm ventral midline incision exposed the facial covering the salivary glands. This was carefully dissected to expose the underlying musculature (Sternomastoid (SM) and Sternohyoid (SH) muscles). (E) Retraction of the SH and Omohyoid muscles allowed access to the common carotid artery which was occluded for 20 minutes and thereafter reperused. (E) The wound was closed with wound closing clips and the rat was put in a recovery cage and monitored.

3.11 BRAIN OEDEMA ASSESSMENT

Twenty four hours after reperfusion, rats were sacrificed under deep anesthesia (Sodium pentobarbital, 150 mg / bw i.p) and decapitated. The brains were harvested and the cerebellum, pons, and olfactory bulbs removed and weighed immediately (wet weight (WW)). Brain sections were then placed in an oven (Memmert, Germany), dehydrated at 105°C for 48 h and reweighed (dry weight (DW)). Brain oedema was estimated as the difference in percentage of brain water and calculated with the formula below (Bigdeli *et al.*, 2007).

$$\text{Brain water content (BWC)} = [(WW - DW) / WW] \times 100.$$

3.12 BLOOD BRAIN BARRIER ASSESSMENT

Disruption of the blood-brain-barrier (BBB) integrity after an ischemic brain injury could be determined following extravasation of plasma content into the brain tissue (Klohs *et al.*, 2009). Four days after reperfusion, the integrity of the BBB was evaluated by studying Evans Blue (Sigma Aldrich, USA) extravasation into the brain parenchyma. Briefly, 4ml/kg of 2% EB solution in PBS was administered to each rat by tail vein injection an hour before sacrifice after which the thoracic cavity was opened under sodium pentobarbital anesthesia. The rats were perfused with cold PBS through the left ventricle to wash out the blood until a colorless perfusion fluid was obtained at the right atrium. The rats were then decapitated and the cerebral hemispheres removed and weighed. The brains were homogenized in 1:10 w/v PBS to extract the EB and an equal volume of 60% trichloroacetic acid (Sigma Aldrich, USA) was also added and mixed by vortex for 30 seconds to precipitate protein. The samples were then centrifuged at 1000×g for 30 min at 4°C. The amount of EB in the supernatants was measured at 610 nm using a POLARstar omega spectrophotometer (BMG Labtech, Ortenberg, Germany). Serial dilutions of EB in PBS were used to prepare a standard curve (see Appendix A for standard curve, page.105) and the result was expressed as µg/g brain tissue (Bigdeli *et al.*, 2007).

3.13 BRAIN TISSUE PREPARATION

Rats were sacrificed 24 h, 4 days and 7 days after BCCAO with an overdose of Sodium pentobarbital injection (150 mg/bw i.p). The skin over the abdomen of deeply anesthetized rat was lifted up with a forceps and cut open to expose the thoracic cavity and the rib-cage cut and retracted to expose the beating heart (Zhang *et al.*, 2012). Once cleared of connective tissue, the heart was held with a forceps and a blunt-ended 16 gauge needle attached to a perfusion fixation set-up was inserted to the base of the heart through the left ventricle and directed towards the aorta. The needle was secured in place with a clamp and the rat perfused transcardially with a 300 ml of cold Phosphate buffered saline (PBS). A pale color of the liver was indicative of a successful perfusion after which, the rats were decapitated and the brains removed, weighed and bisected along the mid-sagittal plane. The right hemisphere was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h for histological and immunohistochemical analysis. The left hemisphere was also quickly immersed in 1:10 w/v 0.1M cold PBS solution and stored at -80°C to be used for biochemical analyses (Zhang *et al.*, 2012).

3.13.1 **Tissue processing**

After fixation of tissues, brains were processed in a Leica-2125 automatic tissue processor (Leica, Germany) to prepare specimen for sectioning, staining and analysis. This standard process consisted of a series of steps which included dehydration (passing tissues through different changes of ethanol to remove water), clearing (the process of removing alcohol) and finally infiltration of tissue with molten paraffin wax. The tissue processing was completed in 7 hours cycle (see table 3.4 below). After which brain tissue were removed from the last change of infiltrating wax and embedded in liquid paraffin wax. After cooling, embedded blocks were sectioned (at 5 microns per section) using a Leica TP-1020 microtome (Leica, Germany) and mounted unto glass slides.

Table 3.4: Tissue processing procedure

Station	Solution	Temperature	Time
1	70 % Alcohol	40°C	30 minutes
2	80 % Alcohol	40°C	30 minutes
3	95 % Alcohol	40°C	45 minutes
4	95 % Alcohol	40°C	45 minutes
5	100% Alcohol	40°C	45 minutes
6	100% Alcohol	40°C	45 minutes
7	Xylene 2	40°C	45 minutes
8	Xylene 3	40°C	45 minutes
9	Paraffin wax 1	58°C	30 minutes
10	Paraffin wax 2	58°C	30 minutes
11	Paraffin wax 3	58°C	30 minutes

3.13.2 **Hematoxylin and Eosin staining**

Glass slides holding the brain sections were stained with hematoxylin and eosin (Sigma Aldrich, USA) to assess for histopathological changes in the hippocampus of the brain. The glass slides were placed on a staining rack and de-paraffinized in 3 changes of xylene. The sections were then rehydrated by immersing the slides in 2 changes of 100% ethanol for 2 minutes followed by 95% and 70% ethanol for 2 minutes each. Thereafter the slides were rinsed in running tap water for at least 2 minutes before staining in hematoxylin for 3 minutes. The slides were then placed in running water at room temperature for 2-3 minutes, differentiated in acid alcohol for 2-3 seconds before being immersed in running water again. Slides were counter stained in eosin for 2 minutes and dehydrated in 3 different 100 % alcohol

baths for 2 minutes each and cleared in 3 changes of xylene at 2 minutes each. Coverslips were then mounted on the slides using DPX mounting medium (Cardiff *et al.*, 2014).

3.13.3 **Cresyl Violet / Nissl staining**

The cresyl violet (CV) staining is a widely used technique to examine brain cytoarchitecture as it provides detailed information about the perikaryon of neurons in comparison to the simple rendition of shape and size of cell bodies provided by hematoxylin and eosin staining (Li, 2012). Briefly, Slides were hydrated in serial concentrations from absolute ethanol to tap water and incubated in 0.5% cresyl violet solution (Sigma Aldrich, USA) for 2 min. Slides were quickly washed in distilled water and differentiated in 95% ethanol and then in two changes of absolute ethanol for 5 minutes. Thereafter, slides were cleared in two changes of xylene for 5 minutes and mounted in a mounting medium.

3.14 IMMUNOHISTOCHEMICAL STUDIES OF APOPTOSIS

Immunohistochemical studies of post-ischemic neurons are valuable for the assessment of the severity of morphological and cytochemical changes in neurons following cerebral ischemia. As described earlier, brains were collected 7 days after BCCAO reperfusion were cut sagittally at 5 microns using a Leica TP-1020 microtome (Leica, Germany). A Terminal dUTP Nick-End Labeling (TUNEL) Assay was subsequently conducted using the in Situ DNA Fragmentation Assay Kit (BioVision, U.S.A.) following manufacturer's instruction described below.

3.14.1 **Deparaffinization and Rehydration**

1. Glass slides holding brain sections were deparaffinized in two changes of freshly prepared xylene at room temperature for 5 minutes.
2. Slides were immersed in two changes of 100% ethanol for 5 minutes at room temperature (RT).
3. Slides were then immersed in graded changes of 90%, 80% and 70% ethanol for 3 minutes at RT.
4. Slides were immersed into 1X PBS and the slides carefully dried carefully.

3.14.2 **Permeabilization, Inactivation of Endogenous Peroxidase and Equilibration.**

Slides were incubated with 100µl proteinase K for 20 minutes and endogenous peroxidases inactivated by 3% hydrogen peroxide in methanol for 5 minutes. Slides were then incubated with 100 µl reaction buffer for 10 minutes at room temperature.

3.14.3 **End Labeling Reaction, Detection and Counterstain**

Slides were incubated with 50 µl of complete labeling reaction mixture containing deoxynucleotidyl transferase (TdT) enzyme in a humidified chamber at 37 °C for 1 h, then a blocking buffer was applied for 30 min at 37 °C. The slides were then incubated with Anti-BrdU-Biotin antibody, and visualized with diaminobenzidine (DAB) substrate. The sections were counterstained with Methyl Green. In the TUNEL stained slides, 3 fields of each section were selected from the hippocampus and the TUNEL-positive cells were quantified by light microscopy at magnification (X400). The total cell numbers and TUNEL-positive cell numbers were obtained in each field. The percentage of TUNEL-positive cells was described as the percentage of the numbers of TUNEL-positive cells to the total numbers of cells in each field.

3.15 MORPHOLOGICAL AND MORPHOMETRIC STUDIES

Since 15 minutes of global cerebral ischemia induces delayed neuronal death selectively in about 80% of CA1 pyramidal neurons, and about 10% in neocortical neurons (Raval and Hu, 2012), morphological and morphometric analysis was carried out on the H&E, cresyl violet and TUNEL -stained cornus ammonis 1 (CA1) region of the hippocampus on images captured at a magnification of x400 using the Zeiss Primo Vert microscope (Zeiss, Germany). In order to cover the area of interest at 400x, 3 captured images were used with each measuring 1159.4 x 869.57 μm . Viable pyramidal neurons of the hippocampus were identified as those exhibiting clear purple cytoplasmic staining with visible nuclei and nucleoli, while ischemic (dead) cells were identified as showing shrunken perikarya, triangular shapes and mostly exhibiting dark-stained nuclei. The data obtained was expressed as percentages of TUNEL-positive cells in total number of cells. Quantification of viable and TUNEL positive cells was done using the NIH Image analysis software (Image J) (Onken *et al.*, 2012).

3.16 NEUROCHEMICAL ASSAYS

3.16.1 Homogenization of tissues

Brain tissues stored at -80°C were thawed and homogenized (IKA Laboratories, Germany) in 10 times (w/v) 0.1M PBS (pH 7.4) in a Teflon glass homogenizer for two periods of 10 seconds each. The homogenate was then centrifuged at 15,000 rpm in a microcentrifuge at 4°C for 10 minutes. The supernatant was collected and transferred into newly marked Eppendorf tubes for different biochemical index (Ahmed *et al.*, 2014).

3.16.2 Lipid peroxidation assay

Assessment of lipid peroxidation (LPO) in the left hemispheric brain was done according to the method described by Wills (1966). This method depends on the formation of lipid peroxidation end product; malondialdehyde (MDA) which reacts with thiobarbituric acid (TBA) to produce

a thiobarbituric acid reactive substance (TBARS) a pink chromogen which can be measured spectrophotometrically at 532 nm. Briefly, 100 µl of supernatant was collected into new 2 ml Eppendorf tubes and 12.50 µl of cold ethanol and 100 µl of 0.2M ortho-phosphoric acid added. The mixture was vortexed for 10 seconds before 12.50 µl of 0.67% TBA (Sigma Aldrich, USA) was added. The reaction mixture was then heated at 90°C for 45 minutes in a water bath (Electrothermal, England). After cooling on ice for 2 minutes and at room temperature for 5 minutes, 1000 µl of n-butanol and 100 µl of saturated sodium chloride (NaCl) were added. The mixture was vortexed and centrifuged at 12,000 rpm at 4°C for 2 minutes after which 300 µl of the top n-butanol phase was collected and used for spectrophotometric measurement at 532 nm. The results were expressed as µmol of MDA per g of wet brain tissue.

3.16.3 **Oxygen radical absorbance capacity (ORAC) assay**

The ORAC assay is one of the most accepted methods for measuring the activity and amount of antioxidants present in biological samples (Cao *et al.*, 1993). The assay works on the principle of adding a sample to a free radical generating system and measuring the degree of protection offered by the sample in preventing free radical damage against a fluorescent molecule.

Briefly, 2,2'-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH) and fluorescein (Sigma Aldrich, USA) were used as the free radical producing system and fluorescent molecule respectively. The oxidation of fluorescein was measured by initiating a reaction following the addition of 50 µl of AAPH to a mixture of 138 µl of fluorescein and 12 µl of sample in a 96-well black plate and the fluorescence read for 2 hours at every 5 minutes interval at an emission and excitation wavelength of 530 nm and 485 nm using a Fluoroskan Ascent fluorescent plate reader (Thermo Fisher Scientific, Waltham, MA, USA). A standard curve was prepared from a 500 µM stock solution of Trolox, an artificial Vitamin E (see Appendix B for standard curve) (Prior *et al.*, 2003). The results obtained were expressed as µM Trolox equivalent (TE) / g of wet brain tissue.

3.16.4 **Ferric reducing antioxidant power (FRAP) assay**

Another assay used for the determination of antioxidant capacity in biological samples is the FRAP assay which uses an oxidation/reduction reaction to measure the ability of antioxidants in a sample to reduce ferric tripyridyltriazine (Fe^{3+} - TPTZ) to a ferrous form (Fe^{2+}) which has an intense blue color which can be monitored by a spectrophotometer (Ndhlala *et al.*, 2010). Briefly, a mixture of 30 ml acetate buffer (300 mM, pH 3.6), 3 ml TPTZ (10 mM in 100 mM HCl), and 3 ml $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (20 mM) was used to prepare the FRAP reagent, from which 300 μl was added to 10 μl of the sample in a clear 96-well plate using a multi-channel pipette. The mixture was then incubated in the incubating oven (Mettler, Germany) at 37°C for 30 minutes and the read at a wavelength of 593 nm in a Multiskan Spectrum automated plate reader (Thermo Fisher Scientific, Waltham, USA). A serial dilution was prepared from a stock solution of Ascorbic acid for the preparation of a standard curve (see Appendix C for standard curve) and expressed as FRAP mg per g of wet brain tissue (Vakili *et al.*, 2014).

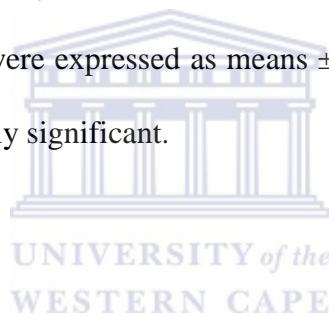
3.17 OPEN FIELD NEUROBEHAVIOURAL TEST

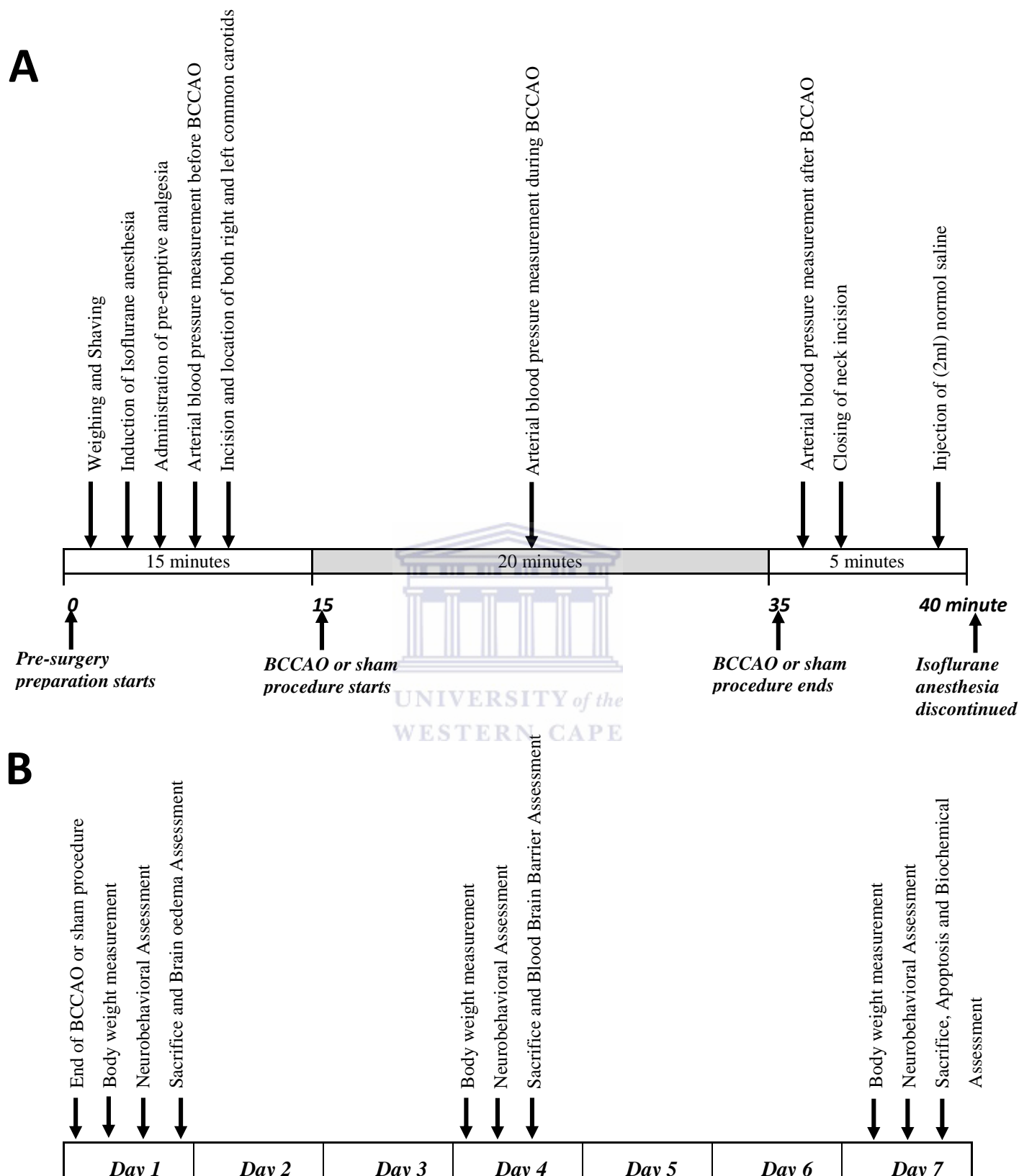
The open field (OF) test is a commonly used neurobehavioral assessment tool that provides simultaneous measurement of locomotion and anxiety in laboratory animals (Kendigelen *et al.*, 2012). The apparatus for the OF assessment involved a square plexi glass box ($72 \times 72 \times 20$ cm), with a digital camera (Samsung HMX-F90, South Korea) mounted directly above it. The open-field arena was divided into 16 equal squares, via a 4×4 grid, to assist in data analysis and animals were tested singly. Briefly, animals were transported from the housing room to the testing room and allowed to acclimatize prior to testing. Testing began by removing each rat from its home cage and placing it in the centre zone of the OF arena. Each session lasted 10 minutes in a single run after which the rat was returned into its home cage and the OF box cleaned with 70% ethanol before testing the next rat. The Smart video tracking software

version 3.0, from Panlab Harvard Apparatus (Massachusetts, USA) was used to measure the locomotor activity of each experimental rat by extracting the total distance traveled in the OF arena. As a measure of anxiety, the total distance traveled in the 12 squares near the walls was compared with the distance traveled in the 4 squares at the centre of the arena. All analysis was done by “blind” observers.

3.18 STATISTICAL ANALYSIS

Results were compared using one-way analysis of variance (ANOVA) test. If a statistically significant difference was obtained, Tukey’s post hoc test was conducted for further comparison among groups. A two-way ANOVA followed by Fisher’s protected least significance difference (Post hoc LSD) test was used for analysis of relative brain weights and open field measurements. Values were expressed as means \pm standard error of mean (SEM). $P < 0.05$ was considered as statistically significant.





CHAPTER FOUR

RESULTS

4.0 INTRODUCTION

In this study, ischemic brain injury was induced in 30 adult male wistar rats (15 pre-treated with fermented rooibos herbal tea and 15 non-treated) by the transient occlusion of the right and left common carotid arteries for 20 minutes. Outcomes from the ischemic group were compared with rats in the control and rooibos sham groups, 24 hours to 7 days post BCCAO. This study was done to evaluate the potential of a 7 weeks administration of fermented rooibos herbal tea in preventing or reducing the severity of ischemic injury to rat brains. Findings from this study are presented below:

4.1 AVERAGE DAILY INTAKE OF FLUID AND FERMENTED ROOIBOS HERBAL TEA

A - Before BCCAO

Throughout the experiment, rats were allowed free access to tap water and fermented rooibos herbal tea *ad libitum*. Recorded values for water and rooibos intake showed that animals in the treatment groups “rooibos sham” and “rooibos + ischemia” significantly drank less fluid (fermented rooibos tea) during the first week of the experiment when compared to the “control sham” and “ischemia groups” which drank tap water. This difference was seen to disappear from the 2nd week onward as rats became familiar with the fluid (fermented rooibos herbal tea). At the end of the 7th week, no significant difference was found in the overall amount of fluid intake across the different experimental groups (Table 4.1).

B - Post BCCAO

Animals continued to receive fermented rooibos herbal tea *ad libitum* after BCCAO or sham surgery. There was however no evaluation of fluid intake post-surgery as animals were kept for only 7 days before sacrifice.

Experimental Groups	Mean Fluid intake (MFI) over 7 weeks period (ml/day/animal)							Average MFI (ml/day/animal)
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	
Control sham (n=15)	46.0 ± 2.5	47.6 ± 7.8	39.0 ± 2.9	38.0 ± 1.8	33.9 ± 1.9	35.1 ± 3.2	40.1 ± 3.8	40.0 ± 2.0
Rooibos Sham (n=5)	30.8 ± 1.2 (a, b)	26.6 ± 1.1 (a)	33.9 ± 3.0	39.0 ± 3.1	35.9 ± 1.3	37.8 ± 1.9	38.9 ± 1	34.7 ± 1.8
Rooibos + Ischemia (n=15)	33.5 ± 0.7 (a, b)	34.1 ± 1.2 (a, b)	35.2 ± 1.1	35.2 ± 5.3	35.2 ± 1.1	39.5 ± 1.4	36.6 ± 1.3	36.07 ± 1.0
Ischemia (n=15)	44.3 ± 3.2	42.7 ± 2.5	40.2 ± 2.3	40.4 ± 3.2	35.0 ± 2.9	36.6 ± 2.4	38.8 ± 2.1	39.7 ± 1.2

Table 4.1: Average daily intake of fluid (ml/day/animal) over 7 weeks in experimental rats. Fermented rooibos herbal tea was given to experimental rats ad libitum, the amount of rooibos intake in the rooibos sham and rooibos + ischemia group was significantly different to the control sham and ischemia group (given tap water ad libitum) at week 1 and 2. This difference was not observed over the remaining weeks and the overall (in average) intake of fluid during the entire experiment did not differ among groups. ^aP < 0.001 when compared with Control sham group, ^bP < 0.01 when compared with the ischemia group. Data expressed as Mean ± SEM.

4.2 EFFECT OF FERMENTED ROOIBOS HERBAL TEA ON POST SURGERY

BODY WEIGHT CHANGES

Following the experimental protocol, rats were sacrificed on different days after BCCAO or sham surgery. Post-BCCAO body weight changes were monitored on day 1, 4 and 7 in rats belonging to the brain oedema, BBB and IHC/LPO sub-groups respectively. Table 4.2 below shows that loss in body weight across all groups on days 1, 4 and 7 was not statistically significant when compared to the pre-surgery weights except in the ischemia group on post-surgery day 4 when body weight was significantly lower ($p < 0.01$) when compared to the pre surgery weight.

PRE-SURGERY WEIGHTS (g) (Day 0)				
DAYS	SUB – GROUPS	C – SHAM	R + I	ISCHEMIA
DAY 0	B.E	389 ± 14.04	378.8 ± 8.31	359 ± 13.14
	BBB	302 ± 2.36	371.6 ± 9.41	409 ± 3.61
	IHC / LPO	365.8 ± 12.75	367 ± 26.82	399 ± 12.99
VERSUS POST-SURGERY WEIGHTS (g) (Day 1 – 7)				
DAY 1	B.E	375 ± 11.91	364 ± 6.93	338 ± 13.43
DAY 4	BBB	296 ± 2.35	360 ± 3.67	$384 \pm 4.56^*$
DAY 7	IHC / LPO	364 ± 12.19	357 ± 26.89	374 ± 11.35

Table 4.2: Body weight changes after bilateral common carotid artery occlusion and sham surgery. There was an immediate body weight loss in all animals that underwent surgery on post-surgery day 1. This loss in body weight was however not significant on post-surgery days 1, 4 and 7 except in the ischemia group on post-surgery day 4. Astericks () indicate significant difference when compared with the pre-surgery weights. C – SHAM: Control sham, R + I: rooibos + ischemia. N = 5, Data is presented as mean \pm S.E.M.*

4.3 EFFECTS OF FERMENTED ROOIBOS HERBAL TEA ON RELATIVE BRAIN WEIGHT

In this study, the neuroprotective effects of FRHT on post-BCCAO relative organ weight was studied on days 1, 4 and 7, following 7 weeks intake of fermented rooibos herbal tea (FRHT). Results show that FRHT did prevent against a significant decrease in relative organ weight in the rooibos + ischemia group when compared to the control group. In contrast, the brain / body weight ratios of rats in the ischemia group were significantly reduced only on day 4 after 20 minutes BCCAO

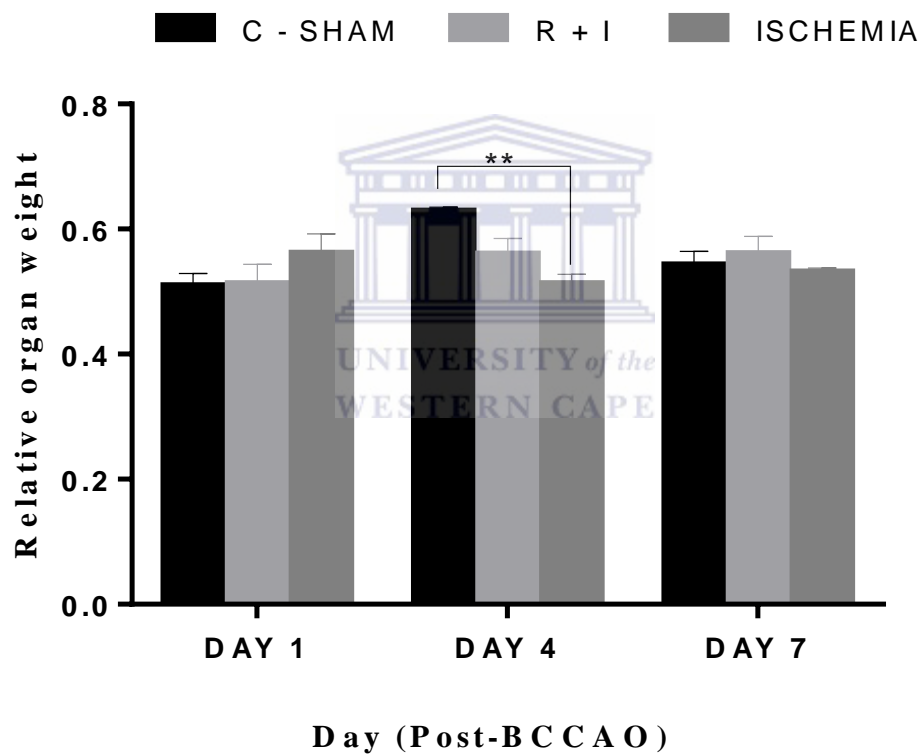


Figure 4.1: Changes in relative brain weights of rats. At different time point, the brain / body weight ratio of experimental rats was assessed after 20 minutes of bilateral common carotid artery occlusion or sham surgery. While the brain volume was mostly insignificantly different across all groups and duration of the experiment post BCCAO, the ischemia group at 4 days after 20 minutes BCCAO presented a reduced brain / body weight ratio. C – SHAM: control sham, R + I: rooibos + ischemia, BCCAO: Bilateral common carotid occlusion. N = 5 **P < 0.01 versus control sham group. Data is presented as mean \pm S.E.M.

4.4 PHYSIOLOGICAL PARAMETERS

Physiological parameters such as systolic blood pressure (SBD), diastolic blood pressure (DBP), mean arterial blood pressure (MAP) and heart rate (HR) were carefully measured before, during and after BCCAO and sham surgery. The recorded values were compared among all groups (see table 4.3) and the result showed no difference.

Physiological Parameter	Group	(N)	Before Occlusion	During Occlusion	After Occlusion
SBP (mmHg)	Control sham	15	108 ± 5.1	N/A	106 ± 6.5
	Rooibos sham	5	96 ± 4.7	N/A	93 ± 7.3
	Rooibos + Ischemia	15	110 ± 3.2	134 ± 3.5	114 ± 3.1
	Ischemia	15	110.4 ± 6.9	127.3 ± 6.5	110 ± 4.2
DBP (mmHg)	Control sham	15	82 ± 4.0	N/A	80 ± 4.3
	Rooibos sham	5	74 ± 3.9	N/A	75 ± 6.1
	Rooibos + Ischemia	15	82 ± 4.3	110 ± 3.4	82 ± 2.6
	Ischemia	15	80.2 ± 4.7	99 ± 5.1	82 ± 3.7
MAP (mmHg)	Control sham	15	90.7 ± 2.4	N/A	88.7 ± 3.2
	Rooibos sham	5	81 ± 3.2	N/A	81 ± 4.6
	Rooibos + Ischemia	15	91.3 ± 1.6	118 ± 3.6	92.7 ± 1.3
	Ischemia	15	90.3 ± 2.3	108.4 ± 6.7	91.3 ± 1.4
HR (beat/min)	Control sham	15	371 ± 6.8	N/A	400 ± 5.8
	Rooibos sham	5	389 ± 10.2	N/A	412 ± 9.1
	Rooibos + Ischemia	15	374 ± 8.0	395 ± 7.4	425 ± 10.4
	Ischemia	15	383 ± 7.1	419 ± 17.5	442 ± 13.3

Table 4.3: Physiological parameters measured before, during and after BCCAO when the animals were underneath isoflurane anesthesia. Measurements were not statistically different across groups ($P > 0.05$). NA: Not Applicable, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, MAP: Mean arterial blood pressure, HR: Heart rate. Data is presented as mean ± S.E.M.

4.5 BRAIN OEDEMA ASSESSMENT

Twenty four hours after reperfusion, the water content of the brain was assessed to evaluate brain oedema following BCCAO. As shown in figure 4.2, hemispheric brain water content was significantly higher ($p < 0.05$) in the ischemia group ($77.80 \% \pm 0.27$) when compared with the control-sham group ($76.68 \% \pm 0.36$). Interestingly, no significant increase in brain water content was observed following pre-consumption of FRHT for 7 weeks as the percentage increase of brain water in the rooibos + ischemia group was not significantly different to the control-sham group ($77.17 \% \pm 0.11$ and $76.68 \% \pm 0.36$, respectively).

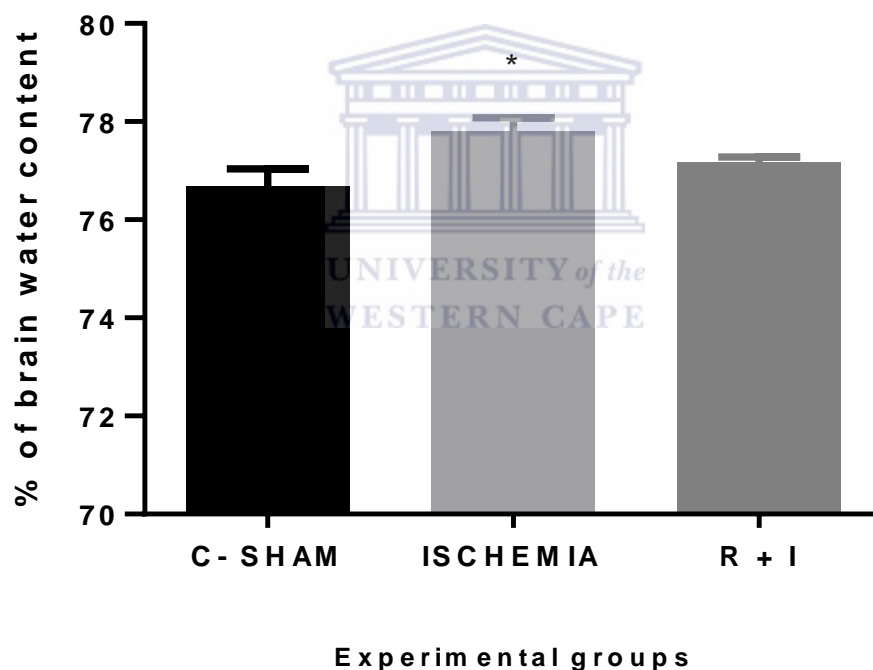


Figure 4.2: Effects of 7 weeks pretreatment with fermented rooibos herbal tea on brain oedema in rats after 24 hours of BCCAO. Brain water content in the R+I group was not significantly greater than control sham value. BCCAO: Bilateral common carotid artery occlusion; C- SHAM: Control sham; R + I: Rooibos + Ischemia. Asterisk (*) = Significant change. $N = 5$, Data is presented as mean \pm S.E.M, $*P < 0.05$.

4.6 BLOOD BRAIN BARRIER ASSESSMENT

Assessment of blood-brain-barrier integrity after 20 minutes BCCAO was done by studying the extravasation of injected Evans blue solution into the brain tissue 4 days after BCCAO. As shown in figure 4.3, BCCAO with 4 days of reperfusion appeared to compromise BBB integrity in the ischemia group was higher in the content of Evans blue in the brain from baseline level in the control sham group ($0.6880 \mu\text{g/g} \pm 0.22$ to $0.9189 \mu\text{g/g} \pm 0.34$); however this difference was not statistically significant. On the other hand, it does appear that 7 weeks pretreatment with fermented rooibos herbal tea did ameliorate the impairment of BBB integrity as Evans blue extravasation values were similar to the control sham group ($0.7341 \mu\text{g/g} \pm 0.25$ and $0.6880 \mu\text{g/g} \pm 0.22$).

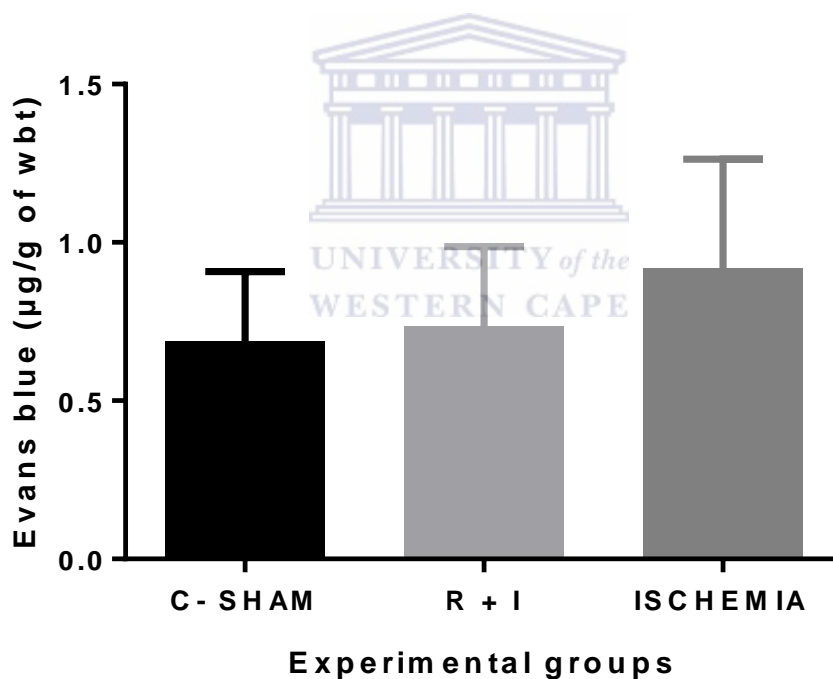


Figure 4.3: Blood brain barrier integrity assessment by Evans blue extravasation into the brain substance 4 days post BCCAO and sham surgery. Twenty minutes of BCCAO did compromise the integrity of the blood brain barrier by increasing the extravasation of Evans blue into the brain parenchyma. However this increase was not statistically significant. wbt = Wet brain tissue.

4.7 HISTOLOGICAL STUDIES

In this study, brain sections were stained with hematoxylin and eosin (H&E) and Cresyl violet stains to evaluate the neuroprotective potential of fermented rooibos herbal tea in preventing or reducing the severity of the delayed neuronal cell death associated with 20 minutes BCCAO in rats. The H&E staining procedure helps to demonstrate the shapes and sizes of cell bodies while the cresyl violet staining helps to give more detailed information about neuronal cell bodies.

4.7.1 **Hematoxylin and Eosin Staining**

Seven days after 20 min BCCAO or sham surgery, neuronal damage in the CA1 region of the hippocampus was evaluated by staining serial sagittal sections of the right cerebral hemispheres with hematoxylin and eosin stains. As shown in Figure 4.4 (B and D), photomicrographs from the control-sham and rooibos-sham groups presented no histopathological changes. In the ischemia group (Figure 4.4 E & F), marked neuronal ischemic damage was observed by cells exhibiting a triangular shape and a dark staining due to the condensation of cytoplasm and karyoplasm. These changes were less frequent in the rooibos + ischemia group (Figure 4.4 H).

4.7.2 **Cresyl Violet / Nissl Staining**

Representative photomicrographs of cresyl violet / Nissl staining of rat brain sections show neurodegenerative changes in the cytoarchitecture of the CA1 region of the hippocampus in the ischemia group at 7 days post-BCCAO. These changes were identified by the presence of shrunken and darkly stained neurons (Figure 4.5 F). Quantitative analysis (see Figure 4.8 B) of viable cells showed a significant decrease in the amount of viable hippocampal neurons in the CA1 region of rats in the ischemia group when values were compared with the control (Figure 4.5 B) and rooibos sham (Figure 4.5 D) groups. However, 7 weeks pretreatment of rats with FRHT (figure 4.5 H) did antagonize the pathogenesis of delayed neuronal death as the percentage of viable cells in the CA1 region of the hippocampus of the rooibos + ischemia group was similar to those in the control and rooibos sham groups (see Figure 4.7 A).

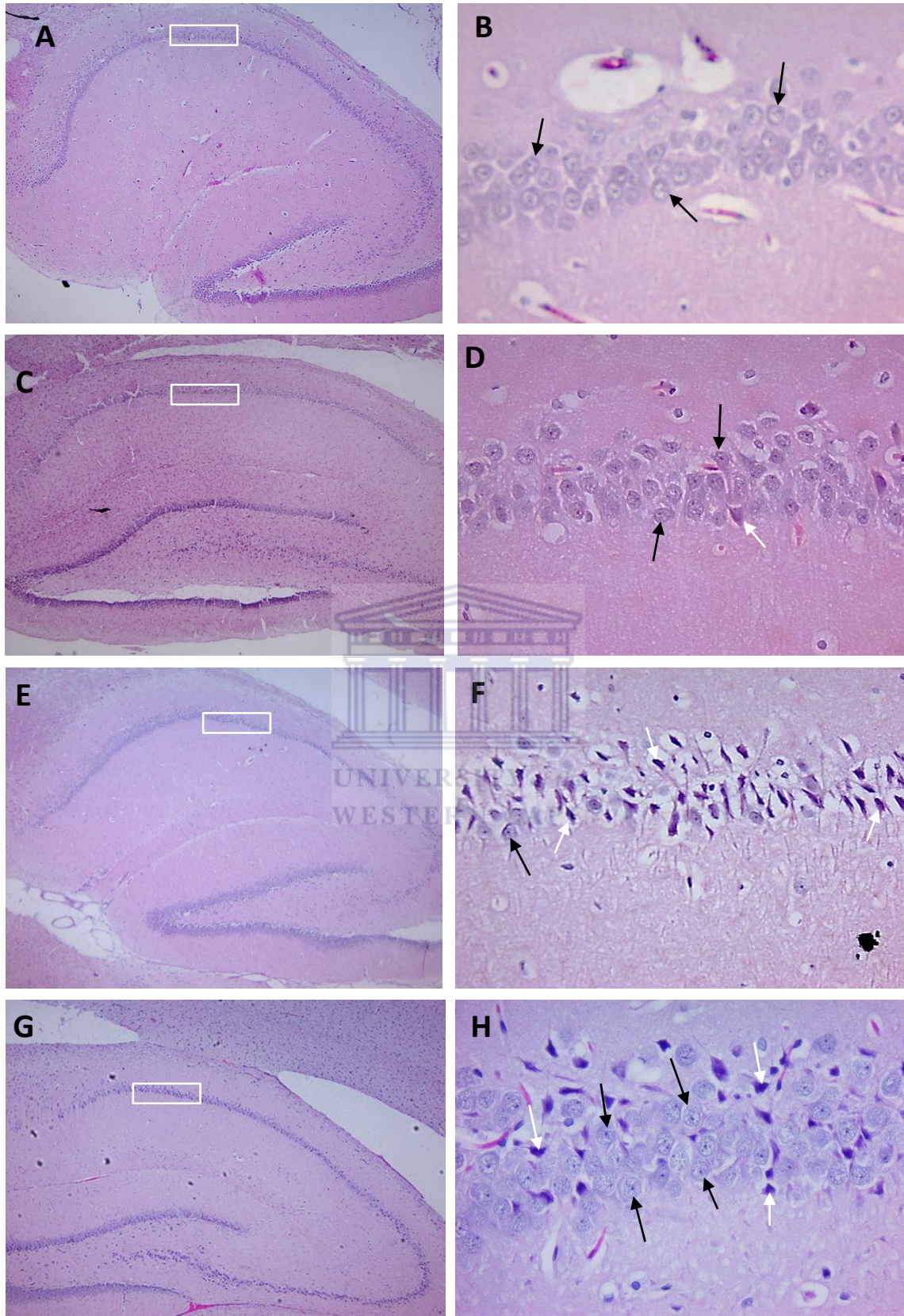


Figure 4.4: Photomicrographs of Hematoxylin and Eosin staining of hippocampal sections of rats 7 days after 20 minutes BCCAO or sham surgery. The left panel depicts the CA1 region displayed on the right panel. The ischemia group shows the presence of numerous shrunken CA1 pyramidal cells with condensed and deeply stained nuclei (white arrows in "F"). Following 7 weeks intake of FRHT, these effects were reduced (black arrows = normal neurons in "H"). A & B=Control sham; C & D=Rooibos sham; E & F=Ischemic and G & H=Rooibos + ischemia groups.

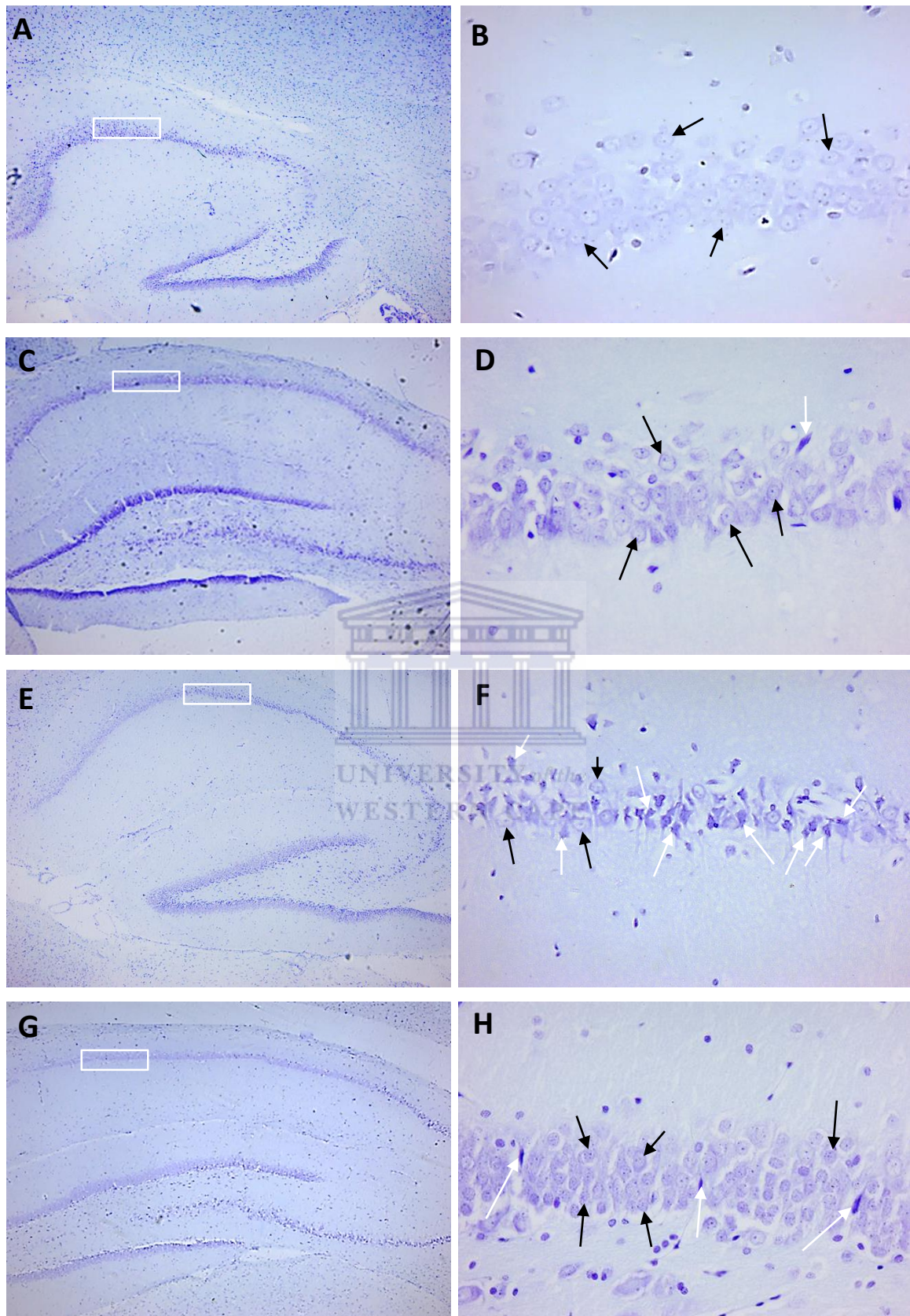


Figure 4.5: Photomicrographs of Cresyl Violet staining of hippocampal sections of rats 7 days after 20 minutes BCCAO or sham surgery. The left panel depicts the CA1 region displayed on the right panel. Neurons appear normal (black arrows) in the Control and Rooibos sham groups, while remarkable damage (white arrows) was seen in the hippocampal CA1 region in the ischemia group (F). The Rooibos + Ischemia group was however similar to both control and rooibos sham groups. (H). A & B: Control sham; C & D: Rooibos sham; E & F: Ischemic and G & H: Rooibos + ischemia groups. Magnification: left panel = X4 and right panel = X40

4.8 IMMUNOHISTOCHEMISTRY (IHC) STUDIES

For an in-depth assessment, cellular changes in rat hippocampus immunohistochemical studies were done to detect disruptions in nuclear DNA which is an established hallmark of apoptotic cell death detection. In this assay, apoptotic cells were recognized by the presence of chromosomal DNA fragmentation into discrete fragments of increasing length using the terminal deoxynucleotidyl transferase mediated UTP nick end labeling (TUNEL) method.

In this assay, TUNEL positive cells are identified as those labelled with a dark or light-brown color or with dark brown granules in the cell nucleus. Evaluation of hippocampal CA1 brain sections from the control sham and rooibos sham groups showed very few TUNEL positive cells on day 7 post-sham surgery ($8.12 \% \pm 2.6$ and $6.21 \% \pm 2.47$) (Figure 4.6 B & D and figure 4.7 B). However, immunohistochemical analysis of CA1 sections of the ischemia group 7 days post-BCCAO showed an increased number of TUNEL positive cells ($46.72 \% \pm 12.8$) (Figure 4.7 B) when compared with the control sham and rooibos sham groups ($8.12 \% \pm 2.6$ and $6.6 \% \pm 6.56$). The number of TUNEL positive cells in the rooibos + ischemia group was also high ($36.54 \% \pm 12.2$) (Figure 4.6 H), but the increase was not statistically significantly when compared to the control sham and rooibos sham groups ($8.12 \% \pm 2.6$ and $6.21 \% \pm 2.47$) respectively (Figure 4.7 B).

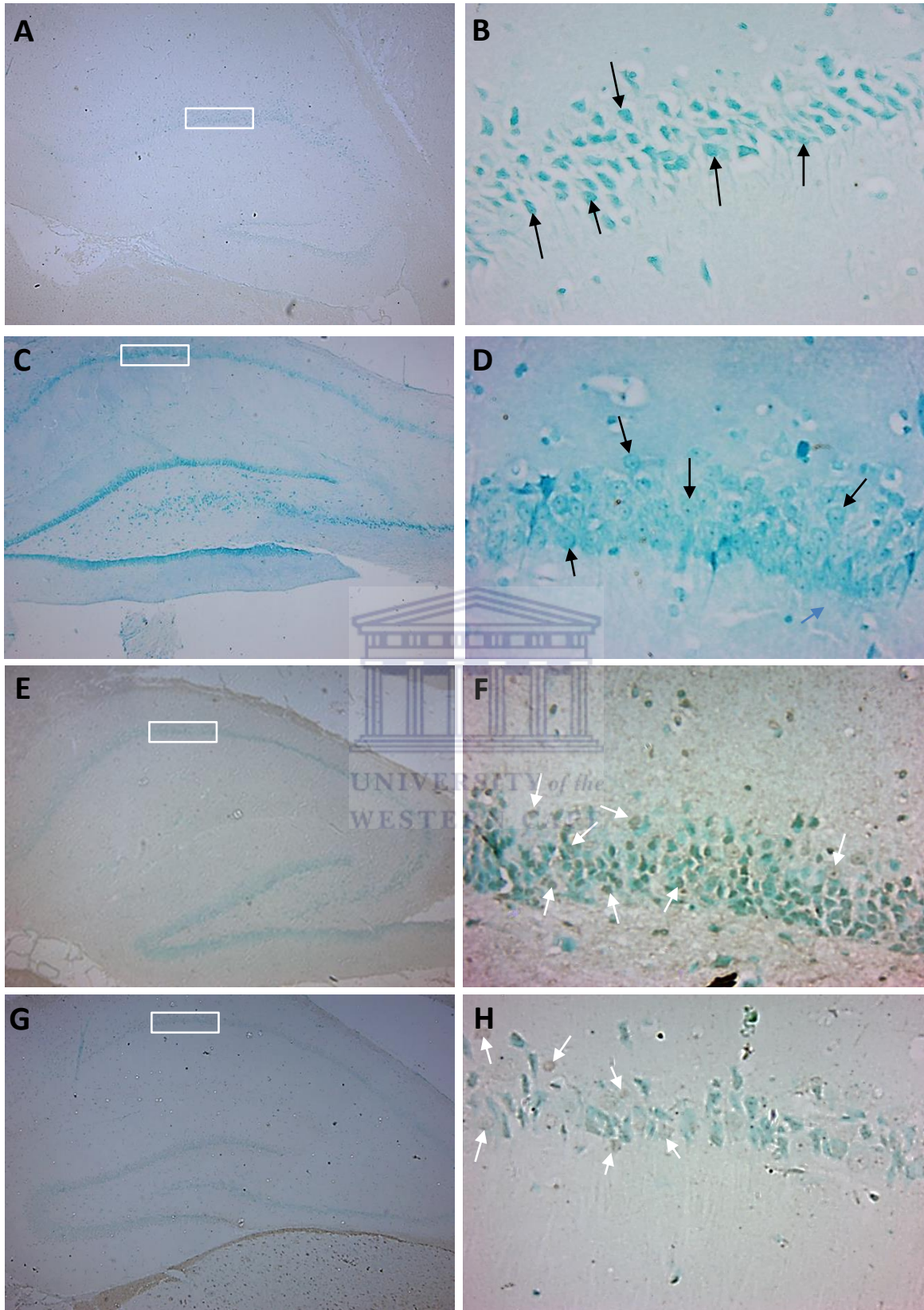


Figure 4.6: TUNEL staining of hippocampal sections of rats 7 days after 20 minutes BCCAO or sham surgery. The left panel depicts the area of the CA1 region displayed in the right panel. Immunohistological evidence of apoptosis in the CA1 region of the hippocampus of rats from the ischemia group (F) is identified by cells labelled with the dark or light brown color (white arrows), normal neurons are colored green (black arrows). Apoptotic neurons are less present in the rooibos + ischemia (H) than in the ischemia group (F). A & B: Control sham; C & D: Rooibos sham; E & F: Ischemic and G & H: Rooibos + ischemia groups. Magnification: left panel = X4 and right panel = X40

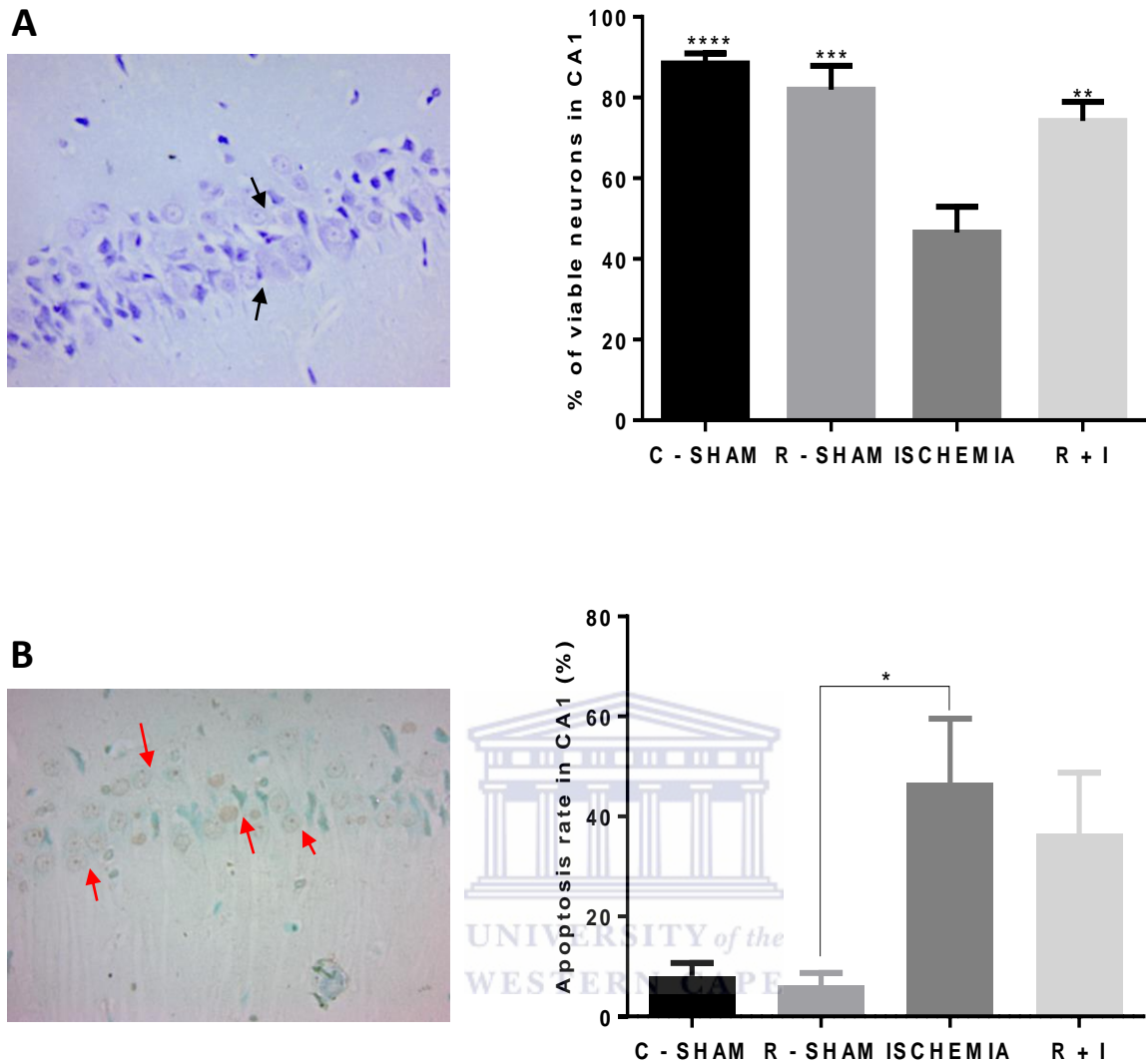


Figure 4.7: Morphometric analysis by cresyl violet and IHC staining. (A) Hippocampal cell survival as assessed by cresyl violet staining at 7 days after 20 minutes BCCAO or sham surgery. The left panel shows photomicrographs of the CA1 region of the hippocampus with viable neurons identified with black arrows. Number of CA1 viable neurons 7 days after 20 minutes BCCAO or sham surgery in the ischemia group was significantly lower in comparison to the C – sham, R-Sham and R + I groups (** $P < 0.0001$ vs control sham, *** $P < 0.001$ vs R-sham and ** $P < 0.01$ vs R + I, $n = 5$ rats per group;). (B) Cellular apoptosis was determined by TUNEL assay. The left panel is a photomicrograph of the CA1 region of the hippocampus showing apoptotic cells labelled in dark and a light brown color. Apoptosis rate in the CA1 region was calculated as follows: neuronal apoptosis rate (%) = $100 \times (\text{number of apoptotic neurons} / \text{total number of neurons})$. At 7 days post BCCAO, the rate of apoptosis was significantly higher in the ischemia group when compared to the R – SHAM group. C - SHAM: Control sham; R-SHAM: Rooibos sham; R + I: Rooibos + Ischemia. $N = 5$ rats per group. Data are expressed as mean \pm SEM; Asterisk (*) = Significant change.**

4.9 NEUROCHEMICAL ANALYSIS

4.9.1 LIPID PEROXIDATION (LPO)

Seven days after BCCAO, the level of lipid peroxidation was determined by measuring malondialdehyde (MDA) levels in the cerebrum. MDA is a specific metabolic tracer molecule for LPO using the thiobarbituric acid reaction (TBAR). Results show that there was a significant increase in the levels of MDA in the cerebral hemispheres of rats in the ischemia group ($0.029 \mu\text{mol/g} \pm 0.0014$) when compared to the control-sham groups ($0.017 \mu\text{mol/g} \pm 0.0014$) and R-sham whereas the rooibos + ischemia group (prior to induction of BCCAO) showed significantly lower MDA levels comparable to the sham levels when compared with the ischemia group ($0.014 \mu\text{mol/g} \pm 0.003$ vs $0.017 \mu\text{mol/g} \pm 0.0014$).

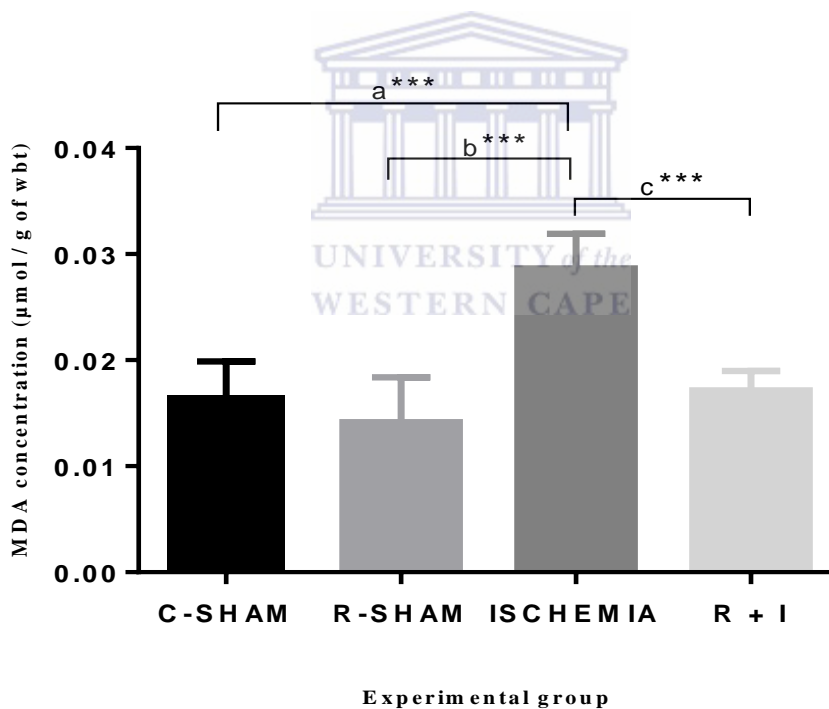


Figure 4.8: Effects of 7 weeks intake of fermented rooibos herbal tea on MDA levels in the cerebral hemispheres of rats 7 days after BCCAO. Fermented rooibos herbal tea prevented increased MDA content following cerebral ischemia induced by BCCAO. Data are presented as mean \pm S.E.M, $n = 5$. $a^{***} = P < 0.001$ vs control-sham group; $b^{***} = P < 0.001$ vs rooibos-sham group; $c^{***} = P < 0.001$ vs R+I group. BCCAO: Bilateral common carotid artery occlusion; C - SHAM: Control sham; R-SHAM: Rooibos sham; R + I: Rooibos + Ischemia. Asterisk (*) = significant difference, wbt = wet brain tissue.

4.9.2 Oxygen Radical Absorbance Capacity (ORAC)

Assessment of total antioxidant capacity is very important in our understanding of how antioxidants protect against reactive oxygen species (ROS). In the human body, peroxy radicals are the most abundant free radicals, which make measurement of antioxidant capacity against peroxy-radical even more biologically relevant. In this study, measurement of antioxidant capacity against peroxy-radical in the rooibos-sham and rooibos + ischemia groups showed that $ORAC_{ROO}$ values were significantly higher ($36.68 \mu\text{mol/g} \pm 1.98$ and $35.16 \mu\text{mol/g} \pm 1.62$ respectively) when compared the ischemia group ($22.26 \mu\text{mol/g} \pm 3.22$) (Figure 4.9).

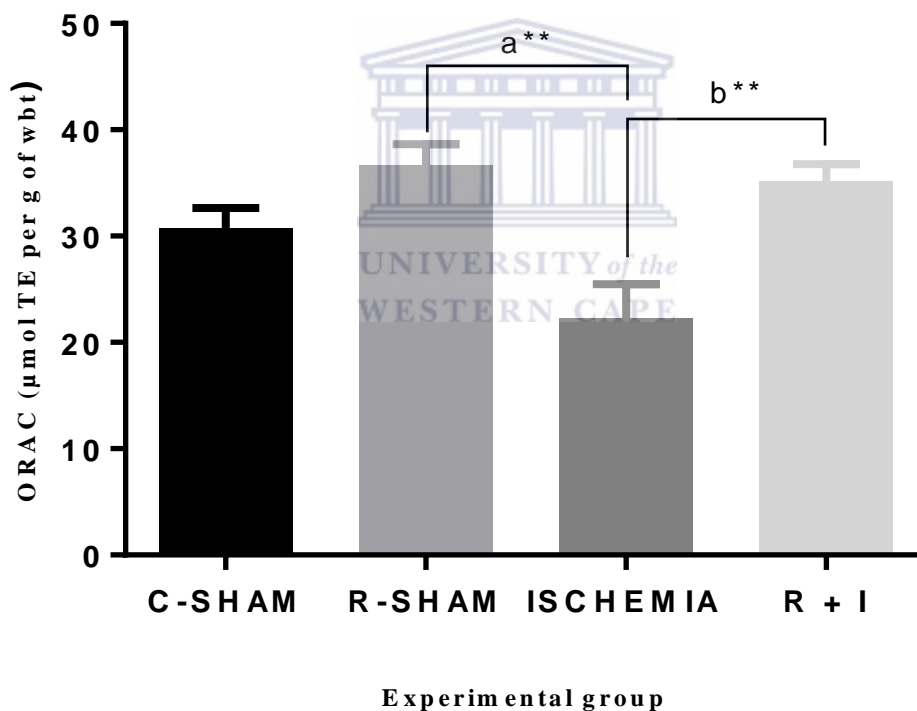


Figure 4.9: Effect of 7 weeks intake of fermented rooibos herbal tea on peroxy radical absorbance capacity ($ORAC_{ROO}$) in the cerebral hemispheres of rats 7 days after BCCAO. Data are presented as mean \pm S.E.M, $n = 5$. $a^{**} = P < 0.01$ vs rooibos-sham group; $b^{**} = P < 0.01$ vs R + I group by student t-test. C-SHAM: Control sham; R-SHAM; Rooibos sham; R + I: Rooibos + Ischemia. Asterisk (*) = Significant difference, wbt = wet brain tissue.

4.9.3 Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay (a measure of the ability of compounds to neutralize free radicals by acting as an electron donors) was done. As showed in Figure 4.10 below, measurement of FRAP levels in the control and rooibos-sham groups presented a higher significant differences (2.36 ± 0.16 and 1.93 ± 0.37) respectively when compared to the ischemia group (0.63 ± 0.11). Interestingly, the FRAP values for samples from the rooibos + ischemia group (2.12 ± 0.16) were significantly higher than the rooibos-sham and rooibos + ischemia groups respectively.

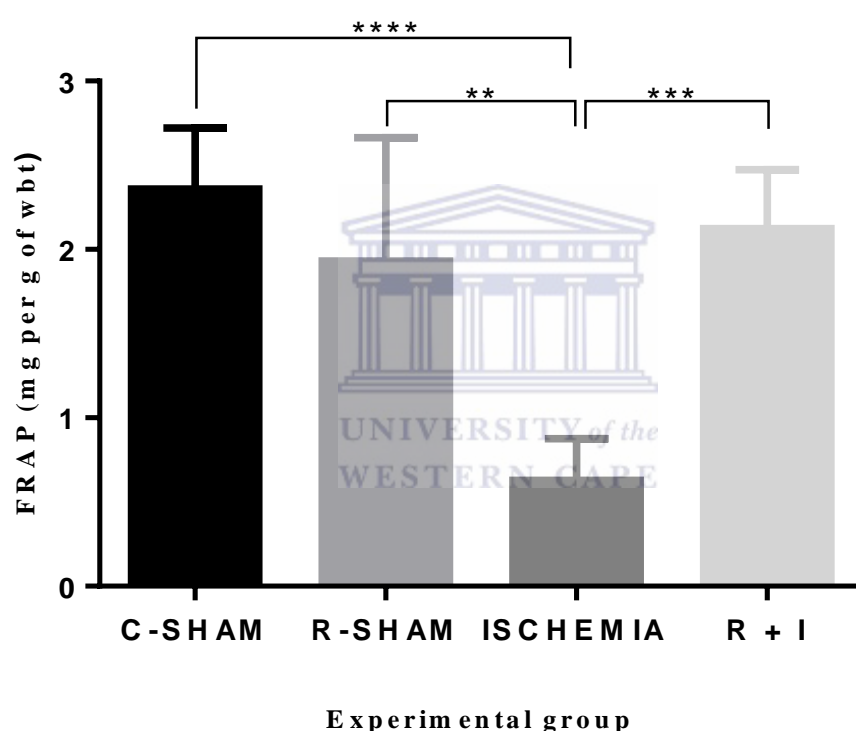


Figure 4.10: Effect of 7 weeks intake of fermented rooibos herbal tea on ferric reducing antioxidant power (FRAP) in the cerebral hemispheres of rats 7 days after BCCAO. The intake of fermented rooibos herbal tea maintained the ferric reducing antioxidant power of in the pretreated ischemia group (R+I) compared to the sham groups. FRAP values were very low in the ischemia group. Values are presented as mean \pm S.E.M, $n = 5$. ** = $P < 0.01$ vs Rooibos-sham group; *** = $P < 0.001$ vs R + I group by student t-test. *** = $P < 0.0001$ vs control sham group. C- SHAM: Control sham; R + I: Rooibos + Ischemia. Asterisk (*) = Significant difference, wbt = wet brain tissue.

4.10 THE OPEN FIELD TEST

The open field test is one of the most widely used measures of animal neurobehavioral deficits. In this study, we investigated neurobehavioural deficits in experimental rats on day 1, 4 and 7 after BCCAO by subjecting animals to a 10 minutes open field test. Results obtained on day 7 show that locomotor / exploratory activity (represented by total distance travelled) of rats in the rooibos + ischemia group was much improved and become insignificantly different at day 7 post-BCCAO when compared with day 1 (Figure 4.11 D), while the exploratory activity of rats in the ischemia group at day 7 post BCCAO was significantly reduced (Figure 4.11 D). The frequency of rearing episodes which is one of the measure of anxiety in rodents during the open field test was seen to have improved across all groups from day 1 to 4, but was significantly lower in both the rooibos + ischemia and the ischemia group at day 7 when compared to the control sham group. However, when compared with the ischemia group, a small improvement in these rearing episodes was noticed in the rooibos + ischemia group pre-treated with fermented rooibos herbal tea but this difference did not reach statistical significance (Figure 4.11 E).

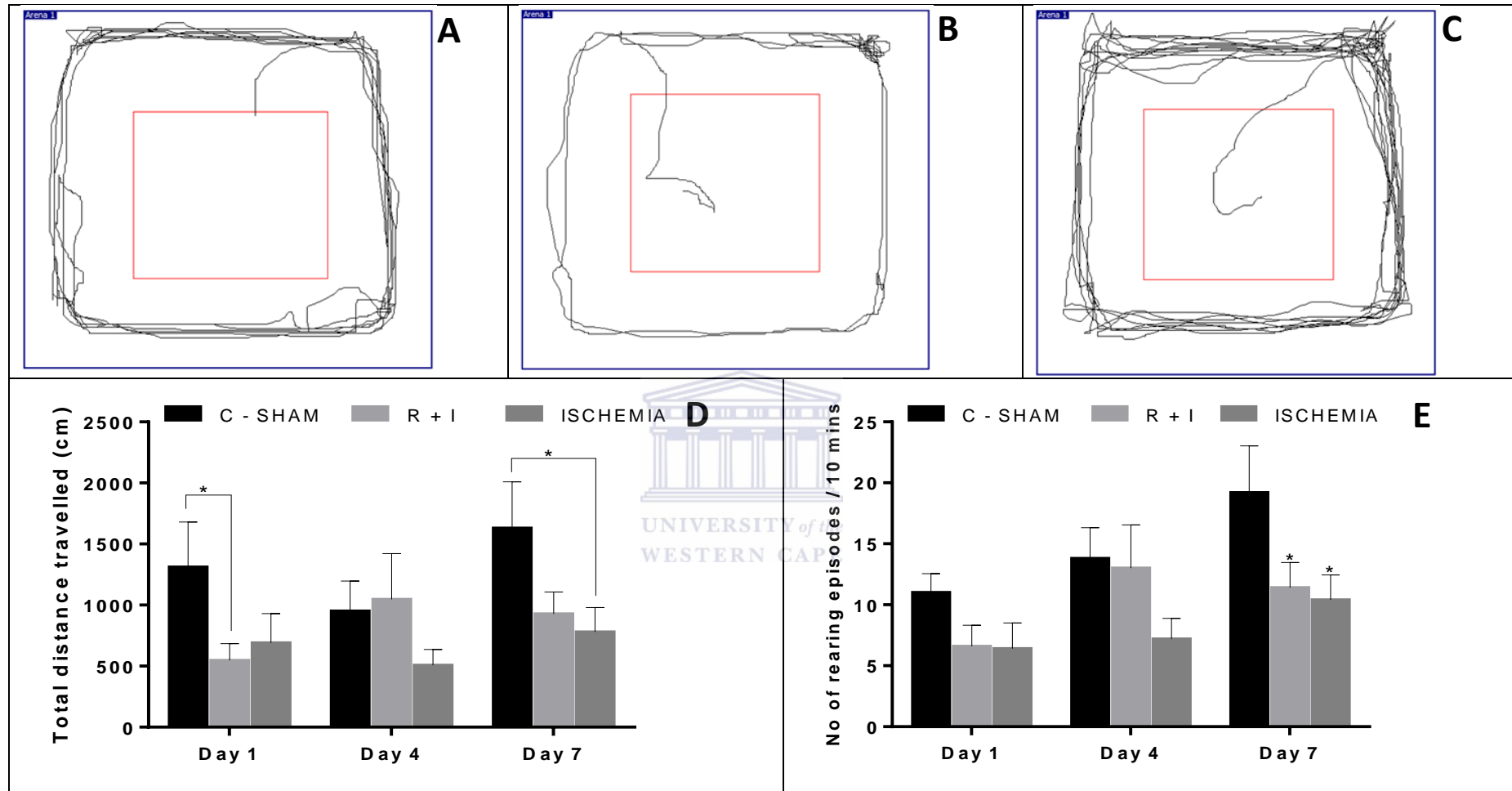


Figure 4.11: Effect of 7 weeks pre-treatment with FRHT on neurobehavioural outcomes. Seven weeks pre-treatment with fermented rooibos herbal tea attenuated neurobehavioral deficits in the 10 minutes Open field test (OFT). Images A, B & C represent the path travelled by the control-sham (C- SHAM), rooibos + ischemia (R + I) and ischemia groups in the OFT D = The significant difference in total distance travelled during the OFT on day 7 post-BCCAO in rats from the ischemia group was significantly reduced by 7 weeks pre-treatment with FRHT (E) The frequency of rearing episodes during the OFT was however not significantly different on days 1 & 4 post BCCAO, while on day 7, the rooibos + ischemia and the ischemia group showed a significant difference when compared to the control sham groups respectively. * $P < 0.05$, Asterisk (*) = Significant difference.

CHAPTER FIVE

DISCUSSION

5.0 INTRODUCTION

The World Health Organization reports that the burden of stroke is expected to rise greatly in the next 20 years since no clinically approved drug is available or licensed for the prevention or treatment of stroke besides thrombolytics which restore blood flow (Johnston, 2008). In addition, the efficacy and safety concerns of thrombolytics have limited their use in medicine (Jin *et al.*, 2010b). A large number of studies are currently ongoing, all in the search for novel neuroprotective agents for stroke.

The current study was done to investigate for the first time, the neuroprotective potentials of fermented rooibos herbal tea (FRHT) in preventing or reducing the severity of an ischemic brain injury to the brain. In this study, seven weeks intake of FRHT by adult male wistar rats remarkably protected the brain against ischemic damage induced by 20 minutes of BCCAO ischemia-reperfusion injury. FRHT was seen to offer protection against brain oedema formation, blood brain barrier impairment, neuronal loss, oxidative stress and the attendant neurological deficits all of which are known to be associated with ischemic brain injury. Many studies have focused on phytochemical analysis of plant extracts to profile their active ingredients (Wu *et al.*, 2010), there is need to also study the mechanisms of action of these phytomedicines. Details about the neuroprotective effects of FRHT are discussed in sections that follow.

5.1 FERMENTED ROOIBOS HERBAL TEA PROTECTS AGAINST OEDEMA FORMATION AND INCREASED RELATIVE BRAIN WEIGHT.

Brain oedema is reported to be one of the two major acute neurologic complications observed following the occurrence of an ischemic stroke in patients (Bansal *et al.*, 2013). Brain oedema is defined as the pathological accumulation of fluid in brain tissue resulting in the expansion of brain tissue volume (Ito *et al.*, 1979; and Kahle *et al.*, 2009). In this study, 7 weeks intake of fermented rooibos herbal tea was found to prevent cerebral oedema by lowering cerebral swelling and water content in the cerebral hemisphere following ischemic injury. Michinaga and Koyama, (2015) reported that the brain oedema observed during the first few hours of ischemia is of the cytotoxic type, characterized by cell swelling in the absence of BBB disruption due to low ATP levels from reduced blood flow to cells. This results in excessive intracellular accumulation of Na^+ followed by accelerated outflow of Na^+ and fluid from blood vessels to compensate for the extracellular decrease. The intra-vascular Na^+ outflows subsequently result in the accumulation of fluid in the brain parenchyma leading to cerebral swelling (Michinaga and Koyama, 2015). Polyphenols from FRHT have been shown to prevent the intracellular accumulation of Na^+ by modulating the activity of the enzyme Na^+/K^+ -ATPase which is responsible for establishing an electrochemical gradients of Na^+ and K^+ across the cell membrane (Vlkovicová *et al.*, 2009). The polyphenols from Fenugreek seeds have also shown similar activities in the red blood cell membrane (Anuradha *et al.*, 2003), adding credence to the possibility that the brain oedema seen in this study was caused by the extracellular accumulation of fluids in the brain from failure of the energy dependent Na^+/K^+ -ATPase gradient during ischemia (Fogarty-Mack and Young, 2006). The evaluation of Aquaporin 4 (AQP4) immunoreactivity (a principal protein involved in intra-extra cellular water balance) (Yang *et al.*, 2012) may have helped confirm how FRHT functioned in preventing brain oedema formation in the rooibos treated brains. Future studies on other animal models of stroke

including the MCAO model may help confirm the exact mechanism involved in the anti-cytotoxic and anti-vasogenic effects of FRHT in the progression of cerebral oedema.

5.2 FERMENTED ROOIBOS HERBAL TEA ATTENUATES BLOOD BRAIN BARRIER IMPAIRMENT

The high mortality associated with ischemic strokes can also be linked to the damage of the blood brain barrier (Knowland *et al.*, 2014), making the maintenance of BBB integrity an important therapeutic approach in the management of ischemic stroke patients (Zhang *et al.*, 2013). Damage to the integrity of the BBB may occur spontaneously during acute stroke or as a result of reperfusion therapy (Nguyen *et al.*, 2013). The exhaustion of ATP, imbalance in ionic homeostasis, oxidative or nitrosative stress signaling and the concomitant phosphorylation of tight junction accessory proteins are processes that could subsequently result in BBB disruption (Kim *et al.*, 2013). BBB damage causes increased vascular permeability which results in vasogenic oedema and cell death, metabolic failure and inflammatory responses (Heo *et al.*, 2005; Rosenberg and Yang, 2007). In the present study, ischemia-reperfusion injury caused increased extravasation of Evans blue into the brain parenchyma, indicating an impairment of the blood brain barrier. Though this increase was not statistically significant, the findings are similar to those reported by Chen *et al.* (2010) as animals pre-administered FRHT showed Evans blue extravasation into the brain parenchyma when compared to the untreated animals.

The protective effects of FRHT on the BBB has not been previously investigated but can be linked to the ability of the active polyphenols in rooibos tea to modulate paracellular permeability and prevent the disruption of tight junctions between brain microvascular endothelia cells. Recent findings by Liu *et al.* (2013), have shown that, alleviating the post-ischemic decrease in expression of tight junction proteins (claudin-5, occludin, and ZO-1) could be the mechanism of action for the neuroprotective benefits conferred by green tea polyphenols on the BBB during early ischemic periods. In addition, Panickar *et al.* (2013) have

also reported the prevention of endothelial cell swelling by dietary polyphenols *in vitro*. The prevention of endothelial degeneration has been suggested by Krueger *et al.* (2015) as a promising therapeutic approach to prevent ischemia-related BBB damage. We suggest rooibos tea polyphenols may have conferred its neurovascular protection along these lines of thought but also suggest future studies to deeply evaluate these claims.

5.3 FERMENTED ROOIBOS HERBAL TEA PREVENTED HIPPOCAMPAL NEURODEGENERATION IN THE ISCHEMIC BRAIN

In the absence of therapeutic intervention during an ischemic stroke, the brain during each hour irreversibly loses as many neurons as it does in almost 3.6 years of normal aging (Saver, 2006). Findings from both animal and human studies have shown that, brief ischemic period could initiate complex processes that ultimately lead to neuronal death (Woodruff *et al.*, 2011; Baron *et al.*, 2014). Because cerebral ischemia-reperfusion injury by the bilateral occlusion of the common carotid arteries does result in delayed neuronal death (DND) in selective regions of the brain, researchers have often used the characteristics of the CA1 cells of the hippocampus to investigate the effects of various treatments therapy (Wang *et al.*, 2009). In this study, we investigated the effects of FRHT on the CA1 region of the hippocampus after 7 days of ischemic-reperfusion. Our findings confirmed that transient global cerebral ischemia does cause neuronal death in the CA1 region of the hippocampus which is consistent with other findings (Abe *et al.*, 1995; Koponen *et al.*, 2000; Nikonenko *et al.*, 2009). Results from the Nissl staining also showed an increase in CA1 hippocampal cell survival in the ischemia group animals pre-administered with FRHT before ischemia. This suggests a protective effect of fermented rooibos herbal tea on neuronal death induced by ischemia. Similarly, the number of TUNEL-positive cells (cells undergoing apoptosis) was also reduced by the consumption of FRHT.

Put together, these results indicate that FRHT could mitigate the delayed hippocampal cell loss and possible neurodegenerative processes induced by BCCAO ischemia–reperfusion injury. The loss of oxidative phosphorylation (necessary for ATP production), mitochondria dysfunction, depolarization of the cytoplasmic potential, calcium overload, etc. are few of the possible complex processes mechanisms by which fermented rooibos herbal tea may have prevented ischemia-induced neurodegeneration. Further studies are required to validate these assumptions.

5.4 FERMENTED ROOIBOS HERBAL TEA PREVENTED LIPID PEROXIDATION IN THE ISCHEMIC BRAIN TISSUES

The role of oxidative stress in the pathophysiology of ischemic stroke has been well documented (Cichon *et al.*, 2015). Cellular damage during and after an ischemic brain injury has been suggested to be due to oxidative damage caused by free radicals (Kinuta *et al.*, 1989). While free radicals are only harmful at high concentrations, their production can be linked to several mechanisms, including mitochondria dysfunction, the activation of nitric oxide synthase (NOS), and the migration of neutrophils and leukocytes, mechanisms known to generate free radicals (such as the superoxide anions (O_2^-)) which are harmful to the brain (Tsai *et al.*, 2014).

The profound effects of oxidative stress in stroke can be attributed to the following reasons; (1) the brain tissue is rich in polyunsaturated fatty acids which are particularly prone to damage by free radicals, (2) low content of antioxidant enzymes and (3) high consumption of oxygen (Allen and Bayraktutan, 2009; Hong *et al.*, 2014). One of the most commonly reported biomarkers of oxidative stress in tissues is Malondialdehyde (MDA), the amount of which shows severity of lipid peroxidation (Serteser *et al.*, 2002). MDA is produced as a by-product

of the reaction of superoxide (O_2^-) and hydroxyl ($-OH$) radicals with unsaturated lipid (Ozkul *et al.*, 2007).

Results from this study show that cerebral ischemia-reperfusion injury by transient BCCAO caused a significant increase in the amount of MDA in the cerebrum but in animals pre-treated with FRHT before ischemia, the amounts of MDA were at physiologic levels. These findings compliment other published reports on the antioxidant benefits of rooibos tea. Inanami *et al.* (1995), Fukasawa *et al.* (2009), Marnewick *et al.* (2011), Awoniyi *et al.* (2012), and Hong *et al.* (2014) have all reported the antioxidative effects of rooibos tea against lipid peroxidation. The activity of the abundant polyphenolic compounds in rooibos tea, particularly aspalathin, has been suggested to be responsible for the remarkable antioxidative benefits reported (Hong *et al.*, 2014). The ability of flavonoids to donate H^+ to the peroxy radical produced as a result of lipid peroxidation has also been discussed as a possible mechanism of protection by Awoniyi *et al.*, (2012). Although findings from the current study appear to support both suggested mechanisms, future studies should investigate the exact mechanism of action by which rooibos tea confers its health benefits.

5.5 FERMENTED ROOIBOS HERBAL TEA MAINTAINED TISSUE TOTAL ANTIOXIDANT CAPACITY LEVELS

Total tissue antioxidant capacity (TAC) levels measure the ability of endogenous systems to resist oxidative damage and current research shows that the TAC assay is a reliable biomarker for many different pathophysiological conditions (e.g. heart and vascular diseases, diabetes mellitus, neurological and psychiatric disorders, renal disorders and lung diseases) (Kusano and Ferrari, 2008). While TAC assays are of more than 2 types (Prior *et al.*, 2005), this study only investigated the antioxidative properties of FRHT using the ORAC and FRAP assays. The ORAC and FRAP assays are of high scientific importance across several disciplines as they

have been used to provide information regarding the antioxidant capacity of many compounds and food samples, with some manufactures now including ORAC values on product labels (Prior *et al.*, 2005). ORAC represents a hydrogen atom transfer reaction mechanism, and is more relevant to human biology than FRAP which analyses the ability of a compound to reduce ferric ion. When total antioxidant capacity levels are low in the body, cells and tissues become more susceptible to disease (Kusano and Ferrari, 2008) and in this case to ischemic brain injury.

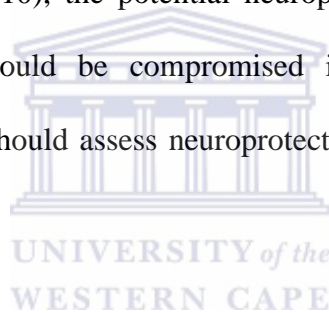
Results from this study show that low levels of ORAC and FRAP observed 7 days after the induction of BCCAO are indicative of the high levels of oxidative stress from the ischemia-reperfusion injury. Consumption of fermented rooibos herbal tea before BCCAO however appeared to have mitigated the oxidative stress by keeping ORAC and FRAP levels at physiological levels. This suggests that long term consumption of FRHT could help maintain the total antioxidant capacity of the brain during an ischemic injury. These findings are similar to the reports from Akinmoladun *et al.* (2015) on the antioxidative properties of Kolaviron against ischemia reperfusion injury and are strongly supported by previous studies by Cao *et al.*, 1998; Vergely *et al.*, 1998 and Jung *et al.*, 2011.

5.6 FERMENTED ROOIBOS HERBAL TEA ALLEVIATED NEUROBEHAVIOURAL DEFICITS

The 2009 stroke therapy academic industry roundtable (STAIR) recommendation for preclinical studies of stroke, suggested that evaluation of neurological outcomes be conducted in preclinical-studies (Minnerup *et al.*, 2012). This is to show that functional neurological outcomes are also improved together with the tissue endpoints since neurological outcomes remain the primary device for clinical assessment of therapeutic interventions (Fisher *et al.*, 2009). To demonstrate the neuroprotective value of FRHT, the open field test (a simple method

for assessment of locomotor activity in rodents) was conducted as suggested by STAIR. Findings from the OF study showed that ischemia-reperfusion injury induced neurobehavioural deficits due to damage to the brain 7 days after BCCAO, whereas pre-consumption of FRHT appeared to attenuate the behavioural impairment induced by ischemia. Since severity of neurologic examinations is often related to histomorphological findings (Hong *et al.*, 2000; Schiavon *et al.*, 2014), our study also show similar correlations.

In this study, a significant amount of neuronal cells survived the ischemic injury, similar to findings by Inanami *et al.* (1995) who reported rooibos tea to prevent age related changes in the brain. Since neuronal death has been reported to occur months after BCCAO ischemic-reperfusion injury (Shin *et al.*, 2010), the potential neuroprotective effects observed in this study (7 days after BCCAO) could be compromised in the long-run. It is therefore recommended that future studies should assess neuroprotective effects of FRHT months after post-BCCAO reperfusion.



CHAPTER SIX

CONCLUSION, LIMITATION AND RECOMMENDATION

6.0 CONCLUSION

Our results provide substantial evidence of neuroprotection conferred by fermented rooibos herbal tea during an ischemic-induced brain injury. A possible explanation for this protection might be the ability of the active polyphenols present in rooibos tea to modulate the oxidative stress and neuroinflammatory pathways which lead to neuronal death, brain oedema and blood-brain-barrier disruption. Our results tend to suggest that long term consumption of fermented rooibos herbal tea could confer multifactorial protective benefits to the brain in the event of ischemic injury especially due to stroke. These findings could potentially inform future clinical trials and public health policy decisions regarding stroke prevention and management, considering the increasing numbers of cases of patients with conditions that are considered risk factors for stroke.

6.1 LIMITATION OF STUDY

Neuroinflammation is a very important component of the ischemic re-perfusion study, hence immunohistochemical studies of microglia activation through GFAP staining could be used to investigate the neuroprotective properties of fermented rooibos herbal tea as seen in this study. Also, the role of astrocytes in the blood-brain-barrier is well documented; hence astrocyte activation studies using the IBA-1 staining could be performed. Both these assays were however not done in this study due to limited funding. In addition, the use of Intraoperative Doppler ultrasonography (IDU) for the measurement and monitoring of changes in cerebral

blood flow during induction of ischemia and reperfusion is important in stroke modeling. The IDU technique could not be employed in this study because the equipment was not available.

Another limitation in this study is the use of only the open field test (OFT) for assessing neurobehavioural deficits due to limited availability of other equipment. Although the OFT test provided useful data in this study, additional neurobehavioural tests (e.g. T-maze test, Water maze test and Object recognition test) could have offered more information on cognitive deficits associated with hippocampal lesions in animals pretreated with fermented rooibos herbal tea before and after the induction of cerebral ischemia-reperfusion injury.

6.2 FUTURE RECOMMENDATION

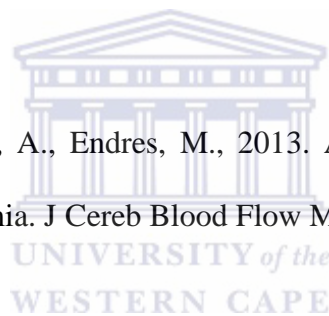
Future studies are recommended to incorporate the following aspects:

- determine the most active compounds in fermented rooibos herbal tea and test their neuroprotective potential against cerebral ischemia
- use of animal models with ischemic co-morbid factors such as atherosclerosis, hypertension which more closely mimic the pathophysiology of cerebral ischemia in humans
- monitoring of biomarkers of inflammation in future experimental research
- administration of fermented rooibos herbal tea before and several months after the induction of cerebral ischemia-reperfusion injury to determine its long-term therapeutic benefits.
- use of additional neurobehavioural tests to corroborate findings on cognitive and motor deficits.

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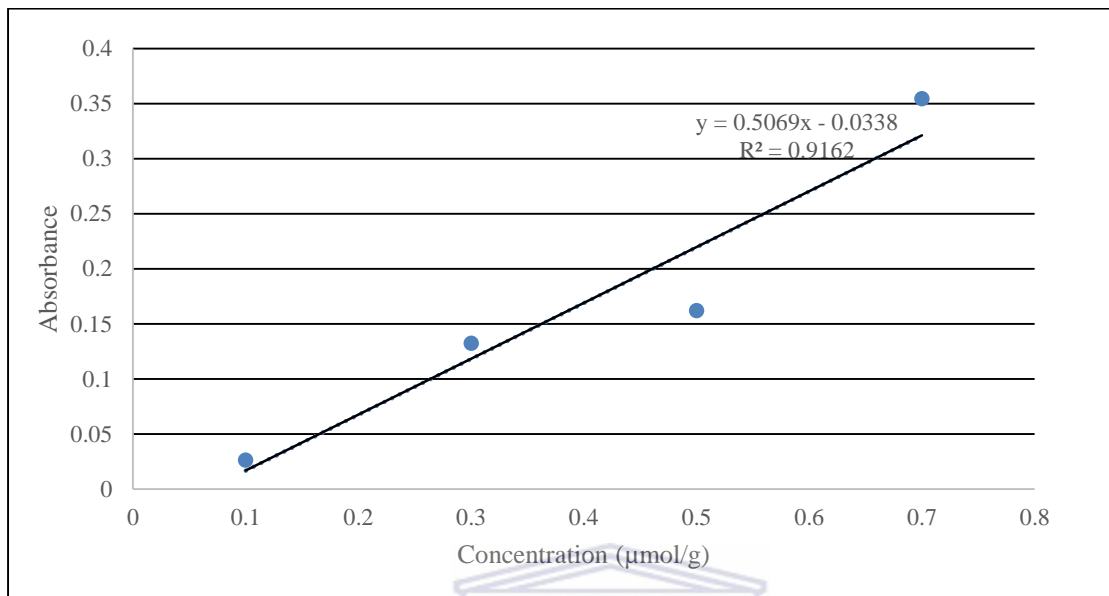
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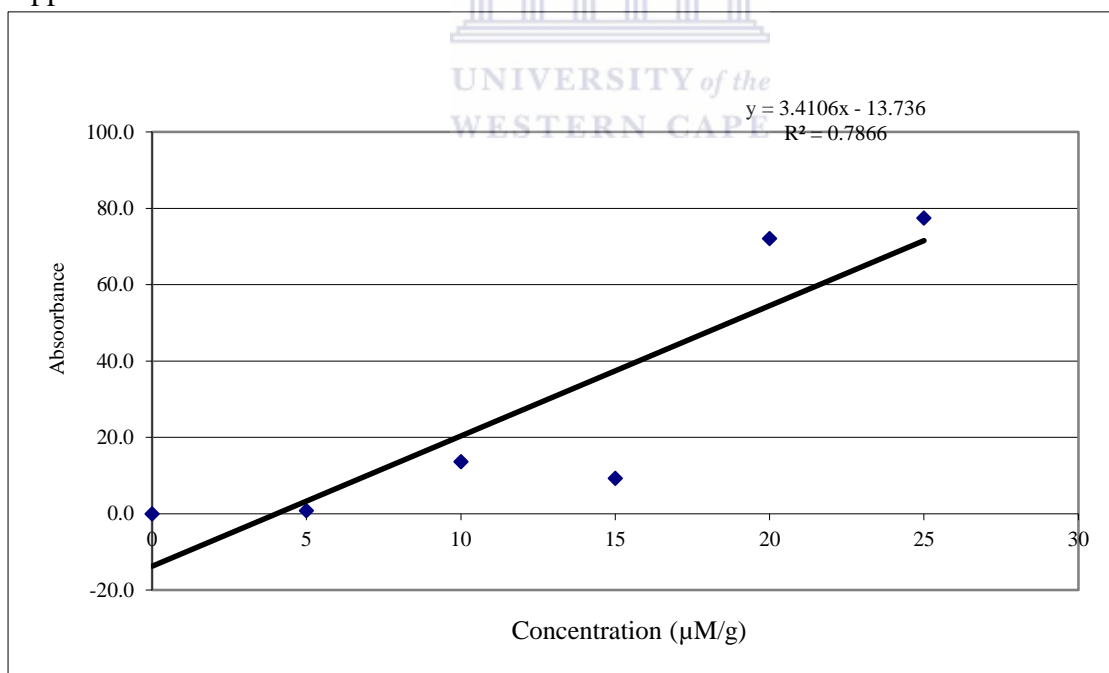


APPENDICES

Appendix A: Standard curve for the determination of Evans Blue extravasation



Appendix B: Standard curve for the determination of ORAC



Appendix C: Standard curve for the determination of FRAP

