RADIOPROTECTIVE EFFECTS OF ROOIBOS HERBAL TEA ON THE DEVELOPING CENTRAL NERVOUS SYSTEM OF WISTAR RATS

By

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DECLARATION

I declare that the thesis titled: ***"Radioprotective effects of Rooibos herbal tea on the developing central nervous system of Wistar rats"*** is my own work submitted for the Master of Science degree in Medical Biosciences at the University of the Western Cape, South Africa, and that this work 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... ***Milod M Ahmed Alrtemi*** **Signed**.....

DEDICATION

This study is dedicated to my parents and my family

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LIST OF ABBREVIATIONS

%	Percent
°C	Degree Celsius
AAF	Acetylaminofluorence
ACE	Angiotensin converting enzymes
AFB	Aflatoxin B
ANOVA	Analysis of variance
ATP	Automated tissue processor
CAT	Catalase
CHO	Chromosome
CNS	Central nervous system
CV	Cresyl Violet
D	Day
DCM	Diabetic cardiomyopathy
DEN	Diethyl nitrosamine
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DPX:	Distyrene Plasticizer Xylene
DV	Daily value
EGCG	Epigllocatechin gallate
ETBR	Ethidium bromide
FFPE	Formalin-fixed, paraffin-embedded

FRAP	Ferric reducing antioxidant power
FRHT	Fermented Rooibos herbal tea
FSH	Follicle stimulating hormone
G	Gram
GP_X	Glutathione peroxide
GR	Glutathione reductase
GSH	Glutathione
Gy	Gray
H	Hour
H&E:	Haematoxylin and Eosin.
HCl	Hydrochloric acid
IHC	Immunohistochemistry
LPO	Lipid peroxidation
M	Molar
Mg/Ml	Milligram per millilitre
MMC	Mitomycin c
MNRET	Micro nucleated reticulocytes
N	Number of samples
Na_2CO_3	Sodium carbonate
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
NAH_2PO_4	Anhydrous monobasic sodium phosphate
NaOH	Sodium hydroxide

NOR	Novel Object Recognition
NS	Normal saline
O^{2-}	Superoxide anion
OF	Open Field
OH	Hydroxyl radical
OSS	Oxidative stress status
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFDB	Paraformaldehyde
pH	Protons of hydrogen
PND	Postnatal days
ROS	Reactive oxygen species
RT	Room temperature
Sec	Second
SOD	Superoxide dismutase
UV	Ultraviolet

KEYWORDS

Gamma Radiation

Brain

Fermented rooibos tea

Ionizing radiation

Neurobehavioural

Central Nervous System

Hippocampus

Cerebellum

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ABSTRACT

Background: Early postnatal radiation exposure from environmental, diagnostic or therapeutic sources is potentially deleterious to the developing nervous system resulting in oxidative stress, structural damage, altered neurochemistry, DNA damage, inflammatory stresses as well as correlating cognitive impairment during adult life. Numerous studies in literature have investigated the radioprotective effects of medicinal plants and beverages. However, only a few studies have focused on the radioprotective effects of rooibos, an indigenous South African herbal tea, well known for its many acclaimed health benefits.

Aims: This study was done to investigate the diverse radioprotective potential of fermented Rooibos herbal tea (FRHT) consumed *ad libitum* by pregnant rats on the adult offspring rats exposed to a once-off 6 Gy dose of gamma irradiation on postnatal day 3.

Methods: Twenty-four (24) adult female rats were equally divided into four groups (6 per group) as control (NS), radiation (X), tea (RT) and their combination. On PND 30, offspring rats were subjected to neurobehavioural assessment for open field and novel object recognition parameters and later sacrificed, the brain tissues removed and processed for histological, immunohistochemical and neurochemical analyses, using standard techniques.

Results: Pre-treatment with FRHT showed overall protection against radiation-induced distortions in offspring rats by significantly improving exploratory activity, the frequency of central square entry, rearing episodes, cumulative freezing time and memory retention as indicated by a relatively higher recognition index. FRHT was also found to significantly improve the antioxidant defence mechanisms in the offspring rats by reversing lowered FRAP levels, increasing superoxide dismutase and catalase enzyme activities and reducing lipid peroxidation. Histological and immunohistochemical analyses showed that morphological alterations were generally attenuated in the RTX group and the high number of caspase-3 and Glial fibrillary acidic protein (GFAP)-positive cells was significantly reduced, indicating protective effects against apoptosis and gliosis.

Conclusion: Taken together, our findings tend to suggest that the potential radioprotective effects of FRHT are multimodal, possibly executed through the anti-apoptotic, antioxidative,

anti-gliosis and other mechanisms, as observed in this study, and this is often attributed to the high polyphenol content in Rooibos tea.

CHAPTER ONE

INTRODUCTION

1.1. Background

Ionizing radiation is produced when electrons are dislodged from atoms and molecules resulting in the formation of charged particles or ions (Elgazzar and Kazem, 2015). It can either be generated spontaneously from atoms of unstable elements (through radioactivity) or artificially by machines. The released radiation may take the form of particles (including electrons, neutrons and alpha particles) or electromagnetic gamma radiation or X-rays, all with different amounts of energy (UNSCEAR Report, 2008).

Living organisms are constantly exposed to one form of background radiation or another, emanating from diverse sources in nature including cosmic radiation, radionuclides in food, water and soil, most of which are low level and not significantly life-threatening. Exposure from effluents and solid waste from military, aviation, nuclear power, industrial or workplace activities as well as from diagnostic radiology, nuclear medicine and radiotherapy could be potentially damaging, depending on dosage (Eisenbud and Gesell, 1997; UNSCEAR, 2008).

For about a century now, ionizing radiation has progressively played an important role in medicine both for diagnostic and therapeutic purposes (Thariat *et al*, 2013). However, studies have shown that exposure to ionizing radiation poses health risks in the form of structural and biochemical lesions which lead to increased lipid peroxidation and the generation of free radicals and reactive oxygen species (ROS) like superoxide ($O_2^{-\cdot}$), hydroxyl radical (OH^{-1}), hydrogen peroxide (H_2O_2) (Cho *et al*., 2003; Spitz *et al*., 2004). Free radicals in turn induce DNA damage eventually leading to cell death (Azab *et al*., 2005).

Rapidly dividing cells in embryos and foetuses are very radio-sensitive, hence pregnancy is considered a critical period for radiation exposure because developmental processes are likely to be disrupted. However, the severity of radiation effects depends on the stage of development and the magnitude of the doses (Schull, 1990). In the same vein, children under 10 are particularly

susceptible and sensitive for the development of thyroid, brain, skin and breast cancer and leukaemia following relatively high levels of radiation exposure (Douple, 2011; UNSCEAR, 2013).

Irradiation-induced central nervous system (CNS) malformations may occur during critical stages of development (organogenesis, differentiation and growth), and lead to teratogenic, carcinogenic, or mutagenic effects (Williams and Fletcher, 2010). Other CNS effects commonly observed include demyelination, disruption of cell proliferation, neuron-glia interactions, endothelial cell loss and capillary occlusion (Belka *et al*, 2001). The tolerance dose in the CNS, as with other tissues, depends on the level of exposure, stage of fetal development and the specific anatomical location irradiated (Schultheiss *et al*, 1995; Williams and Fletcher, 2010).

Reactive oxygen species (ROS) are continuously produced in mammalian cells including neurons, as by-products of normal metabolic processes and neurotransmitter metabolic processes, potentially threatening neuronal integrity and survival the induction of lipid peroxidation, protein oxidation, and DNA damage (Chan, 1994). Exposure to radiation has been found to further increase the severity of free radical formation in the brain (Gutteridge, 1995). To counteract the adverse effects of ROS.an endogenous antioxidant defence system exists in body tissues, comprising of the enzymes superoxide dismutase, glutathione peroxidase, catalase and glutathione, among others (Wilson, 1997). Brain and neurons have been shown to be particularly vulnerable to oxidative stress as a result of the limited antioxidant level but high content of poly unsaturated fatty acid (PUFA) and consumption of oxygen; hence, ROS detoxification is especially important for the brain

Developmental studies have shown that as a foetus develops from an *in utero* hypoxic to a relatively hyperoxic environment with an approximate 4-fold elevation in oxygen concentration, antioxidant enzyme levels are elevated possibly as compensatory mechanisms aimed at protecting the newborn from oxidative stress (Khan and Black, 2003). One previous study showed that ROS attacks terminally differentiated neuronal cells which are sensitive to oxidative stress therefore leading to nerve damage (Gilgun-Sherki *et al.,* 2001), while another study has shown that electromagnetic radiation had a negative effect on the rat brain by increasing total

antioxidative capacity and oxidative stress levels in the frontal cortex, brain stem and cerebellum (Eser *et al.*, 2012).

Ionizing radiation is known to have significant effects on a variety of neurobehavioral outcomes in mice (Greene-Schloesser *et al.,* 2012; Rola *et al.*, 2004) and humans alike (Bar *et al,* 2004). In on another study, gamma radiation was found to reduce spontaneous locomotor activity whereas motivated behaviour of social exploration was un-impacted (York *et al*., 2012). A significant disturbance in the normal behavioural pattern as well as dose-dependent decrease in open-field locomotor and exploratory activities of mice were observed in expo (Devi, Hossain and Bisht, 1999; Hossain and Devi, 2000). Exposure to ionizing radiation has been considered a factor in the genesis of depression in humans (Loganovsky and Vasilenko, 2013).

Modulation or elimination of radiation-induced adverse effects has attracted a number of studies in the last two decades, including suggestions of radioprotection using plants and herbs (Jagetia, 2007). More than 80% of the world's population has been reported to use complementary and alternative medicines (Mainardi *et al.,* 2009) which include herbalism and botanical medicine use. Herbalism is the medical use of preparations that contain exclusively plant material (Ernst, 2003); botanical medicines contain a number of active ingredients which could have similar molecular targets as pharmaceutical drugs (Treasure, 2005). Thus, herbal and botanical medicines present a readily available, accessible and affordable medicinal resource at a fraction of the cost of conventional medicine.

A number of plants have been utilized successfully for the treatment of free radical-mediated humans diseases such as rheumatoid arthritis, atherosclerosis, cancer, Alzheimer's disease, Parkinson's disease, aging and several other conditions including inflammatory diseases (Das, 2002; Singh *et al.*, 2000). This tends to suggest that some herbs and botanicals contain active ingredients or compounds that could confer protection against radiation-induced ROS-mediated damage in nervous tissue, possibly through their potent antioxidant, immunostimulant, cell proliferation stimulation, antiinflammatory and anti-microbial effects.

Rooibos (*Aspalathus linearis*) is a legume plant indigenous to Southern Africa and is known to contain a plethora of bioactive compounds. Its leaves are used to prepare a herbal tea called rooibos or bush tea or sometimes red bush tea. This tea has been very popular in Southern Africa

for generations and is now being consumed in many countries due to its well acclaimed health benefits (Mahomoodally, 2013). Rooibos tea is also an important source of dietary antioxidants, including flavonoids, dihydrochalcone glucoside and aspalathin (Koeppen *et al.,* 1966). Rooibos tea has also been shown to possess potent antimutagenic and anticancer properties (Marnewick *et al*., 2000; Marnewick *et al*., 2005; Marnewick *et al*., 2009). This study was therefore designed to ascertain whether rooibos tea, commonly consumed as beverage in South Africa, could have radioprotective effects in the developing brains of exposed to rat pups.

1.2. Significance of Study

Application of ionizing radiation, over and above surgery and chemotherapy has been the treatment of choice for solid malignancies (Kinsella, 2011) but there remains substantial concern about toxicity to normal tissues and organs from accidental exposure, making protection planning very crucial (Arora *et al.*, 2005; Jagetia, 2007). Thus, the use of nontoxic radioprotectors in the form of herbal supplements or complementary and alternative medicines is of considerable interest especially in less developed countries as Africa.

Rooibos tea has gradually become the tea of choice in South Africa (Fukasawa *et al.*, 2009) and its antioxidant and free-radical scavenging properties have been previously reported (Marnewick *et al.*, 2011). Despite the large percentage of the South African population consuming rooibos tea with its many acclaimed health benefits, not much is known in literature about the potential of this herbal product to protect nervous tissue against early postnatal irradiation effects arising from either accident exposure or medical diagnosis or therapy. Even if this tea does show potent radioprotective effects, the underlying mechanisms involved in such actions also need to be elucidated.

1.3. Hypothesis

It is expected that frequent intake of fermented Rooibos herbal tea will protect the developing nervous tissue against damage resulting from ionizing gamma irradiation. This hypothesis is based on the fact that Rooibos tea (possibly due to its flavonoid contents) has been reported to

show antioxidant properties which could be protective against free radicals generated from radiation injury.

1.4. General Aim

The general aim of this research was to determine whether maternal consumption of Rooibos tea during gestational as well as postnatal consumption via breastfeeding would provide general radioprotection to the developing nervous tissue, following early postnatal exposure to ionizing gamma radiation.

1.5. Objectives

i. To investigate histomorphological injury to the developing nervous tissues following cephalic exposure of pups to ionizing radiation

ii. To determine the radioprotective effects of fermented rooibos herbal tea against irradiation-induced histomorphological injury to the developing nervous tissue.

iii. To investigate the antioxidant effects (SOD, LPO, CAT, FRAP and GSH) of fermented rooibos herbal tea following irradiation-induced oxidative stress in developing nervous tissue.

iv. To determine the protective effects of fermented rooibos herbal tea in modulating lipid peroxidation in the developing nervous tissue following gamma irradiation.

v. To evaluate the effects of radiation injury on neurobehavioral outcomes of mature Wistar rats exposed to radiation on postnatal day 3.

CHAPTER TWO

LITERATURE REVIEW

2.1. RADIATION

Radiation is energy that comes from a source and travels through some material or through space. Light, heat and sound are sources of radiation. Radiation has been all around us since inception of planet earth as it is naturally present in our environment as well as from industrial and medical sources. Basically, radiation is also the phenomenon by which energy is transmitted from one place to another. Radiation also covers the electromagnetic spectrum, which includes static fields like the earth's magnetic field, fields generated by alternating currents, radio waves, microwaves, infrared, visible and ultraviolet (UV) light, and non-ionizing and ionizing radiation (IR).

2.2. Types of Radiation

2.2.1. Non-ionizing radiation

Non-ionizing radiation possess less amount of energy and consist of radiations from ultraviolet and visible white light, radio wave, micro wave as well as infrared (Ng, 2003). They lack the capacity to induce ionization but could dissipate energy though increased molecular movement and heat (Hall, 2000).

2.2.2. Ionizing Radiation

Ionizing radiation consisted of both particles and electromagnetic radiation. The particles are further classified as electrons, protons, neutrons, beta and alpha particles depending on their atomic characteristics. The most common electromagnetic radiation with enough energy to produce ions, break chemical bonds and alter biological function is x-rays and gamma rays and other subtypes are followed below.

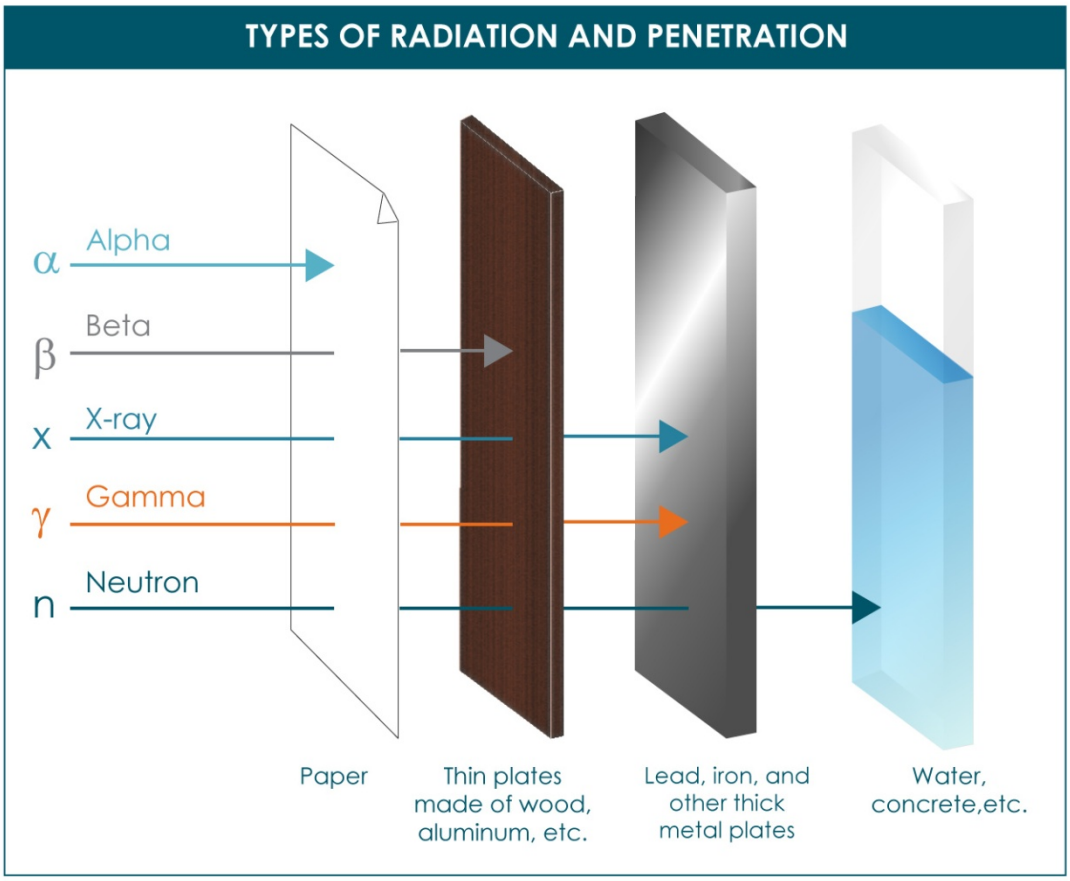

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Figure 2.1: Diagram showing stages of penetration all ionizing radiation.

2.2.2.1. Alpha particle

Alpha particles are made up of two protons and two neutrons packed together into a particle similar to that of helium nucleus. Alpha particles are produced during the course of radioactive decay and they are quite large in terms of mass and this makes it difficult for them to move fast in matter. Considering this alpha particles tend to dissipate all their energy within the shortest distance thus leading to a heavy ionization. More so, due to their short range of absorption and lack of penetrating power, they lack the ability to go through the outer layers of skin and as such less threat to life unless when they are swallowed or inhaled. The consequence of this is extremely deleterious and could pose a serious health challenge (Christensen *et al*., 2014).

2.2.2.2.Beta particles

Beta particles are termed high energy particles which could either be positive or negative coming from the nucleus of an atom. A positive charge beta particle is referred to as positron why a negatively charged is termed negatron (Keith *et al*., 2012). Beta particles could also be referred to as beta ray and they are usually emitted in the course of radioactive decay of the nucleus of an atom. Medically, beta particles find application in the treatment of health conditions like cancer as well as their application as radioactive tracers. Unlike alpha particle, beta particles possess the ability to penetrate living matter to some extent thus altering the structure of such matter (Keith *et al*., 2012). In many cases such alteration could lead to medical conditions like cancer as they could target DNA towards leading to a mutation and in some cases it could also lead to death.

2.2.2.3. Neutrons

Neutrons are uncharged ionizing radiation particles which have almost a similar mass of a proton and they constitute the nucleus of an atom. Nuclear reactors are the most common source of neutrons as they are emitted during splitting of the nucleus of plutonium and uranium. Neutrons possess the ability to go through the tissues and organs of the human body and could pose serious threat to man if inhaled or ingested. Neutrons have found application in their use as lenses in neutron microscopy and neutron/gamma ray tomography (Kumakhov and Sharov 1992).

2.2.2.4. Gamma rays

Gamma rays are high-energy photons ionizing electromagnetic radiation which arises as a result of radioactive decay of the nuclei of an atom. They occur naturally in the earth from radioisotopes as well as from cosmic ray particles interaction with the atmosphere. They are highly penetrating and can easily spread around the body thus causing a lot of cellular damage. In comparison with the alpha and beta particles, gamma rays are less ionizing but more penetrating. Gamma rays even at low concentrations could pose a threat as during radiation dose assessment such concentrations might be seeing to induce cancer as well as genetic damage (American Cancer Society 2015). During gamma radiation induction of DNA damage, a cell

might be able to repair the damage genetic material depending on the intensity of the radiation. Rothkamm and Löbrich, (2003) reported that the repair process is likely to be better in the high dose exposure as compared with the lower dose exposure which is slower to repair.

2.2.2.5. X-rays

X-Rays electromagnetic radiations are similar to gamma radiations but with longer-wavelength and lower energy. They also differ from gamma radiations as they originate from electron cloud which is caused by a change in the energy of electrons which involves energy moving from a level of higher energy to a lower on and thus causing excessive release of energy. X-rays are quite harmful to living tissues as their photons possess substantial energy enough to alter molecular bonds and ionize atoms. A very high exposure to x-rays within a short period of time could lead to radiation sickness, while lower doses probably could increases the likelihood of radiation-induced cancer. X-rays have found strong application in medical imaging whose benefit greatly surpasses the risk of its cancer inducing effect. However, the ionizing potentials of X-rays can also be annexed in and used in cancer treatment in the form of radiotherapy to malignant cells. X-rays have also found application in x-ray crystallography and in microscopy as it is possible to examine structure smaller than what the normal microscope can see thus making it possible to acquire images with high resolution. They are very adaptable for these techniques as they possess shorter wave length. Ever since Röntgen's discovered that X-rays can be used to identify bony structures, X-rays have been used for medical imaging (Spiegel 1995).

2.3. Biological Effects of Radiation

Humans are exposed to ionizing radiation from natural sources and human-made sources through external and internal exposure (Ramachandran, 2011). The external exposure is considered when the body is exposed to ionizing emitted from an outside sources and radiation energy is absorbed in the body from outside to inside. Living organisms can be affected in two ways when they are exposed to ionizing radiation. The direct effect of radiation occurs when radiation targets DNA molecules directly thus leading to a single or double-stranded DNA damage. On the other hand, the indirect effect of radiation occurs when radiation targets water molecules thus leading to a split of the water molecule which in turn generates free radicals (Kawamura *et al*., 2018).

2.3.1. Direct Effects of Radiation

The direct effect of radiation occurs when radiation targets DNA molecules directly thus leading to a single or double-stranded DNA damage. More so, other cellular components of a cell critical for survival might be affected (Nelson, 2003). This interaction with the cell could alter the ability of the cells to replicate and eventually survival. This might also give rise to several lesions associated with double- stranded DNA damage which could threaten the existence of a cell as the chromosome content might have been altered (Karbownik and Reiter, 2000).

2.3.2. Indirect Effect of Radiation

The indirect effect of radiation is as a result of the radiolytic decomposition of water. When cells are exposed to radiation, they sometimes don't target the DNA but have a direct effect on water as water makes up about 60% of the cell. This interaction of radiation with the water molecule could in turn split the water molecule into hydrogen ions (H^+) and hydroxyl ions (OH^-) which is known as hydrolysis. The components of the splitted water molecule could interact again to form water or hydrogen gas. However, they could also react to form hydrogen peroxide free radicals which in turn could lead to lipid peroxidation as well as free radical induced cell death as they adversely affect other components of the cells like proteins, lipids and carbohydrates (Lehnert and Iyer, 2002; Spitz *et al*., 2004). Some of the reactions involved are as follows:

1. H_2O (molecule) + ionizing radiation $\longrightarrow H^+ + OH^-$ (hydroxyl, free radical)
2. $H^+ + H^+ \longrightarrow H_2$ (Hydrogen gas)
3. $H^+ + OH^- \longrightarrow H_2O$ (water)

Considering the above, exposure cells to radiation could lead to; destruction of the cell membrane through lipid peroxidation, single or double stranded DNA damage as well as generation of free radicals which subsequently leads to damage in cells. Thus a substance that improves the body antioxidant defense system would help ameliorate the effects of radiation.

2.4. Mechanism of Radiation-induced Brain Injury

Radiation therapy is used widely for the treatment of diffuse primary and metastatic brain tumors (Tsao *et al*., 2005). Most times after radiation therapy in brain tumours condition, patients usually suffer from a condition termed "Radiation-induced brain injury (RIBI)", which encompasses a variety of clinical manifestations ranging from elevated intracranial pressure, mental and behavioural disorders, focal neurological deficits, secondary epilepsy as well as progressive deterioration of the hippocampal-associated learning and memory functions (Roman and Sperduto, 1995). These conditions can be very devastating to patients and caregivers and can be classified into acute, early delayed and late delayed injury as shown in figure 2.2 (Tofilon and Fike, 2000). Acute effects usually occur shortly after exposure to radiation ranging from days to while early delayed effects occur about 1 – 6 months after radiation and are characterized by generalized weakness and transient demyelination and are sometimes reversible. On the other hand, the late effects usually kick start from 6 months and beyond after radiation it is at this point numerous irreversible neurological deficits set in. It is characterized by cognitive deficits (Taphoorna and Martin 2004) as well as necrosis of the brain parenchyma and vascular abnormalities (Schultheiss and Stephens, 1992). Crossen *et al*., (1994) also reported radiation affected neurobehavioral outcomes in humans.

The underlying mechanisms of radiation-induced brain injury are not well understood but Hopewell (1979) proposed two theories. The first was that; most severe consequences of radiation exposure emanates from direct impairment of brain parenchymal cells, while vascular changes were considered to be less importance. On the other hand, the second theory was that radiation exposure which leads to destruction of the vascular system is of grave significance as it results to brain ischemia. However, reactive oxygen species generations in cells, blood-brain barrier destruction, apoptosis and neurogenesis inhibition as well as non-specific inflammation in brain cells are ways radiation exposure could affect the brain.

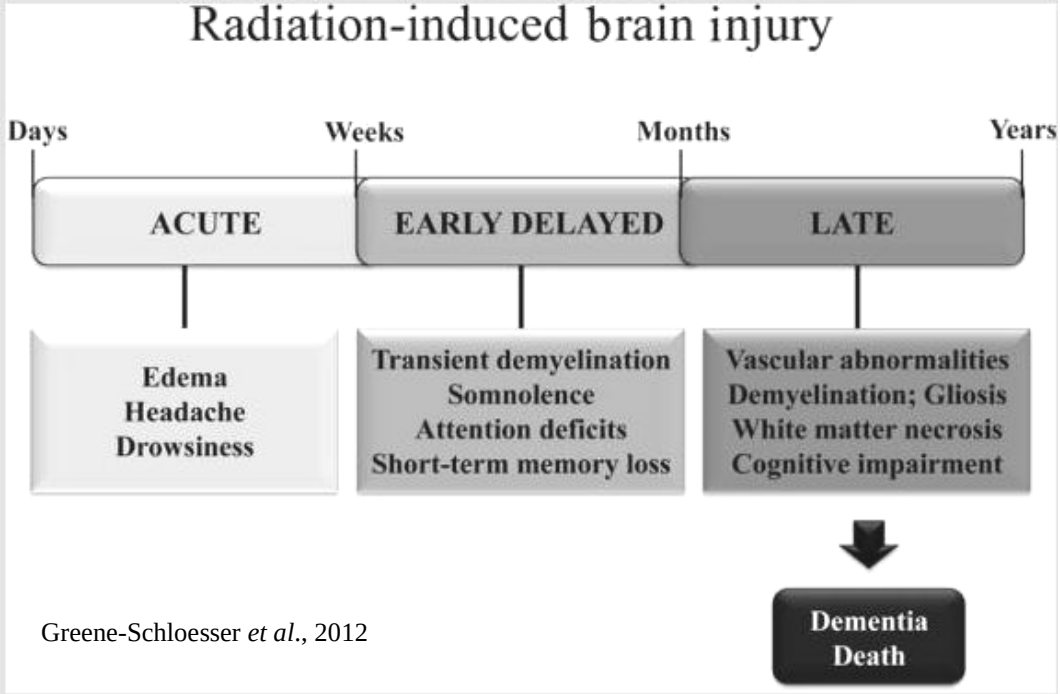

Figure 2.2: Figure showing the stages of radiation-induced brain injury

2.4.1. Reactive Oxygen Species

Following radiation therapy, microglia system is activated and it's closely followed by infiltration of immune cells to the brain. These cells then produce ROS and an imbalance between ROS production and ROS clearance may lead to oxidative stress (Zhao, *et al*, 2013). The generated ROS can cause disruption to several cellular components including, DNA, lipids and proteins and finally culminating to apoptosis (Gorman *et al*., 1996)

2.4.2. Blood Brain Barrier (BBB) disruption and Nonspecific inflammation

Exposure to radiation can lead to the disruption of the blood brain barrier due to apoptosis of the endothelial cells. Apoptosis of endothelial cells can also lead to chronic hypoxia and peritumoral tissue oedema (Li *et al*., 2003). Nakata *et al*., (1995) reported that changes in BBB permeability has been thought to be the most sensitive and reliable index for detection of early RIBI.

Furthermore, BBB disruption triggers other nonspecific inflammation cascades. It has been reported that radiation might induce astrocytes proliferation and which could lead to secretion of

a high amount of pro-inflammatory mediators which aids infiltration of leucocytes into the brain through the blood-brain barrier (BBB) (Zhou *et al*., 2011). More so, mmicroglia activation is usually followed after radiation exposure and this could lead to a surge in ROS generation as well as other cytokines involved in neuroinflammation (Conner *et al*, 2010).

2.4.3. Inhibition Apoptosis and neurogenesis

RIBI may also trigger neuronal apoptosis and inhibition of neurogenesis. It has been reported that radiation-induced apoptotic cell death appears in the rodent CNS between 3 and 4 h after exposure and peaks at 6–12 h (Shinohara *et al*., 1997 and Pena *et al*., 2000). The affected cells were reported to include oligodendrocytes, sub-ependymal cells, certain types of neurons, endothelial cells as well as neural precursor cells in the dentate gyrus (DG) hippocampal region.

2.5. Medicinal Plants with Radioprotective Properties

The brain is a very delicate and highly vulnerable to oxidative stress as the neurons and glial cells contain relatively low levels of antioxidants enzymes like SOD, gluthathione peroxidase and catalase compared with other tissues (Dringen *et al*., 2000). Furthermore, the membranes of myelin also possess high levels of peroxidizable fatty acids, which contribute greatly to their vulnerability to ROS (Smith *et al*., 1999). Considering this, medicinal plants and plant metabolites have been demonstrated to serve as potential radioprotectant as they help to build up the body antioxidant defence system.

In a study by Naika *et al*. (2004), the potential antioxidant effect of *Terminalia chebula* was evaluated on γ-radiation-induced lipid peroxidation in rat liver microsomes and damage to superoxide dismutase enzyme in rat liver mitochondria. The DPPH radical was used as a marker and for antioxidant status and it was observed that the plant extract was able to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals. They further concluded that aqueous extract of *Terminalia chebula* could be a probable radioprotector as it protected cellular organelles from radiation induced damage.

Goel *et al*., (2002), investigated the radioprotective effect of *Hippophae rhamnoides* L. (RH-3) for its radioprotective effect in whole body irradiation in mice. Whole body survival, spleen

colony forming units (CFU), haematological indices as well as free radical scavenging potentials were investigated. Their results show that RH-3 lead to 82% survival in mice as well as normalizing spleen colony forming units count, haematological indices. Also, RH-3 was able to inhibit generation of hydroxyl and superoxide free radicals and this was found to be dose dependent and they concluded that the free radical scavenging activity as well as immune-modulatory effect of the extract might attribute to its radioprotective properties.

Again, Goel *et al*., (2004) also investigated the radioprotective effect *Tinospora cordifolia* (RTc) in whole body gamma-irradiation in mice for its radioprotective potentials in terms of whole body survival, spleen colony forming units (CFU), hematological parameters, cell cycle progression, and micronuclei induction. Pre-treatment with RTc lead to 76.3% survival after 30 days as well as prevented weight loss when compared with 100% mortality. It was also observed that CFU counts in spleen as well as total lymphocyte counts which were decreased following irradiation were seeing to be restored towards normalcy at day 15 post irradiation. S-phase cell population that was reduced following 2 Gy irradiation were seeing to be on the rise while irradiation-induced increase in micronuclei was seeing to be reducing by a pre-irradiation treatment of RTc.

In another study, the mechanism of radioprotection by *Podophyllum hexandrum* was investigated in whole body gamma irradiated male Swiss albino mice. The levels of Glutathione S-transfers (GST), catalase, superoxide dismutase (SOD) activities and lipid peroxidation (LPx) were determined in the liver, jejunum and ileum at various time points in the presence of or absence of aqueous extract of *P. hexandrum* rhizome. Pre-treatment with plant extracts significantly elevated SOD and GST levels in liver and intestinal issues but no change was found in catalase level. They concluded that the radioprotective effect of *Podophyllum* sp. against whole body lethal irradiation might be attributed to the high level antioxidant activity present in the plant extract (Mittal *et al*., 2001)

Again, the effect of aqueous extract of triphala (an Ayurvedic herbal medicine) was studied on radiation-induced mortality in mice exposed to 10 Gy of γ-radiation. Pre-treatment of mice with the plant extract prior to irradiation delayed the onset of mortality and also reduced the symptoms of radiation sickness (Jagetia *et al*., 2002).

The *in vitro* radioprotective potential of *Ficus racemosa* ethanolic extract (FRE) was studied in Chinese hamster lung fibroblast cells (V79) using micronucleus. They previously demonstrated FRE was able to inhibit DPPH, ABTS, hydroxyl radical and superoxide free radicals as well as lipid peroxidation. Pre-treatment with different doses of FRE prior to 2 Gy γ-radiation resulted in a significant decrease in the percentage of micronucleated binuclear V79 cells. The also showed that at increased concentration and increased radiation intensity of 4 Gy γ-irradiation the radioprotection was still seeing to be significant compared with the respective radiation controls. They concluded that ethanolic extract of *F. racemosa* acts as a potent antioxidant and a probable radioprotector (Veerapur *et al.*, 2009).

2.5.1. Rooibos Tea

Rooibos or *Aspalathus linear* is a shrub-like leguminous plant predominantly found in the Cederberg mountains and neighbouring area of the Western Cape Province of South Africa (Standley *et al.*, 2001). It is characterized by a strong two metre-long taproot, reddish-brown branches and bright green, needle-shaped leaves (McKay *et al.*, 2007; Van Niekerk, 2008). It is very well cultivated around this area for commercial purposes as an herbal tea. The leaves are used to make an herbal tea called rooibos or bush tea or sometimes red-bush tea. This herbal tea is very popular and unique to the people of South Africa and may be considered as the country's unofficial beverage (Wilson, 2005). In South Africa, rooibos tea has been documented to have many functions, ranging from appetite stimulation, enhanced gastrointestinal motility as well as controlling mental condition (Morton, 1983; Nakano *et al.*, 1997). Rooibos tea can be categorized in to two based on preparation; the first is the unfermented product remains green in colour and is referred to as green rooibos tea. While the second is the red tea or red rooibos tea which is the fermented rooibos tea as fermentation leads to colour change from green to red due to oxidation of the constituent polyphenols.

Figure 2.3: Rooibos plantation in the Clanwilliam area (Joubert and de Beer, 2011).

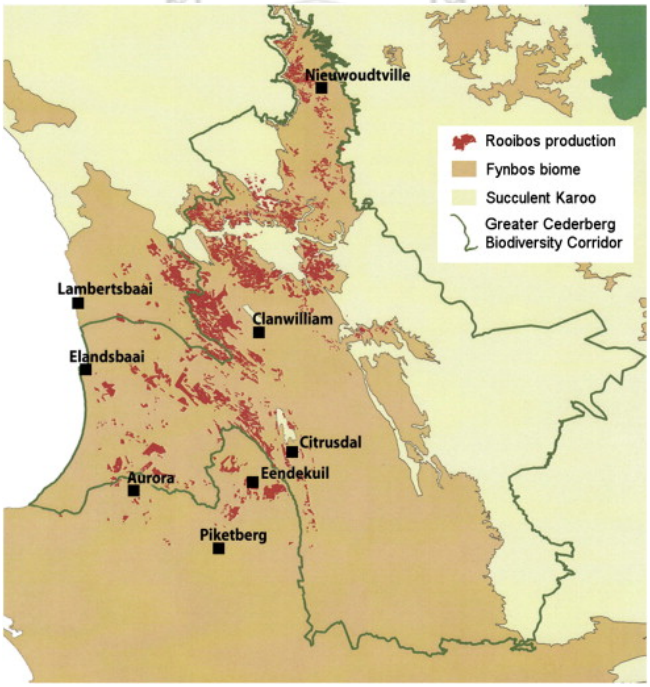

Figure 2.4: Production areas of *Aspalathus linearis* in and around the Greater Cederberg Biodiversity Corridor (Joubert *et al.*, 2011)

2.5.2. History of Rooibos Tea

The first recorded use of rooibos tea can be dated back as early as the 17th century when the harvested leaves and stems of rooibos plant was served as beverage among the mountain-dwelling tribe of Khoi in the Clanwilliam region of the Western Cape (E Joubert *et al.*, 2008). Traditionally, the khoi people climbed the mountains to cut the fine, needle-like leaves from wild rooibos plants. They prepared fermen ted rooibos by chopping the leaves with axes as well as bruising with hammers, before being left in the sun to dry (E Joubert *et al.*, 2008). This process is believed to be the basis of the industrialized process for fermented rooibos that is common today. The major hurdle in growing rooibos commercially back then was that farmers could not germinate the rooibos seeds and as such the tea was harvested from wild rooibos until the 1930's. In 1930, rooibos was developed into a crop plant by district surgeon and botanist Dr Pieter Le Fras Nortier who began conducting experiments which lead to the cultivation of the rooibos plant. Dr Nortier also saw the vast commercial potential the tea held for the region. Ever since then, rooibos plantations are now a common sight in that region.

Today rooibos is a household name in South Africa and concerted effort has been made to rebrand and package rooibos tea in so many different forms. A very trending package in the rooibos market is the unfermented rooibos which has high antioxidant potential with other health benefits and as such high in demand. It has been reported that unfermented rooibos was first introduced for a scientific study on antioxidant activity of rooibos in the mid-1990s (Von Gadow *et al.*, 1997). Presently, rooibos has grown past being utilized as just a herbal drink but has also gain popularity in the cosmetic industry, brewery and food industry (Mahomoodally, 2013). Furthermore, as a result of its antioxidant potential, the consumption rate of rooibos has been reported to have increased globally (Mahomoodally, 2013).

2.5.3. Taxonomy

Aspalathus linearis belongs to the family of the *Fabaceae* and about 278 species in the genus *Aspalathus* are restricted to South Africa (Dahlgren, 1988; McKay and Blumberg, 2007. Aspalathus linearis ssp. linearis is the most common of the subspecies and includes those types used for tea production. 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).

2.5.4. 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	Aspalathus L.	Aspalathus
Species	Aspalathus linearis (Burm. f.)	

2.5.5. Active Ingredients and Chemical composition

The range of compounds present in a rooibos infusion is responsible for the flavour, colour and functional properties of the tea. A number of research groups have analyzed the chemical composition of the tea as well as the changes in the chemical profile that take place during fermentation. Brewed rooibos tea (1 tsp/cup) contains 300 mg protein with the amounts of Cu, Fl and Mn present in this single serving providing 7.8%, 5.5–7.3% and 1.7–2.2% of the U.S. Daily Value (DV), respectively (Erickson, 2003) while Ca, Fe, K, Mg, Na, PO4 and Zn (Hesseling *et al*., 1979; Morton, 1983) constitute less than 1% DV.

Several phenolic compounds are present in the brewed teas of both green and red rooibos, but the total concentration of flavonoids in each can differ by more than 10-fold (Bramati *et al*., 2003; Bramati *et al*., 2002). Of the several components, rooibos has been shown to be composed of two unique phenolic compounds, which are, aspalathin and aspalalinin. Aspalathin is a dihydrochalcone *C*-glucoside while aspalalinin is a cyclic dihydrochalcone (Shimamura *et al*., 2006). Currently rooibos is the only known natural source of aspalathin and one of only two known sources of nothofagin (Joubert, 1996). Other major phenolic compounds present in

rooibos include flavones (orientin, isoorientin, vitexin, isovitexin, luteolin, chrysoeriol), flavanones (dihydro-orientin, dihydro-isoorientin, hemiphlorin) and flavonols (quercetin, hyperoside, isoquercitrin, rutin) (Open *et al*., 1962, Marais *et al*., 2000, Rabe *et al*., 1994, Ferreira *et al*., 1995 and Shimamura *et al*., 2006). A summary of the active compounds in rooibos is shown in the table below.

Table 2.1: Bioactive compounds present in Rooibos and their antioxidant activity. (Joubert and de Beer, 2014)

	Free Radical Scanvenging Assay			Lipid Peroxidation Assays			
Compounds	**ABTS (IC50, µM) a**	**ABTS (TEAC) b**	**DPPH (% Inhibition)c**	**Superoxide (% Inhibition) d**	**Microsomes (IC50, 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* (-)	-	-	-	-

2.6. Bioactivity of Rooibos

2.6.1. Anti-inflammatory properties

The in vivo anti-inflammatory effect of rooibos tea was demonstrated by Haruna *et al*., 2009 in Dextran Sodium Sulphate induced colitis in seven weeks old Wister rats. Rooibos administration lead to a significant ($P < 0.05$) increase in serum SOD levels and a decrease in urine levels of 8-hydroxy-2′-deoxyguanosine. They showed that showed that unfermented rooibos tea was able to regulate serum levels of SOD which remained significantly higher in the rooibos group when compared to the controls after induction of colitis. They also suggested that Rooibos tea may reduce DNA damage from oxidation stress by its anti-oxidative activity *in vivo*.

2.6.2. Anti-mutagenic properties

Rooibos has been shown to possess antimutagenic properties and this has been reported in a number of research output. Marnewicka *et al*., (2000) reported that aqueous extracts of fermented and unfermented rooibos tea (*Aspalathus linearis*) as well as honeybush tea (*Cyclopia intermedia*) possess antimutagenic activity against 2-acetylaminofluorene (2-AAF) and aflatoxin B_1 (AFB_1)-induced mutagenesis using tester strains TA98 and TA100 in the presence of metabolic activation. Their result suggest two potential mechanism of action which are, interference of tea with cytochrome P450-mediated metabolism of these mutagens and the direct interaction between the tea constituents, presumably the polyphenolic compounds, with the promutagens and/or the active mutagenic metabolites.

In another study, the antimutagenic properties of the most prevalent flavonoids contained in rooibos (*Aspalathus linearis*) were compared in the *Salmonella typhimurium* mutagenicity assay using tester strains TA98 and TA100 with, respectively, 2-acetamido-fluorene (2-AAF) and aflatoxin B_1 (AFB_1) as mutagens in the presence of metabolic activation. It was demonstrated from their findings that Aspalathin and nothofagin as well as their structural flavonoid analogues displayed moderate antimutagenic properties while luteolin and to some extent, chrysoeriol, showed activities comparable to those of the green tea flavonoid (−) epigallocatechin gallate (EGCG) (Snijman *et al*., 2007).

Furthermore, a comparative study of the antimutagenic activity of aqueous extracts of the South African herbal teas, *Aspalathus linearis* (rooibos) and *Cyclopia* spp. (honeybush) with that of *Camellia sinensis* (black, oolong and green) teas in the *Salmonella* mutagenicity assay using aflatoxin B_1 (AFB_1) and 2-acetylaminofluorene (2-AAF) as mutagens had been done by Van der Merwe *et al*., 2006. The herbal teas demonstrated protection against both mutagens in the presence of metabolic activation as the antimutagenic activity of "fermented" (oxidised) rooibos was significantly ($P < 0.05$) less than that of *Camellia sinensis* teas against AFB_1, while for 2-AAF it was less ($P < 0.05$) than that of black tea and similar ($P > 0.05$) to that of oolong and green teas. Unfermented rooibos was less effective than the *C. sinensis* teas and fermented rooibos, but had similar ($P > 0.05$) antimutagenicity to that of fermented *C. sessiliflora* against AFB_1 and fermented *C. subternata* against 2-AAF (van der Merwe *et al*., 2006).

The chemoprotective potentials of unfermented and fermented rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*) herbal teas, and green and black teas (*Camellia sinensis*) has been investigated against fumonisin B_1 (FB_1) promotion in rat liver utilizing diethylnitrosamine (DEN) as the cancer initiator. Data show that the teas differentially affected the clinical chemical markers associated with liver and kidney damage associated with FB_1. Green tea enhanced ($P < 0.05$) the FB_1-induced reduction of the oxygen radical absorbance capacity, while fermented herbal teas and unfermented honeybush significantly ($P < 0.05$) decreased FB_1-induced lipid peroxidation in the liver. The teas also modulated activities of antioxidant markers like; catalase, glutathione peroxidase (GPx) glutathione reductase (GR) as well as the glutathione (GSH) (Marnewick *et al*., 2009).

Again, the effect of ethanol/acetone (E/A) soluble fractions of processed and unprocessed rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*) as well as green (*Camellia sinensis*) teas was investigated in a two-stage mouse skin carcinogenesis assay. It was revealed that topical application of the herbal tea fractions prior to the tumour promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), on ICR mouse skin initiated with 7,12-dimethylbenz[*a*]anthracene (DMBA) significantly suppressed skin tumorigenesis (Marnewick *et al*., 2005).

In another study, the effect of 3 herbal teas green tea (GT) from Japan, Po-lei tea (PT) from China, and Rooibos tea (RT including rooibos was investigated on the induction of chromosome aberrations in cultured CHO cells and mice. Exposure of CHO cells to the

various tea extract in the presence of rat liver microsomal enzymes (S9 mix) together with benzo[*a*]pyrene (B(a)P) or mitomycin C (MMC), lead to a reduction in the frequency of chromosome aberrations observed (Sasaki *et al*., 1993). Also oral administration of tea extracts again inhibited the formation of micronuclei by benzo[*a*]pyrene as well as mitomycin C and they concluded that, intake of tea might suppress the mutagenic activity of certain potent mutagens in human beings.

2.6.3. Hepatoprotective properties

Hepatoprotective activity of rooibos tea *(Aspalathus linearis)* was investigated in carbon tetrachloride (CCl_4) induced liver damage in experimental rat models. Rooibos tea administration leads to a reduction of steatosis and cirrhosis in the liver tissue as well as significantly inhibiting a rise in liver tissue concentrations of malondialdehyde, triacylglycerols and cholesterol. In the same vain, rooibos tea significantly suppressed the increase in plasma activities of liver function markers like; aminotransferases, alkaline phosphatase and billirubin. And they concluded that rooibos might serve as a plant hepatoprotector in the diet of patients with hepatopathies (Ulcina *et al.*, 2003).

2.6.4. Reproductive properties

Unfermented and fermented rooibos contain high levels of antioxidants which inhibit reactive oxygen species (ROS) and this was tested in male rats as spermatoazoa are highly prone to damages from reactive oxygen species. Male rats were administered 2% or 5% unfermented or fermented rooibos tea as the only source of drinking for 52 days and it was observed that no marked difference were observed in serum antioxidant capacity, body and reproductive organ weight of animals. However, testosterone levels were slightly lowered but spermatogenesis was not affected as abundant sperm was contained in the lumen. Simultaneously, a significant decrease in the height of the germinal epithelial layer and tubule diameter was noticed. They also observed sperm motility, viability and concentration to be on the increase as a result of RT administration and this was followed with slight increase in acrosome reaction (Monsees and Opuwari, 2013).

2.6.5. Anti-ageing properties

Rooibos has been demonstrated to be a potential anti-ageing agent as its anti-ageing activity was investigated in oxidative stress and mitochondrial dysfunction *in vitro* culture models. It was observed that RT was able to improve cell viability in the presence of toxins. Consequently, rooibos also protected against mitochondrial DNA depelteion in preadipocytes cells 3T3-L1 ρ^0 deficient in Mitochondrial DNA (mtDNA) as a result of continuous exposure of cells to ethidium bromide (EtBr). Depletion of the mtDNA lead to a significant reduction of mitochondrial membrane potential and rate of proliferation in culture, as well as an increased glucose utilization and lactate production. All these were seeing to be reversed following rooibos treatment and they concluded rooibos extracts exhibit effects which preserve the functional capacity of pre-adipocytes exposed to ageing related insults (Hattingh *et al*., 2014).

2.6.6. Cardiovascular properties

The effects of RT as well as other herbal teas have been investigated on Angiotensin Converting Enzymes (ACE) activity in Seventeen healthy volunteers. After oral intake of a single dose of rooibos tea ACE activity was analysed with a commercial radioenzymatic assay while ACE genotype was determined using a PCR method. Result show that all herbal teas significantly inhibited ACE activity after 30, 60 and 180 minutes ACE activity and this may affect blood pressure regulation and thereby preventing cardiovascular diseases (Persson *et al*., 2009).

A study involving human subjects to evaluate the effect of rooibos tea on biochemical and oxidative stress parameters in adults at risk for cardiovascular disease was done in 40 volunteers who drank six cups of fermented/traditional rooibos daily for 6 weeks, followed by a control period. Results show that plasma antioxidant capacity remained the same but an increase in polyphenol levels was observed. Also, lipid peroxidation as well as serum lipid levels were decreased while reduced glutathione levels was increased after rooibos intake compared with control values (Marnewick *et al*., 2011).

In another study, the cardioprotective effects of the main polyphenolic compounds contained in rooibos were investigated against ischaemia/reperfusion injury in male Wistar rats. Rooibos (2%) and green tea was administered for 7 weeks and after which their hearts were harvested and perfused in a working heart perfusion apparatus. They observed that rooibos

administration markedly enhanced aortic output recovery after reperfusion. In addition, there was a significant reduction of cleaved caspse-3 and PARP pro-apoptotic proteins during reperfusion suggesting an inhibition of apoptosis (Pantsi *et al*., 2011).

2.6.7. Antidiabetic properties

The protective potential of the green rooibos tea component aspalathin was investigated on glucose metabolism both *in vitr*o in L6 myotubes as well as RIN-5F pancreatic β-cellsand and type 2 diabetes mice model *in vivo*. Aspalathin in a dose dependent manner significantly increased glucose uptake by L6 myotubes and insulin secretion from cultured RIN-5F cells. Dietary aspalathin (0.1–0.2%) also suppressed the increase in fasting blood glucose levels and improved impaired glucose tolerance of experimental mice models. They concluded that aspalathin might possess a positive effect on glucose metabolism in type-2 diabetes as it promotes uptake of glucose in muscle tissues and secretion of insulin from pancreatic β-cells (Kawano *et al*., 2008).

Furthermore, the protective effect of rooibos against Diabetic cardiomyopathy (DCM) was tested in cultured cardiac myocytes of heart muscles excised from streptozotocin induced diabetes in rats. Cultured cardiomyocytes that were treated with rooibos showed improved activity of glutathione activity and amelioration of apoptosis as compared to myocytes exposed to Hydrogen peroxide or an ischemic solution. This activity was attributed to the antioxidant effects of rooibos (Dludla *et al*., 2014)

2.6.8. Radioprotective effect

Rooibos tea has also been shown to have radioprotective properties which may be attributed to the radical scavenging properties of their flavonoid content (Shimoi *et al*., 1994 and Shimoi *et al*., 1996). Flavonoids are a class of polyphenol compounds present in a large number of plants including rooibos tea. In other studies involving whole body gamma radiation exposure resulted in increased lipid peroxidation, altered enzyme levels, damage to blood and other cellular DNA, most of which were ameliorated by pre-consumption of gallic acid (Gandhi and Nair, 2005; Manu, Leyon and Kuttan, 2007; Nair & Nair, 2013). A study in rats showed potential radioprotective ability of 17-β-estradiol (βE) possibly via an antioxidant mechanism of action Caceres *et al*., (2011). Studies on the radioprotective properties of plant extracts or compounds on the nervous system are scanty in literature, which was a motivation for the present investigation.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Ethical Considerations

Ethical clearance for this study was approved by the Faculty of Natural Science Research Ethics Committee of the University of the Western Cape, Cape Town, South Africa. Ethical and project registration numbers were assigned to the research project before commencement (Ethics Registration No: 13/10/94).

3.2. 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.

3.3. 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; Pantsi *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).

3.4. Animals

Twenty-four (24) female Wistar rats approximately two months old, with average weight between 180-280 g were used for this study. Animals were procured from the University of Stellenbosch animal facility, Cape Town, South Africa and maintained under standard laboratory conditions at the animal house of the Department of Medical Bioscience, University of the Western Cape, South Africa. Daily body weights of rats were taken using a

weighing balance (Adam Equipment Ltd, Milton Keynes, United Kingdom) and progressive weight changes relative to the initial weights were noted. Mating took place by keeping one male rat with one female rat (1:1) in a cage for seven days. During mating, pregnancy and post-partum periods, animals were allowed free access to standard rat chow and tap water. Pregnancy was confirmed by the presence of vaginal plug as well as weight gain as previously described (Heyne *et al*, 2015). Pregnant dams were randomly assigned to four treatment groups of six dams each (n = 6; total = 24) as shown in Table 3.1.

Table 3.1: Animal Grouping

Groups	No. of dams	No. of pups sacrificed
G-1: Normal saline	6	12
G-2: Irradiation	6	12
G-3: Rooibos tea	6	12
G-4: Rooibos tea + irradiation	6	12
Total	24	48

3.5. Treatment Protocol

Throughout pregnancy, dams were randomly assigned to four different groups of six rats each (n=6) and housed in separate cages, and water in the drinking bottles was replaced in all but one group – G-2 as summarized below:

G-1 received 20 % normal saline (NS) *ad libitum*
G-2 received tap water *ad libitum*
G-3 received 20 % fermented rooibos tea *ad libitum*
G-4 received 20 % fermented rooibos tea *ad libitum*

After parturition, pups remained with mothers in cages maintained at temperature 21–24ºC under a 12 hours light and 12 hours darkness cycle. In order to expose the pups in G-2 and G-4 to irradiation, eligible 3-day old pups were removed from their maternal cages and placed exposed to a once-off 6 Gy dose of irradiation before being returned to the cages for further maternal grooming and breastfeeding. All treatment with rooibos tea and normal saline continued throughout breastfeeding, hence weaned pups also drank from these treatments until sacrifice on postnatal day (PND) 30. A total of 12 pups per group were sacrificed after

30 days and tissues were processed for routine histological staining and immunohistochemical staining

3.6. Preparation of Rooibos Tea

A concentration of 0.02 g/ml of fermented rooibos herbal tea reported to be the routine amount used by rooibos consumers for tea-making purposes (Marnewick *et al*., 2003; Pantsi *et al*., 2011) was used throughout this study. Briefly, 1000 ml freshly boiled tap water was added to 20 g of fermented rooibos leaves and stems for 5 minutes followed by filtration with cheese cloth and Whatman's filter papers (no 4 and 1 respectively). The aqueous extract was then allowed to cool off to room temperature before administration rats *ad libitum*. Fresh tea was prepared every second day. Daily intake of rooibos tea and water was measured throughout the experimental period by subtracting the volume of the remaining fluid from the initial volume. No major fluid leakage from water bottles was observed and the average consumption rate per cage was 40 ml/day.

3.7. Radiation facility and procedure

All eligible Wistar rat pups were exposed at the radiation facility of ithemba Laboratories Fourie, South Africa on PND 3. Animals were immobilized using a clear plugged restraining tube and a selective cranial irradiation setup was employed to expose the pups using a Cobalt-60 source (Theratron 780). The posterior field margin (5cm x 30cm) was positioned to include the cranium up to the occipital protuberance so that the whole skull was irradiated. The dose specified at the skull surface in the central axis of the beam corresponded to 6 Gy (0.5 Gy/min) at SSD 75 cm. Calibration and dose verification on the Theratron 60Co γ-ray teletherapy unit was performed with a NE farmer-type 0.6cc ionization chamber and matched electrometer.

3.8. Chemicals and Equipment

All chemicals used for this study were of analytical grade and were obtained from certified suppliers (Table 3.2). Equipment for this study was also of approved quality and obtained from certified suppliers (Table 3.3).

Table 3.2 List of chemical and supplier

Product	Supplier
DPK	Kimix (SA)
Ethanol	Merck Chemicals (Germany)
Haematoxylin and eosin (H & E) stain	Merck Chemicals (Germany)
Parafine wax	Merck Chemicals (Germany)
Paraformaldehyde (4%) (PFD)	Merck Chemicals (Germany)
Phosphate Buffered Saline (PBS)	Merck Chemicals (Germany)
Potassium chloride(KCl)	Merck Chemicals (Germany)
Sodium dihydrogen (Na_2HPO_4)	Merck Chemicals (Germany)
Sodium chloride (NaCl)	Merck Chemicals (Germany)
Sodium dihydrogen(NaH_2)	Merck Chemicals (Germany)
Sodium hydroxide(NaOH)	Merck Chemicals (Germany)
Xylene	Kimix (SA)

Table 3.3 List of equipment used and supplier

Instrument	Manufacturer
Centrifuge (5417R)	Germany
Cimarec+™ Stirring Hotplates Series	Canada
Dehydration	Leica tp (1020) Germany
Microtome	Leica rm (2125) RT Germany
RADWAG Wagi Elektroniczne	Poland
Ultra turrax t25 homogenizer	Germany
Tissue Embedding	(TEK) Germany
Water bath	Electrothermal (England)

3.9. Neurobehavioral Test

Neurobehavioral studies are often used to assess behavioural changes and gene expression effects induced by exposure to treatments of varying types, concentrations, dosages (Seale *et al*, 2012). Commonly assessed neurobehavioral parameters may include locomotor activity, cognitive function (e.g. memory deficit, etc.), gait, depression, anxiety, equilibrium, etc. In this study, only the open field test and the novel object recognition test were done.

3.9.1. Open Field

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 was a square plexi glass box (72 × 72 × 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, for ease of data analysis. Animals were tested singly, each transported from the housing room to the testing room and allowed to acclimatize in the OF apparatus. Pre-testing was done for 2 days to prepare the

animals. On the third day, each rat was placed in the centre zone of the OF arena and observed for 5 minutes. This was repeated twice after which the rat was returned into its home cage and the OF box cleaned with 70% ethanol before testing the next rat. Video recording of all tests was done using the overhead camera. The recordings were analysed using the Smart video tracking software version 3.0, from Panlab Harvard Apparatus (Massachusetts, USA) to measure the locomotor activity of each rat by extracting the total distance travelled in the OF arena. As a measure of anxiety, the total distance travelled in the 12 squares near the walls was compared with the distance travelled in the 4 squares at the centre of the arena. All analysis was done by researcher and two other "blind" observers and the results were averaged.

3.9.2. Novel Objective Recognition (NOR) test

This test is based on the main assumption that access to novelty (e.g. an object or an environment) can elicit approach behaviours in animals (Ennanceur and Delacour, 1988). The NOR test is a valid task that can be used to assess working memory as seen in the ability of animals to recognize a novel object in a familiar environment. The apparatus for the NOR test included a square plastic/glass box (40x40x25 cm), with a digital camera (Samsung HMX-F90, South Korea) mounted directly above it. The object test arena was divided into 4 equal squares via a 2 × 2 grid, to assist in data analysis and animals were tested singly before test. In our study, two dissimilar "test" objects (in shape, size, colour) were put inside the animal cage as "enrichment" and left for two days to enable the animal familiarize with the objects.

On the day of testing, the objects were removed from the cages and placed inside the box in the testing room and one of the test objects was replaced with a new (novel). The test animal was then put into the box and left for 3 minutes before being returned to its home cage. The interaction of the animal with the objects was recorded by the overhead camera and analysed by the researcher and two "blind" observers. It is expected that if the animal 'remembers' the previous exposure to the familiar object, it will explore the novel object to a greater degree than the familiar one. The testing box was cleaned with 70% ethanol before testing the next animal.

The recognition index (RI) was our chosen measure of preference for the novel object and has been considered the main index of retention (Botton *et al*. 2010; Gaskin *et al*. 2010)

3.10. Animal sacrifice

The final body weight of each animal was taken before injection of 150 mg / bw (i.p) sodium pentobarbital followed by decapitation under the deep anesthesia. The sacrifice was done 3 hours after the final neurobehavioral testing. The brains were removed and weighed immediately (wet weight = WW). Six brains per group per sacrifice day were carefully fixed in 4% paraformaldehyde solution for histology and the other six brains were preserved in phosphate buffered saline (PBS) for neurochemical analysis.

3.11. Histological preparation of brain samples

3.11.1. Automated tissue processing

After two days, brain specimens were put in appropriately labelled cassettes before processing in a Leica-2125 automated tissue processor, ATP (Leica, Germany) for 7 hours in preparation for sectioning, staining and microscopic analysis. The programme selected for the brain specimens consisted of 4 hours of dehydration (through different grades of ethanol to remove water), 1 hour 30 minutes of xylene clearing (to remove ethanol) and 1 hour 30 minutes of infiltration with molten paraffin wax (to replace xylene) (Table 3.4).

Table 3.4 Tissue processing protocol

The following table shows the concentrations of various solutions:

Station	Solution	Temperature	Duration
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 1	40°C	45 minutes
8	Xylene 2	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

3.11.2. Embedding

The cassettes of brain specimens were removed from the last change of paraffin wax in the ATP and samples manually removed from cassettes and carefully placed inside steel moulds containing liquid paraffin wax in the desired orientation and more liquid wax added until the samples are completely covered by paraffin wax. The moulds were then transferred to the refrigerating plate of an embedding machine to allow the wax to solidify. Tissue blocks were later removed from the moulds and refrigerated until sectioning.

3.11.3. Sectioning

Tissue blocks were removed from refrigeration and allowed for two hours before trimming to appropriate dimensions followed by sectioning at 5 microns using a Leica TP-1020 microtome (Leica, Germany). Each section was placed on warm water (in a water bath with regulated temperature) to allow for spreading before mounting on a labelled glass slide.

3.11.4. Staining

Standard haematoxylin and eosin (H&E), cresyl violet as well as immunohistochemical staining for glial fibrillary acidic protein (GFAP) and Caspase-3 were done for morphometric studies.

3.11.4.1. Haematoxylin and Eosin

Haematoxylin and Eosin (H & E) staining was done with an autostainer machine (Leica Auto Stainer XL) at the Histology Laboratory of the University of Stellenbosch, South Africa using the standardized protocol in Table 3.5.

Table 3.5 Haematoxylin and Eosin staining protocol

Chemical solution	Time	Repetitions
Deparaffinization and Rehydration		
Oven (60C)	2 minutes	x 1
Xylene	5 minutes	x 2
99 % Ethanol	2 minutes	x 2
96 % Ethanol	2 minutes	x 1
70 % Ethanol	2 minutes	x 1
Tap water	2 minutes	x 1
Haematoxylin Staining		
Haematoxylin	8 minutes	x 1
Running tap water	5 minutes	x 1
Eosin Staining and Dehydration		
Eosin - counterstaining	4 minute	x 1
Running tap water	1 minute	x 1
70% Ethanol	30 minutes	x 1
96 % Ethanol	30 minutes	x 2
99% Ethanol	30 minutes	x 1
Xylene	1 minute	x 1
Mounting for observation was done using DPX and slide cover slips.		

3.11.4.2. Cresyl Violet / Nissl staining

This staining method 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 H & E staining (Li, 2012). Briefly, sections were deparaffiniZed in xylene twice for 10 minutes each and then hydrated in 100% ethanol twice for 5 minutes each, then in 95% ethanol for 3 minutes and again in 70% ethanol for 3 minutes. Hydrated sections were then rinsed in tap water and again in distilled water before staining in 0.1% Cresyl violet (Sigma Aldrich, USA) for 5 minutes and rinsed quickly in distilled water. Sections were further differentiated in 95% ethanol for 2 minutes and then dehydrated in 100% ethanol for 10 minutes, cleared in xylene for 10 minutes before coverslip mounting with DPX and drying for 48 hours. Mounting helps to improve the visual quality of the slide under a microscope.

3.11.4.3. Immunohistochemical (IHC) staining

All immunohistochemical (IHC) staining procedures were done using automation with the Leica Bond Autostainer and the Bond Polymer at the Histology Laboratory of the University

of Stellenbosch, South Africa using a standardized protocol for both antibodies. The tissue sections were then manually rehydrated in ethanol grades and cleared in xylene (Table 3.6).

Table 3.6 IHC staining procedure

Step	Type	Incubation Time	Temperature	Dispense Type
1	Peroxide Block	5 min	Ambient	selected vol.
2-4	Bond Wash Solution	0 min each	Ambient	selected vol.
5	Primary Antibody	15 min	Ambient	selected vol.
6-8	Bond Wash Solution	0 min each	Ambient	selected vol.
9	Post Primary	8 min	Ambient	selected vol.
10-12	Bond Wash Solution	2 min each	Ambient	selected vol.
13	Polymer	8 min	Ambient	selected vol.
14-15	Bond Wash Solution	2 min each	Ambient	selected vol.
16-17	Deionized Water	0 min each	Ambient	selected vol.
18	Mixed DAB Refine	10 min	Ambient	selected vol.
19-21	Deionized Water	0 min each	Ambient	selected vol.
22	Haematoxylin	5min	Ambient	selected vol.
23-25	Deionized Water	0 min	Ambient	selected vol.

Rehydration and clearing

Step	Solution	Duration
1	70% alcohol	5 dips
2-3	96% alcohol	5 dips each
4-5	99% alcohol	5 dips each
6-7	Xylene	Dip for 1 min each
Mounting for observation was done using DPX and slide cover slips.		

3.12. Neurochemical Assays

Neurochemical assays are often done to determine the quantities of biological molecules and compounds of interest, ranging from neurotransmitters, neuromodulators, reactive oxygen species, neurosteroids, ions, neurohormones, neuroenzymes, immunogens, ingested drugs, neurotoxins, etc. The neurochemical assays done in this study include thiobarbituric acid reactive substance (TBARS) assay for lipid peroxidation (LPO); superoxide dismutase (SOD) and catalase (CAT) assays for determination of the levels of the main antioxidant enzymes; the glutathione (GSH) and ferric reducing antioxidant power (FRAP) assays for determination of brain antioxidant capacity.

3.12.1. Homogenization of tissues

Six brain specimens per group/day were stored at -80°C and used for this study. The tissues were thawed and homogenized in 10 times (w/v) 0.1M PBS (pH 7.4) in a Teflon glass homogenizer (IKA Laboratories, Germany) 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 assays.

3.12.2. Lipid peroxidation

Lipid peroxidation is the oxidative degradation of lipids to release free radicals, including the major oxidative stress biomarker, malondialdehyde (MDA). The lipid peroxidation (LPO) assay, first described by Wills (1966), involves the reaction of MDA with thiobarbituric acid (TBA) to produce thiobarbituric acid reactive substance (TBARS), a pink chromogen measureable 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 orthophosphoric acid added. The mixture was vortexed for 10 seconds before 12.50 µl of 0.67% TBA (Sigma Aldrich, USA) was added. The resulting reaction mixture was then heated to 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.

3.12.3. Superoxide dismutase activity (SOD)

Total superoxide dismutase enzyme activity was determined by the method of pyrogallol autoxidation according to Marklund and Marklund (1974) with a slight modification. Briefly, 100 µl of supernatant was mixed with Tris-EDTA-HCl buffer (1.5mL, pH 7.4) as well as pyrogallol (100 µL, 15mM in dH_2O) and incubated at 25^{o}C for 10 min. The reaction was determined by the addition of HCl (50µL, 1N), and the activity measured at 440 nm. One unit was determined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50%. The result obtained was expressed as units per milligram of protein.

3.12.4. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay is designed to quantitatively measure antioxidant status in a variety of samples, including the supernatant of brain homogenates in our study. The assay 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 colour that can be monitored spectrophotometrically (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 $FeCl_3 \cdot 6H_2O$ (20 mM) was used to prepare the FRAP reagent, from which 300µl was added to 10 µl of the sample in a clear 96-well plate using a multi-channel pipette. The mixture was then vortexed and incubated in an oven (Memmert, Germany) at 37°C for 30 minutes and absorbance was read at a wavelength of 593 nm in a Multiskan Spectrum automated plate reader (Thermo Fisher Scientific, Waltham, USA). The level of FRAP in the supernatant sample was deduced using a standard curve prepared with a serial dilution of solution of Trolox used as the standard. The results obtained were expressed as µM Trolox/ mL of supernatant (Benzie and Strain 1996).

3.12.5. Catalase activity (CAT) assay

This assay is based on the principle that the ubiquitous antioxidant enzyme, catalase will catalyse the decomposition of the reactive oxygen species (ROS), hydrogen peroxide (H_2O_2) into water and oxygen in tissues. H_2O_2 is a toxic product of normal aerobic metabolism and unconverted H_2O_2 often reacts with a probe to yield a product that can be measured colorimetrically. In this study, catalase activity was measured by mixing 100 µL of supernatant with 400 µL PBS (pH 7.4) and 500 µL of 20mM H_2O_2 at 25^{o}C, followed by reading at 240nm after 2 minutes as previously described (Aebi, 1984). The extinction coefficient of 43.6 $mol^{-1}cm^{-1}$ for H_2O_2 was used for calculation. One unit of CAT is defined as the activity of the enzyme that catalysed the reduction of 1 µmol of H_2O_2 per min per milligram of protein (enzyme).

3.12.6. Glutathione (GSH)

Glutathione is a tripeptide which consists of glutamine, cysteine and glycine and is known to help prevent cellular damage caused by reactive oxygen species (ROS) (Lushchak, 2012).

Total glutathione level was evaluated by reacting 5, 5'- dithiobis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent) with the $^{-}$SH groups of glutathione to give a yellow complex which is absorbed at 412 nm (Ellman, 1959). In brief, 3 mL of Ellman's reagent (4.96mg in 250mL potassium phosphate buffer (0.1M, pH 6.5) was added to 20 µL of supernatant, homogenized and incubated for 15 min and absorbance was measured at 412 nm against blank. Extinction (coefficient of 13,600 mol^{-1} cm^{-1} was used for calculation and results obtained were expressed in millimolar thiol per milligram of protein.

3.13. Statistical analysis:

The results in this study were compared using one-way analysis of variance (ANOVA) test and p-value of less than 5% ($p<0.05$) were considered statistically significant. GraphPad Prism, version 6.0, Graph Pad software, Inc., San Diego Cap2130, USA, was used for further comparison between mean values. A two-way ANOVA followed by Dunnett's multiple comparison tests were used for analysis of relative brain weights, neurobehavioral tests and neurochemical assays. Values were expressed as means ± standard error of mean (SEM).

CHAPTER FOUR

RESULTS

4.1. Introduction

In this study, two groups of pregnant Wistar rat dams (RT and RTX) were administered fermented rooibos herbal tea (FRHT) *ad libitum* during pregnancy and lactation and offspring from one of the rooibos groups (RTX) were exposed to irradiation on postnatal day (PND) 3. A third group of dams (NS) was administered normal saline *ad libitum* and offspring served as control group while another group (X) received tap water *ad libitum* and offspring were irradiated on PND3. Only mature rats at PND30 from each group were subjected to neurobehavioural testing (NBT). Finally, all animals were sacrificed on the respective specified days and the brain samples were removed for laboratory analysis of developmental changes in neurohistomorphology and neurohistochemistry.

Results of the control group (NS) were used as non-pathological reference for all other groups while ordinary one-way ANOVA multiple comparisons test and Uncorrected Fisher's LSD of the (GraphPad Prism version 6.0) was used to compare differences between respective treatment groups for all parameters. This study was done to evaluate the potential benefits of continuous consumption of FRHT in preventing or reducing the severity of radiation injury in developing nervous tissue. Findings from this study will contribute to existing body of knowledge in radioprotective agents present in the large array of accessible and affordable natural products.

4.2. Average Daily Intake of Fluid and Fermented Rooibos Herbal Tea

Daily intake of rooibos tea and water was measured throughout the experimental period by subtracting the volume of the remaining fluid from the initial volume. Throughout the experiment, rats were allowed *ad libitum* access to tap water, normal saline and FRHT respectively depending on the treatment groups. Initially, animals in NS and RT and RTX significantly drank less of the FRHT during the first week of the experiment when compared to the X animals given filtered tap water. As rats became familiar with both fluids from the 3rd week of administration, the rate of consumption approximated tap water consumption such that by the 6th week, no significant difference was observed in the overall amount of

fluid intake across the different experimental groups. No major fluid leakage from the water bottles was observed and the average consumption rate per cage was 40ml/day throughout the study period.

4.3. Neurobehavioural Tests

4.3.1.1. The Open Field Test

The open field test (OFT) is one of the most widely used measures of animal neurobehavioural deficits (NBDs). In the present study, video recordings of offspring rats were used to evaluate total distance travelled, frequency of central square entry (CSE), the frequency of rearing episodes as well as total freezing time. The recordings were analysed by 3 different observers and results were averaged to obtain the final values and statistically analysed for comparisons between the respective groups.

4.3.1.2. Locomotor or exploratory activity

Locomotor behaviour includes all of the acts in which an animal moves from one place to another usually involving movement initiation (warm-up), turning, walking and running, exploration, etc. The total distance travelled by the rats in the open field arena is often used as an estimate of locomotor or exploratory activity. Results obtained in this study (Figure 4.1) showed significantly reduced locomotor or exploratory activity in offspring rats for the X and RT groups respectively. This effect however appeared to be reversed in the RTX groups.

The representative trajectory maps in Figure 4.2, show locomotor activity similar to the results obtained in the graphs (in Figure 4.1).

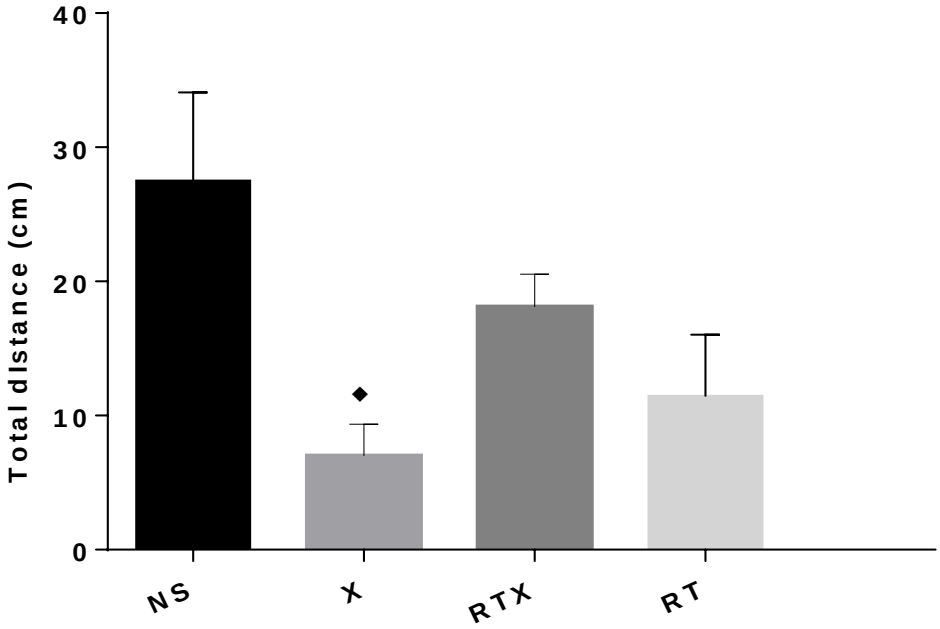

Figure 4. 1: *Average total distance travelled inside the Open Field apparatus. Test duration is 5 minutes per offspring rat. NS: Normal saline, X: Irradiation, RT: Rooibos tea, RTX: Rooibos tea + irradiation. Statistically significant difference at P < 0.05;* ◆ *= X compared with NS; N =7.*

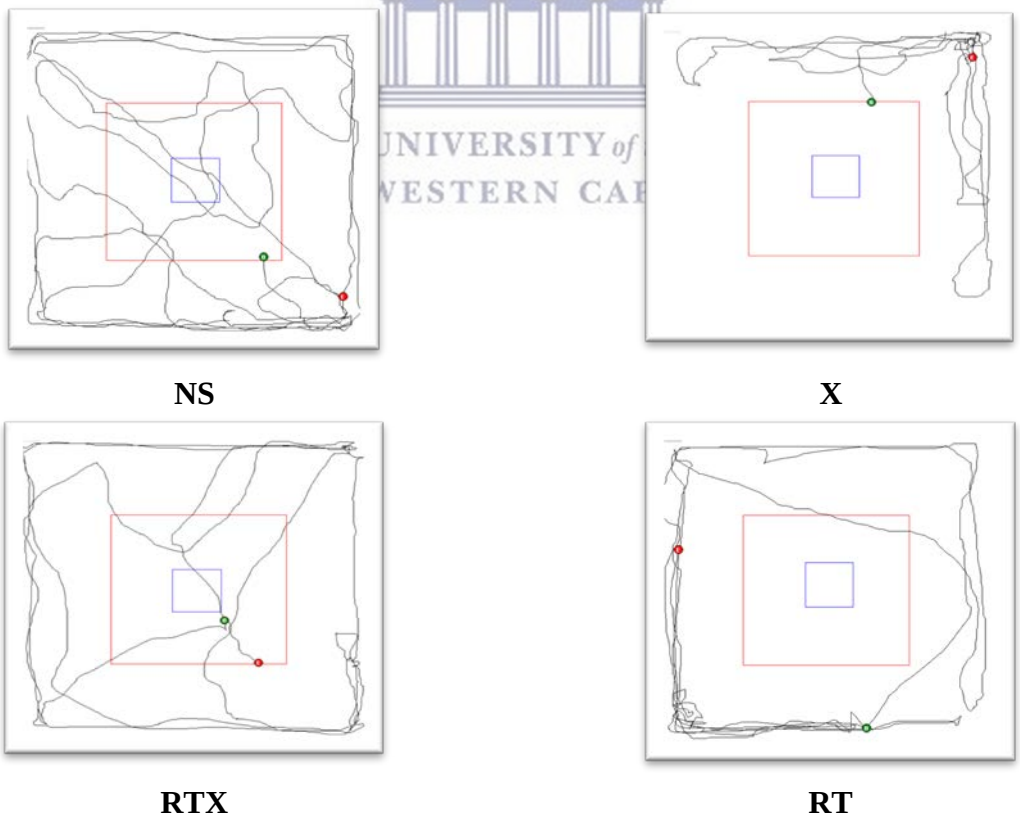

Figure 4. 2: *Representative trajectory maps of changes in locomotor activity inside the open field apparatus. Test duration was 5 minutes per offspring rat*

4.3.1.3. Central Square Entry (CSE)

Central Square Entry (CSE) frequency refers to the number of times the rats entered the centre square of the open field apparatus with all four paws. Results obtained (Figure 4.3) showed that offspring rats from treatment groups generally had fewer entries with groups X and RT having significantly fewer entries compared to the NS group. The frequency of CSE was higher but not significant following treatment with rooibos tea as seen in the RTX groups.

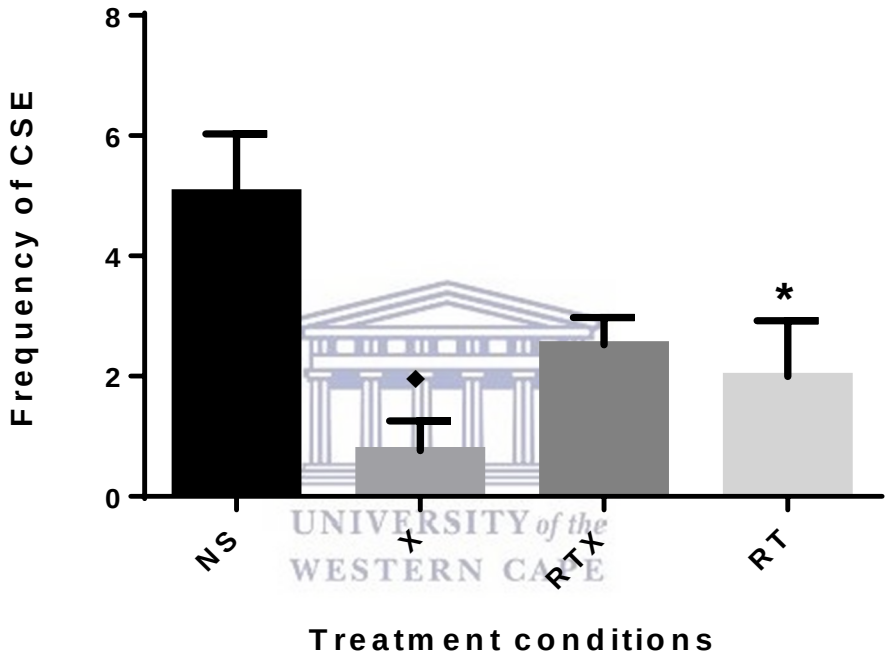

Figure 4. 3: *Average central square entry in the Open Field apparatus. Test duration is 5 minutes per offspring rat as determined by counting. NS: Normal saline, X: Irradiation, RT: Rooibos tea, RTX: Rooibos tea + irradiation. Statistically significant difference at P < 0.05;* ◆ *= X compared with NS;* ✱ *= RT compared with NS; N =7.*

4.3.1.4. The frequency of rearing episodes

This is the frequency with which an animal stands on hind limbs or leans against the walls of the open field apparatus with front paws. It is considered one of the measures of anxiety in rodents and higher rearing frequencies have been said to indicate increased exploratory behaviour. Figure 4.4 shows that exposure to X resulted in significantly reduced rearing episodes whereas RT and RTX were not significantly lower.

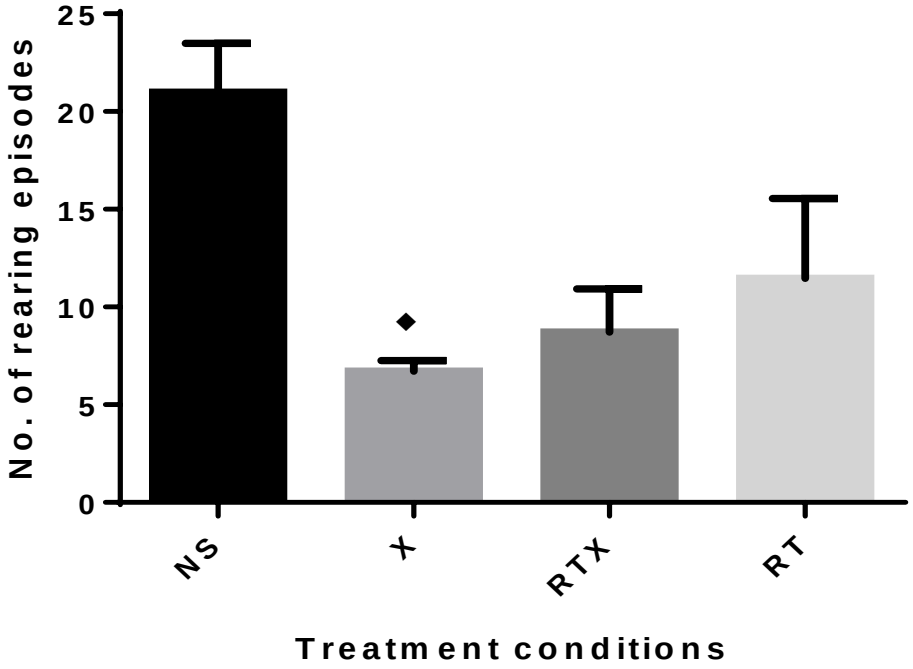

Figure 4. 4: *Average number of times offspring rats stood on hind limbs with raised forelimbs. Test duration is 5 minutes per offspring rat. NS: Normal saline, X: Irradiation, RT: Rooibos tea, RTX: Rooibos tea + irradiation. Statistically significant difference at P < 0.05; ◆ = X compared with NS; N = 7.*

4.3.1.5. Total Freezing Time

Freezing (complete absence of body movements) was scored with a stopwatch by reviewing the video recordings per rat during the test period and the total freezing time was determined. Figure 4.5 shows that both RT and X treatment appeared to cause significant cumulative freezing whereas it was significantly reduced in the RTX treatment group.

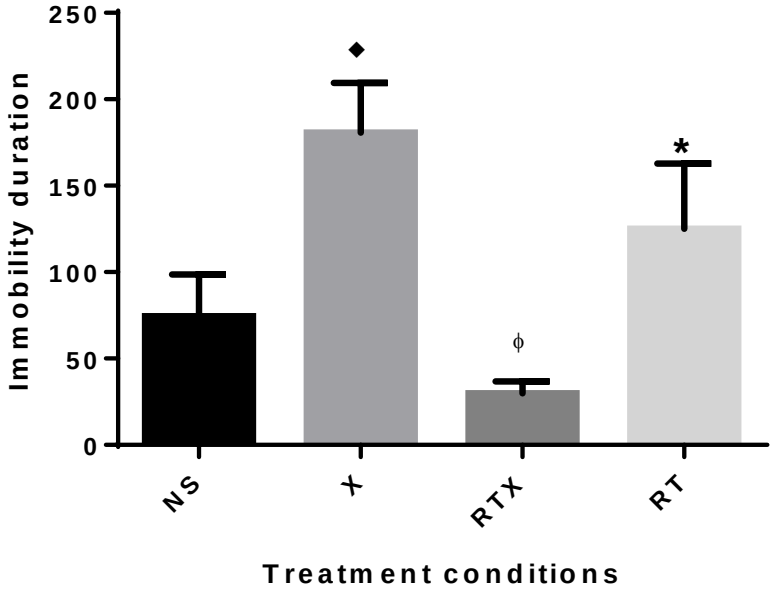

Figure 4.5: *Average total freezing time observed per group of offspring rats during the 5 minutes test period. NS: Normal saline, X: Irradiation, RT: Rooibos tea, RTX: Rooibos tea + irradiation. Statistically significant difference at P < 0.05;* ◆ *= X compared with NS; ϕ = X compared with RTX;* ✱ *= RT compared with NS; N = 7.*

4.3.2. Novel Object Recognition (NOR) Test

In this study, rats were exposed to both new and old objects for 3 minutes, and the time spent exploring the novel object relative to the total time spent exploring both objects (or Recognition Index, RI) was calculated using the formula RI = TN ÷ (TN + TF), where TN = time spent with new (novel) object and TF = time spent with familiar (old) object.

Figure 4.6 shows that both X and RT treatments resulted in significantly lower RI values compared to the control and the RTX group had a relatively higher RI value.

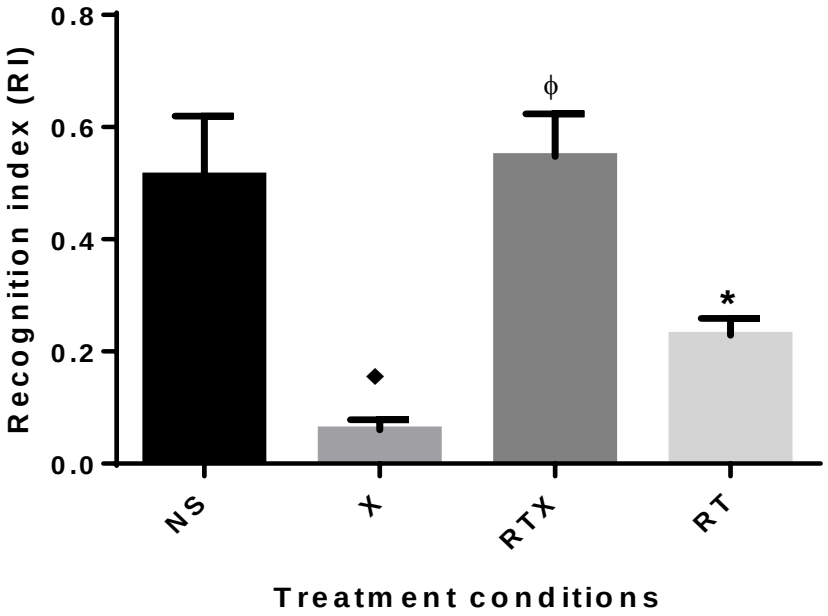

***Figure 4.6**: Average RI per group of offspring rats during a 3 minutes test period of exposure to a new "novel" object. NS: Normal saline, X: Irradiation, RT: Rooibos tea, RTX: Rooibos tea + irradiation. Statistically significant difference at P < 0.05; ◆ = X compared with NS; ϕ = X compared with RTX; * = RT compared with NS; N = 7.*

4.4. Neurochemical Analysis

4.4.1. Ferric Reducing Antioxidant Power (FRAP) assay

Ferric Reducing Antioxidant Power (FRAP) is an assay that measures the ability of compounds or plant extracts to neutralize free radicals by acting as electron donors. The assay helps to determine the antioxidant content present within a sample. In this study, FRAP levels were measured in all homogenized brain samples and the results show that FRAP (antioxidant content) was significantly reduced in the X group animals compared to the NS group. However, rooibos treatment in the FRHT group appeared to modulate the low FRAP levels to normal values significantly in the RTX and less significantly in the RT group when compared to the NS group (figure 4.7).

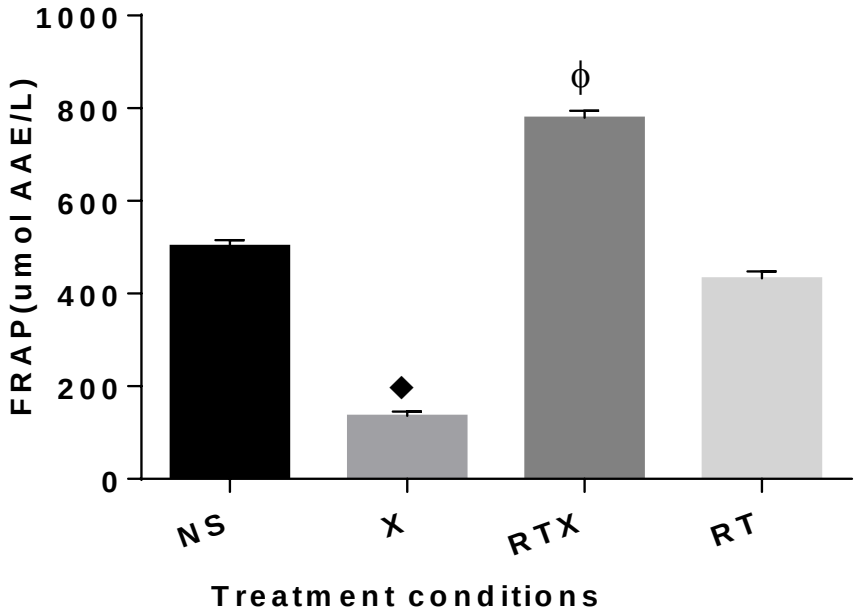

Figure 4.7: *Effects of different treatments on Ferric Reducing Antioxidant Power (FRAP) in cerebral homogenates of offspring rats. NS: Normal saline, X: Irradiation, RT: Rooibos tea, RTX: Rooibos tea + irradiation. Statistically significant difference at $P < 0.05$;* ◆ *= X compared with NS; ϕ = X compared with RTX; N = 7*

4.4.2. Lipid Peroxidation (LPO)

Lipid peroxidation is the degradation of lipids in cells or tissues and is a useful marker of oxidative stress. Malondialdehyde (MDA) is a specific metabolic tracer molecule for LPO in the thiobarbituric acid reaction (TBAR). In this study, LPO levels were determined by estimation of MDA levels in the homogenates of brain samples. Results obtained showed LPO significantly increased in the RT and X groups compared to the NS group indicating an increase in oxidative stress, whereas the RTX group had significantly low LPO levels, a possible indication that the free radicals generated by X were cleared by FRHT (Figure 4.8).

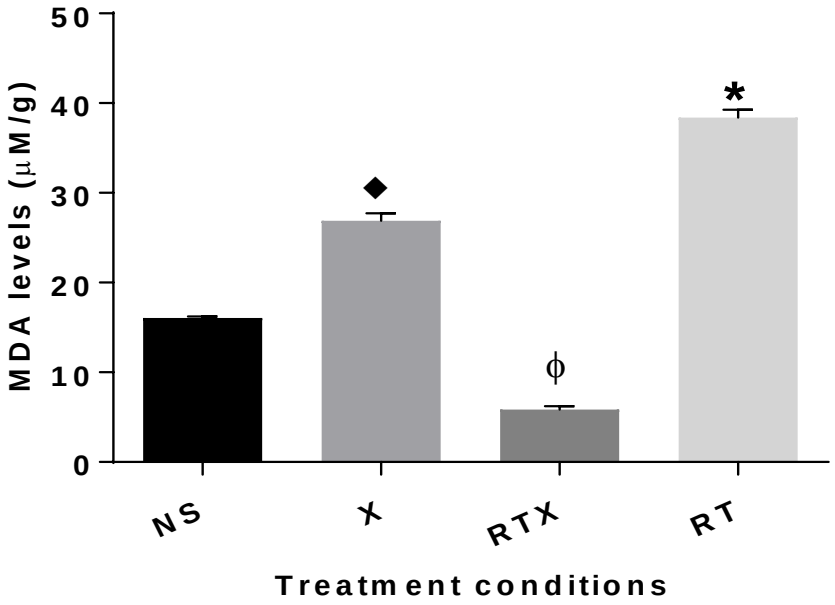

Figure 4.8: *Effects of different treatments on malondialdehyde (MDA) levels in cerebral homogenates of offspring rats. NS: Normal saline, X: Irradiation, RT: Rooibos tea, RTX: Rooibos tea + irradiation. Statistically significant difference at P < 0.05;* ◆ *= X compared with NS; ϕ = X compared with RTX;* * *= RT compared with NS; N = 7*

4.4.3. Superoxide Dismutase Activity (SOD)

Superoxide dismutases are enzymes that eliminate the superoxide radical (O^{2-}) from cells to counteract oxidative damage. SOD activity assays measure the amount of enzyme that inhibited the oxidation of pyrogallol, expressed as units per milligram of protein. Our results show that there was significant reduction in SOD activity in the X group compared to the NS group. Treatment with FRHT (in the RTX group) appeared to cushion SOD levels towards normal and no change in SOD was observed when the RT group was compared to the control (figure 4.9).

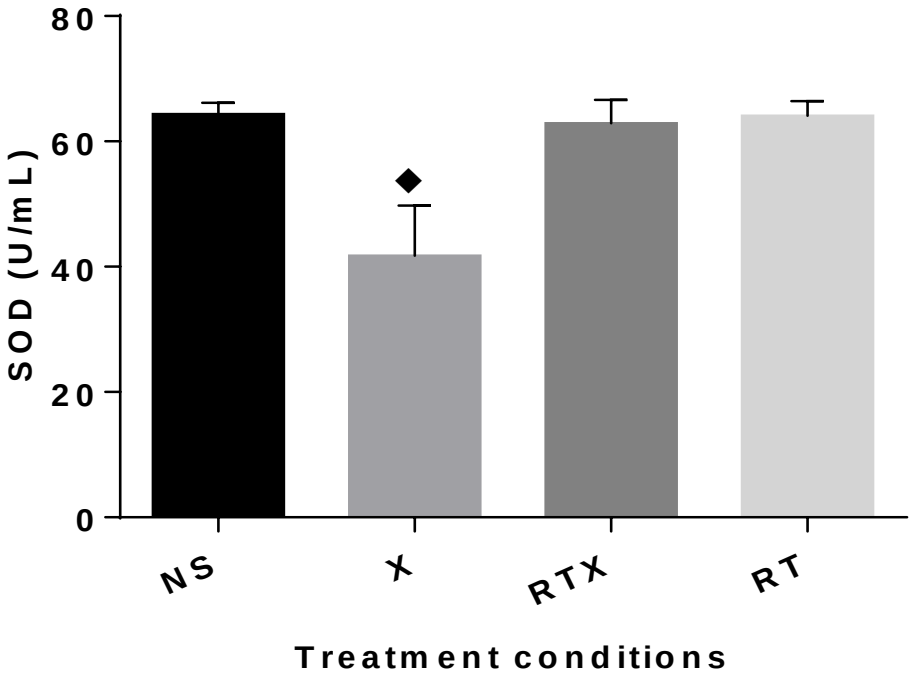

Figure 4.9: *Effects of different treatments on Superoxide Dismutase (SOD) enzyme activity levels in cerebral homogenates of offspring rats. NS: Normal saline, X: Irradiation, RT: Rooibos tea, RTX: Rooibos tea + irradiation. Statistically significant differences: ◆= X compared with NS at P < 0.05: N = 7.*

4.4.4. Catalase Activity (CAT)

This assay is based on the principle that the ubiquitous antioxidant enzyme, catalase will catalyze the decomposition of hydrogen peroxide (H_2O_2) into water and oxygen in tissues and the rate of H_2O_2 disintegration is directly proportional to catalase concentration. Hence, higher CAT concentrations indicate highly potent antioxidant effects and vice versa. In this study, CAT activity was lowered in the X group compared to the NS group but the treatment RTX group had slightly higher CAT activity, with the RT group having the highest CAT activity. All the differences were not statistically significant (figure 4.10).

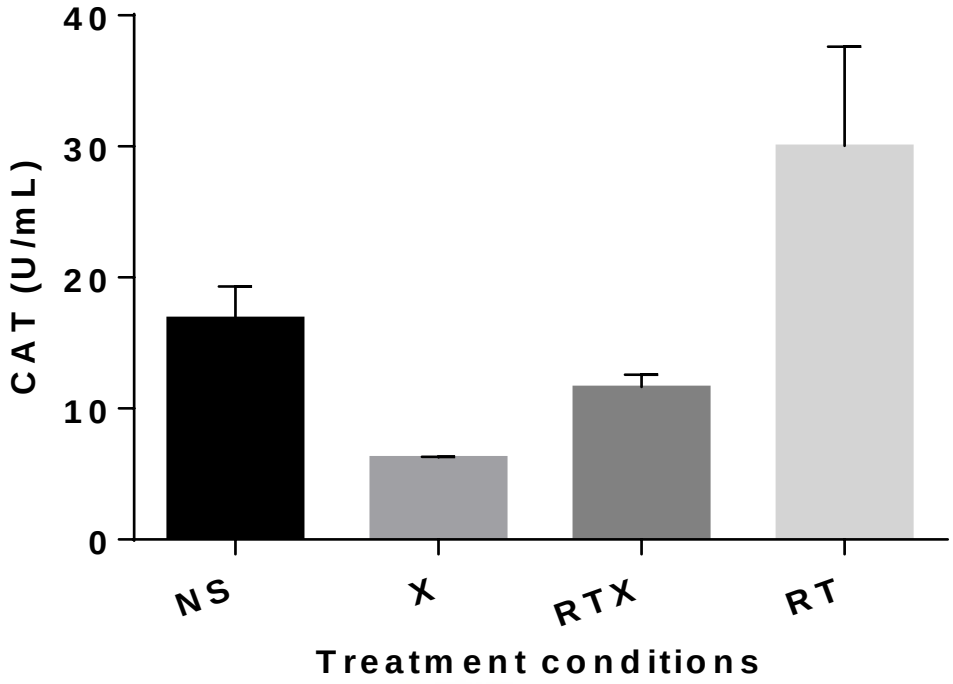

Figure 4.10**:** *Effects of different treatments on CAT enzyme levels in cerebral homogenates of offspring rats Irradiation, RT: Rooibos tea, RTX: Rooibos tea + irradiation. N = 7.*

4.5. Histological Studies

In this study, coronal sections of the cerebrum were made and stained to study the effects of the respective treatments on hippocampal neuronal integrity. Haematoxylin and Eosin (H & E) as well as Cresyl violet (CV) staining were done to assess morphological and morphometric changes in hippocampal cornu ammonis (CA-1). Immunostaining with Caspase-3 and GFAP was also done for analyses of apoptosis and gliosis respectively.

4.5.1. Length and thickness of the CA-1 region of hippocampus

A total of 7 rats were assigned per group and 3 sections from each of 3 slides per specimen were prepared per group and images captured for analysis using the Zeiss Axio Imager motorized histology microscope. H & E as well as CV staining showed clearer outlines of the pyramidal cells and the cell layer in the hippocampus and were therefore used to determine hippocampal CA-1 length and thickness based on the landmarks shown in Figure 4.11.

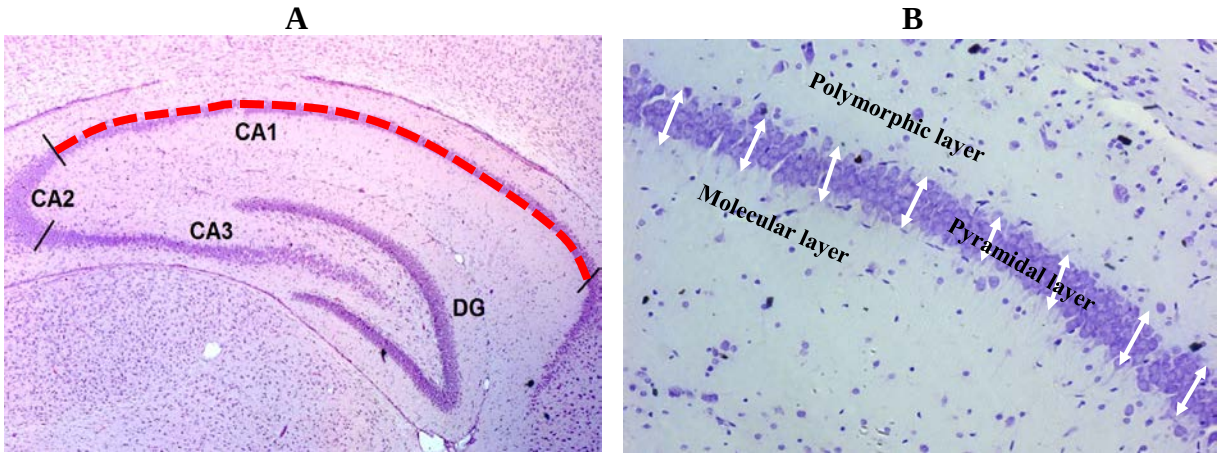

Figure 4.11: *Diagram of the hippocampus showing the landmarks used for determining hippocampal length (A) and thickness (B) in the cerebri of offspring rats. CA - cornu ammonis (with regions CA-1, CA2 and CA3 indicated); DG – Dentate gyrus.*

Graph Pad Prism software version 6 was used to determine the average values of the measured parameters and plotted into bar graphs. Results of the measurements showed that CA-1 length was reduced in all treatment groups and significantly so in the X group, compared to the NS group (figure 4.12). The CA-1 thickness was found to be significantly increased in the X group which appeared to be modulated by the significant reduction observed in the RTX compared to the control.

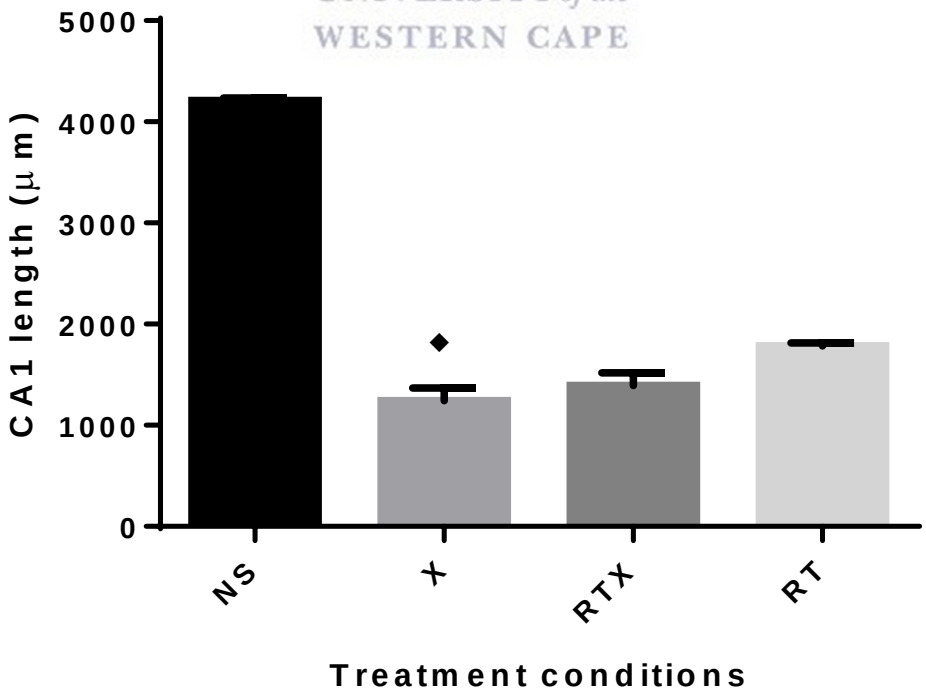

Figure 4.12: *Average length of the CA-1 region of the hippocampus in offspring rats. NS: Normal saline, X: Irradiation, RT: Rooibos tea, RTX: Rooibos tea + irradiation. Statistically significant difference at P < 0.05;* ◆ *= X compared with NS. N = 7.*

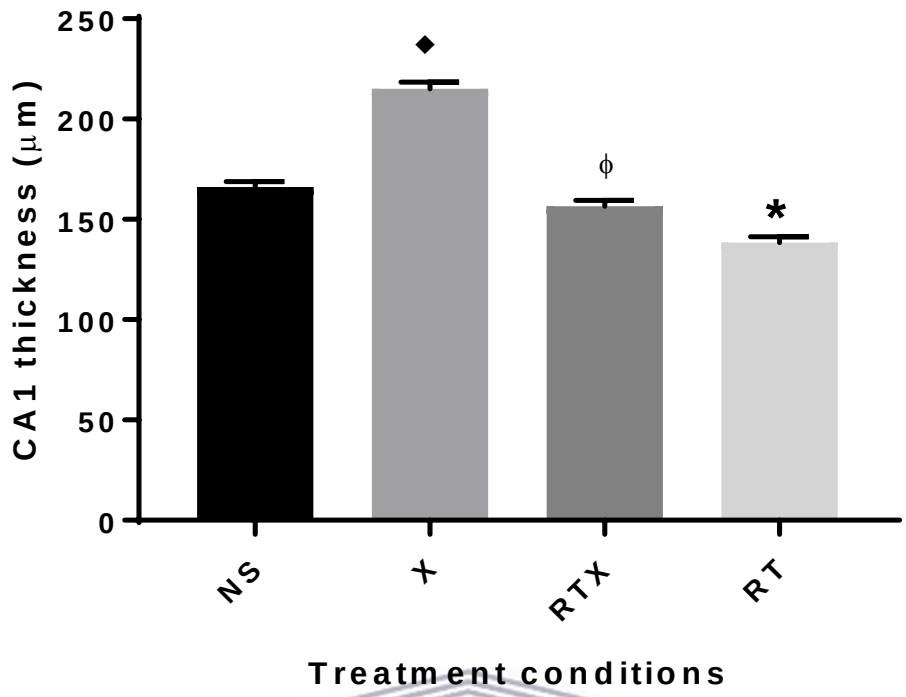

Figure.4.13: *Average thickness of the CA-1 region of the hippocampus in offspring rats aged PND30. NS: Normal saline, X: Irradiation, RT: Rooibos tea, RTX: Rooibos tea + irradiation. Statistically significant difference at P < 0.05;* ◆ *= compared with control;* ***ϕ*** *= compared with irradiation;* ******* *= compared with rooibos tea;* ***N = 7.***

4.5.2. Caspase 3 immunostaining

A total of 7 rats were assigned per group and brain sections were processed for Caspase 3 immunostaining. All neuronal nuclei that were positive for Caspase 3 stain were determined in six symmetrical counting boxes along the CA-1 region of the hippocampus per slide as shown in Figure 4.14. Average values were calculated, plotted and compared between the different treatment groups using the Graph Pad Prism software version 6.

Our results show that animals in the irradiation group (X) had a significantly higher number of caspase-3 immunopositive cells in the CA-1 area than all other groups followed by the RTX group, whereas RT group showed cell count values similar to the controls (figure 4.14).

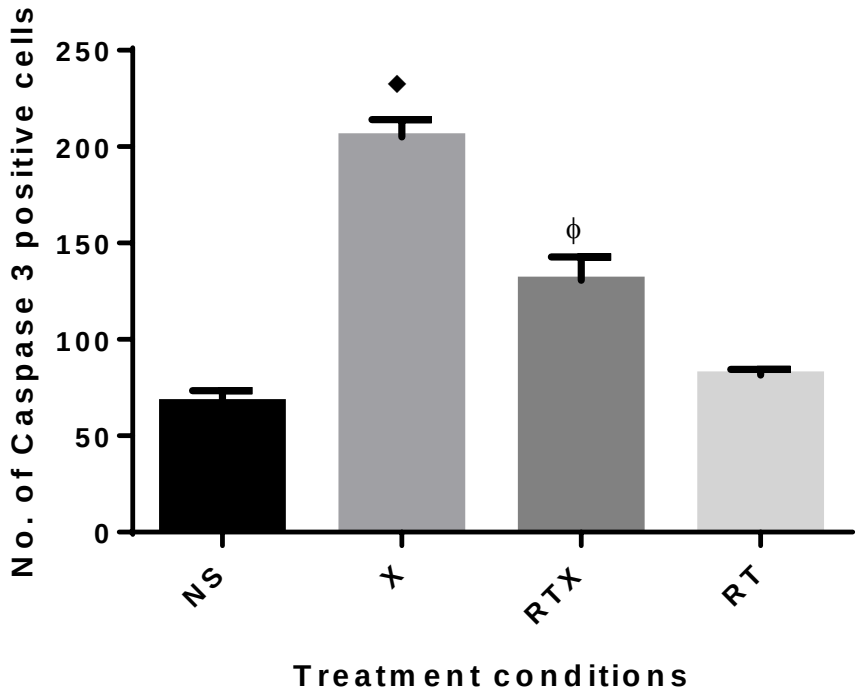

Figure 4.14: *Number of Caspase-3 positive cells in the CA-1 region of the hippocampus in offspring rats. NS: Normal saline, X: Irradiation, RT: Rooibos tea, RTX: Rooibos tea + irradiation. Statistically significant difference at P < 0.05; ◆ = X compared with NS; ϕ = X compared with RTX; N = 7.*

4.5.3. GFAP immunostaining

A total of 6 rats were assigned per group and brain sections were processed for immunostaining to determine the astrocytic marker, glial fibrillary acidic protein (GFAP). All GFAP-immunoreactive astrocytes were determined in six symmetrical counting boxes per slide along the CA-1 region encompassing parts of the polymorphic and molecular layers adjoining the pyramidal layer. The average values were computed, plotted and compared between the different treatment groups using the Graph Pad Prism software version 6. The results show a significant increase in GFAP-immunoreactive astrocytes in the irradiation group (X) compared to the control (NS), while rooibos treatment (RTX group) significantly reduced GFAP levels compared to the X group. Similarly, RT also significantly reduced GFAP levels as shown in figure 4.15.

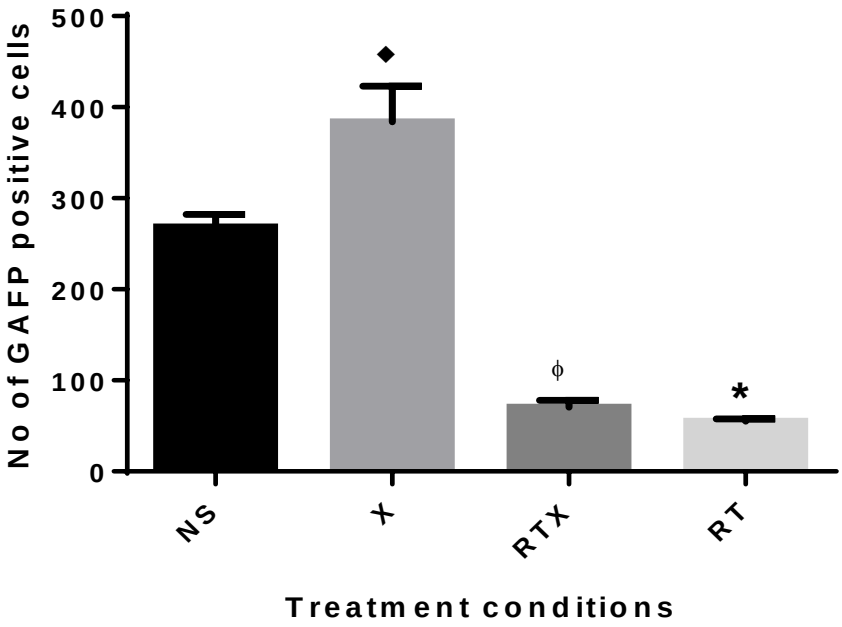

Figure 4.15. *Number of GFAP-positive cells in the CA-1 region of the hippocampus in offspring rats. NS: Normal saline, X: Irradiation, RT: Rooibos tea, RTX: Rooibos tea + irradiation. Statistically significant difference at P < 0.05;* ◆ *= X compared with NS,* ϕ *= X compared with RTX;* ✱ *= RT compared with NS; N = 7.*

4.5.4. Morphological studies of the hippocampus

Morphological assessment of stained slides showed that animals in all treatment groups have relatively normal histomorphological structure of the hippocampus except the X group as seen in Figure 4.16 showing the panoramic view (X25). At this magnification, the tapered structure of the CA-2 genu and the dentate gyrus was altered into a curvature (indicated with thick white arrows) in five of the specimens processed in this group. At higher magnifications (X100), the CA-1 region in all the experimental groups can be seen as a distinct layer of packed pyramidal cells hence CA-1 thickness was easily measured. However in most of the specimens in the group X, the CA-1 was not uniformly thin and localized as cells could be seen migrating away from the pyramidal layer into the adjoining polymorphic and molecular layers, accounting for the generally thicker CA-1 observed in the irradiated animals (Figure 4.16).

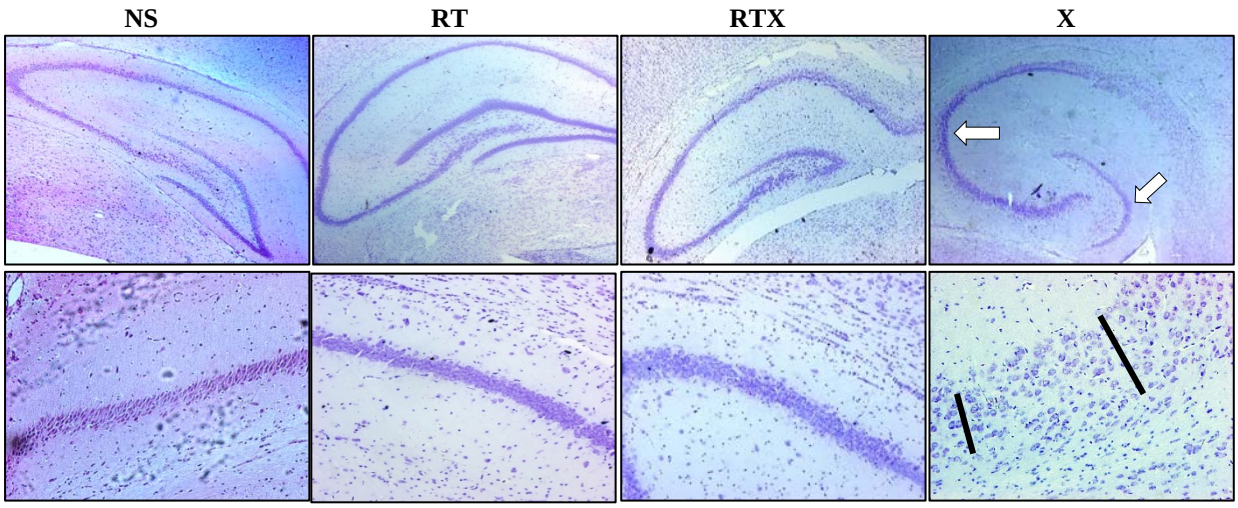

Figure 4.16: *Representative photomicrographs of the hippocampus (upper panels) and CA-1 region (lower panels) at X25 and X100 magnification respectively. NS =Normal saline; RT = rooibos tea; RTX = rooibos and irradiation; X = irradiation.*

Irradiation effects in the hippocampus are highlighted in the images below (Figure 4.17). In the panel 'A', neurons appeared closely packed in a relatively distinct layer and had rounded nuclei with very clear outlines (red arrows). In contrast, cells in panel 'B' were much smaller and mostly amorphous (white arrow), lacked clear outlines and a clear pyramidal cell layer cannot be distinguished as cells appeared to be migratory (red stars). Cells clusters were observed in most of the slides (black arrow) and cell nuclei appeared very fragmented (red arrows). These features could be due to the prolonged effects of early postnatal irradiation.

Panels 'C' and 'D' show representative images of Caspase -3 positive cells in the control and irradiation groups respectively. Results show relatively fewer brown-stained Caspase-3 positive cell nuclei (white arrows) in the control group and a lot more in the irradiation group which also had many shrunken nuclei (red arrows). The blue arrow indicates artefact stains.

Panels 'E' and 'F' show representative images of GFAP-positive cells in the control and irradiation groups respectively. Results show fewer brown-stained astrocytic cytoplasm and processes in the control specimens and relatively more stained neuroglia in the irradiated specimens (red arrows).

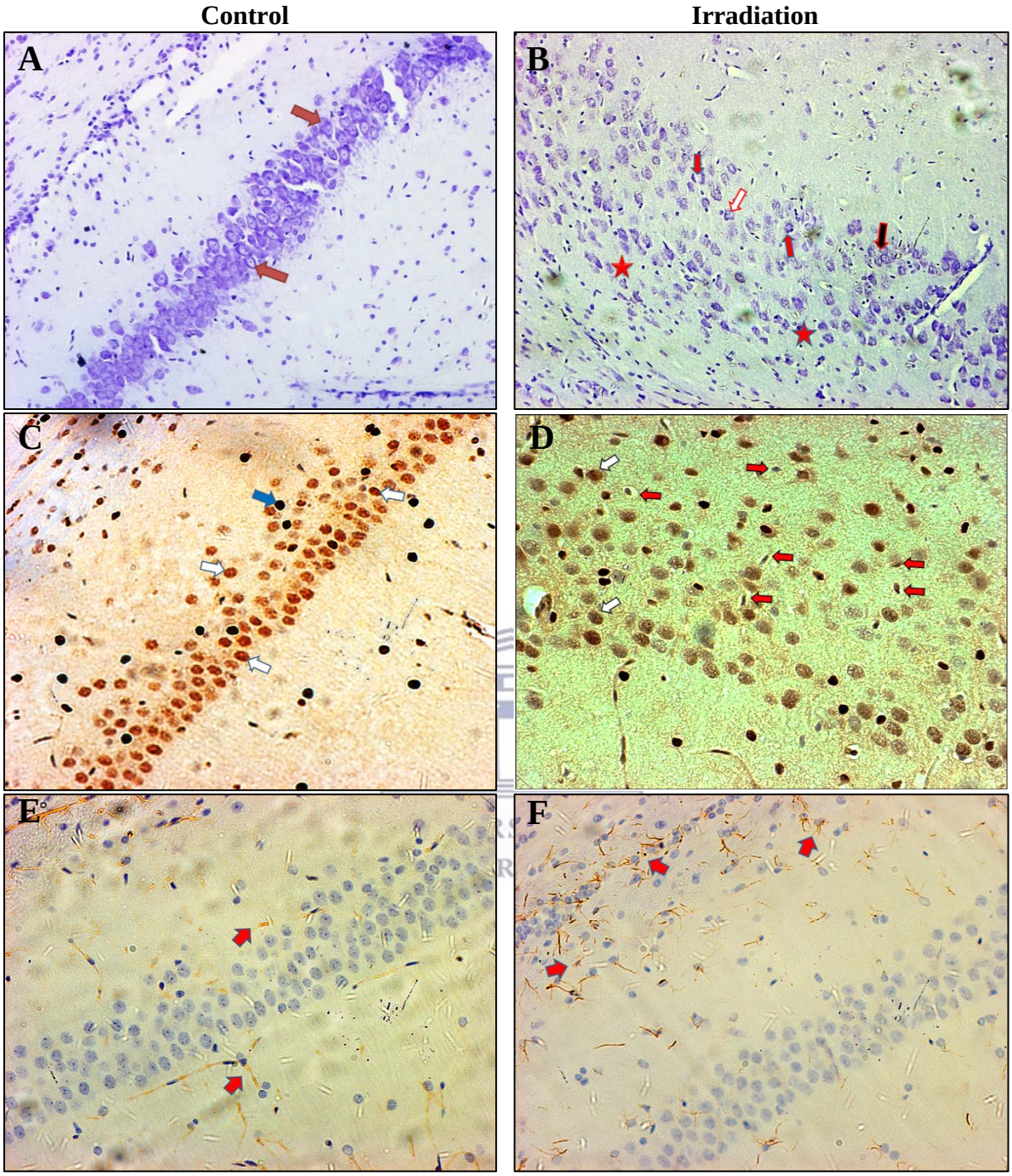

Figure 4.17: *Representative photomicrographs of hippocampal CA 1 sections compared between the control and irradiated rats at X200 magnification. The arrows and symbols represent features of note. Staining: A&B = Cresyl violet; C&D = Caspase-3-positive cells; E&F = GFAP-positive cells.*

CHAPTER FIVE

DISCUSSION

5.0. Introduction

The increase in applications utilising radiation in healthcare, agricultural and industrial activities poses a threat to our environment and the human race (Makhlouf and Makhlouf, 2012). Medical imaging and cancer radiotherapy are some of the major sources of irradiation exposure (Lin, 2010; Linet *et al*., 2012). Ionizing irradiation has been shown to cause injury to the developing human brain which worsens over time (Mulhern *et al*., 2004). Treatment of paediatric central nervous system (CNS) cancers sometimes involves radiotherapy, and there have been reports of increased risk of late cognitive dysfunction (Pollack *et al*., 1995; Mulhern *et al*., 2004) possibly due to damage to normal neurons and glial cells. Even the use of radiotherapy for cutaneous haemangioma in young children aged 18 months, has been shown to cause cognitive impairments in adulthood (Hall *et al*., 2004). Computerized tomography (CT) is one diagnostic and imaging technique that is now widely used for medical purposes even in children, with potential for increased radiation exposure *(Bernier et al*., 2012). Even an acute, once-off radiation exposure to the nervous system during critical stages of development has been reported to cause persistent neurotoxicity (Canfield *et al*., 2003; Lanphear *et al*., 2005). Since the application of irradiation especially in medicine cannot be avoided, the search for potential radioprotective agents is plausible (Smith, *et al*, 2017).

This study was undertaken to investigate if continuous consumption of fermented rooibos herbal tea (FRHT) could offer diverse protection against potential damage to the developing CNS of offspring Wistar rats exposed to a once-off 6 Gy dose of irradiation on PND 3, following *ad libitum* maternal consumption before, during and after pregnancy as observed after PND 30. The offspring were therefore considered to have ‘formed’ and ‘developed’ in the presence of *ad libitum* rooibos tea consumption including postnatal consumption through breastfeeding and direct consumption post-weaning. After the assessment of neurobehavioural activity, rats were sacrificed and samples were examined for changes in antioxidant activities, immunohistochemistry and histomorphological. The results obtained from this study are discussed in the following sections.

5.1. Rooibos tea alleviated irradiation-induced neurobehavioural deficits

In this study, the open field test was used to evaluate selected neurobehavioral tests such as total distance travelled, locomotor or exploratory activity, central square entry (CSE), and frequencies of rearing episodes, total freezing time and novel object recognition (NOR) test. The open field test is designed to evaluate emotionality and anxiety-linked behaviour (Hall, 1934, Prut and Belzung, 2003) and its principle is based on the tendency of rodents to evade brightened, illuminated, open and new spaces, thus making the open field environment an anxiogenic stimulus that permits the evaluation of anxiety-triggered locomotor/exploratory activity (Habr *et al*., 2011). Through exploration, most rodents are able to gather information about their environment (Lynn and Brown, 2009, Habr *et al*., 2011), leading to improved awareness of the environment and better chances of locating food, help and shelter when in the face of danger (Alves *et al*., 2012).

Findings from the present study showed that locomotor activity was significantly reduced in the X group but increased in the RTX and RT groups compared to the NS control. The lowered locomotor activity in the RXT group showed FRHT had a protective effect in rats exposed to radiation. This is in line with earlier findings demonstrating the ability of RT to improve locomotor activity in rats (Akinrinmade *et al*., 2017). Another indicator of anxiety investigated was CSE in offspring rats. Increased CSE frequency signifies high exploratory activity and reduced anxiety in rodents (Ekong *et al*., 2008) and findings from our study showed that offspring rats had fewer CSE in the X group when compared to the NS control group while rats in the RT group had higher CSE when compared to rats in the X group. These results indicate that rats treated with RT demonstrated a lower level of anxiety than rats exposed to irradiation, further suggesting that RT is protective against the adverse effects of radiation (Osman *et al*., 2011, Balentova and Adamkov, 2015), as indicated by the higher RTX value.

Rearing behaviour is believed to be an exploratory activity triggered by new stimuli (Van Abeelen, 1977, Alves *et al*., 2012). Variations in the rate of rearing have been previously demonstrated under experimental conditions, such as drug treatment (van Lier *et al*., 2004) and brain stimulation (Racine, 1972, Hannesson *et al*., 2001). Other authors have also shown the presence of a positive association between the total rearing duration and locomotor activity in rats (Borta and Schwarting, 2005). This correlation is in line with our findings which revealed similar patterns of reduction in rearing and locomotor activity in rats exposed

to radiation. Conversely, the increase in rearing and locomotor activity in rats treated with RT demonstrates its protective effect against radiation in Wistar rats.

In the same light, freezing activity in an open field test is also considered a potent indicator of anxiety in rodents (Díaz-Morán *et al*., 2014). Freezing is regarded as the complete lack of movement except respiration, as commonly seen in conditions of highly elevated stress levels (Walsh and Cummins, 1976). Freezing occurs in response to apparent danger, environmental displacement, sudden changes and fright stimuli (Brandao *et al*., 2008). Our findings revealed that freezing was significantly increased in the X group which is in agreement with previous studies which showed increased freezing activity in stress conditions (Ranjbar *et al*., 2017) and in conditions of impaired brain function (Saikhedkar *et al*., 2014). We however observed a significantly reduced freezing in the RTX group, which confirms the radoiprotective effects of FRHT.

The NOR test was introduced by Ennanceur and Delacour (1988) to evaluate short-term memory in rodents (Moscardo *et al*., 2012). The short-term memory highlights the capacity of the rodent to actively and readily remember information for a short amount of time (Mathiasen and DiCamillo, 2010). The benefits of the NOR test over other behavioural or cognitive function tests in rodents include its promptness and its application without prior training, food denial or enforcement stimuli (Mathiasen and DiCamillo, 2010). Thus, the NOR test can be used to study memory and learning, preference for novelty, brain mapping of memory functions, assessment of different drugs effects in many animal disease models, etc. (Zhang *et al*., 2012; Antunes and Biala, 2012).

In this study, we evaluated the recognition index (RI) which measures an animal's ability to recognize a novel object in a familiar environment as described in section 4.3.2. During the first few minutes of the retention test, more time will usually be spent by normal animals exploring the novel object since the other object is already recognized (Mumby *et al*., 2002). A higher RI may indicate a preference for the novel object and previous studies have shown reduced RI in stress conditions (Nagata *et al*., 2009; Eagle *et al*., 2013). In our findings, rats in the RT and X group had significantly lower RI when compared to the controls. The RTX group showed RI values which were comparable to the control, possibly indicating significant attenuation of irradiation effects and radioprotection by FRHT.

An interesting finding in this study was the unexpected reduction in RI value for the RT group which tends to suggest that FRHT treatment alone could adversely affect memory retention. The exact reason for the low RI value is not understood and this adverse finding was found to correlate only with increased lipid peroxidation but not behavioural studies, and could be due to a number of confounding factors, including experimental techniques. All previous studies consistently indicate beneficial effects of rooibos and other teas in improving memory loss. One such study showed that pre-treatment with rooibos tea improved memory deficit following ischemic injury (Akinrinmade *et al*., 2017), while another showed that green tea polyphenols have been reported to inhibit cognitive impairment caused by chronic cerebral hypoperfusion (Xu *et al*., 2010). Furthermore, the flavonoid vitexin was found to be capable of attenuating scopolamine-induced memory impairment in rats (Abbasi *et al*., 2013) while a systematic review of 28 studies showed that acute or chronic polyphenol consumption could greatly improve cognition (Lamport *et al*., 2012). From the above studies, it appears flavonoids and other polyphenols account for the observed effects, hence further elaborate studies are necessary to evaluate the effects of rooibos tea on memory retention including the molecular mechanisms involved.

5.2. Rooibos tea restored antioxidant activity and attenuated irradiation-induced lipid peroxidation in brain tissues

Irradiation exposure has been reported to lead to the generation of reactive oxygen species (ROS) and concomittant cellular injury and impairment of organ function (Breen and Murphy, 1995, Berroud *et al*., 1996, Kamat *et al*., 2000, Fang *et al*., 2002, Jagetia *et al*., 2003). The initiation of oxidative stress in cells inhibits antioxidant capacity and leads to the inactivation of such endogenous antioxidant enzyme systems such as SOD, CAT and GSH in the cells (Koc *et al*., 2003a, Koc *et al*., 2003b, Halliwell and Whiteman, 2004). A study by Ahlersova *et al*., (1998) reported a reduction of serum antioxidant enzymes five days after radiation exposure to 14.4 Gy irradiation. Thus, the antioxidant capacity in cells demonstrates the ability of the endogenous system to attenuate oxidative injury which has been reported in many previous studies (Fukasawa *et al*., 2009, Marnewick *et al*., 2011,)..

Previous studies have reported the routine use of the FRAP assay for the evaluation of antioxidant capacity in tissues (Katalinic *et al*., 2005, Nakhaee *et al*., 2009, Changizi-Ashtiyani *et al*., 2017) as this assay evaluates the iron-reducing capability of a sample. A

reduction in FRAP indicates possible oxidative damage and cell death (Benzie and Strain, 1996, Kusano and Ferrari, 2008). In this study, FRAP levels were significantly reduced in rats exposed to radiation which suggests reduced ability by cells in these animals to defend against oxidative stress. Conversely, FRAP levels were significantly increased in the RTX group, suggesting that consumption of RT could help preserve the total antioxidant capacity of the brain after radiation injury. These findings are in agreement with previous reports demonstrating radiation-induced reduction in the total antioxidant capacity in rat brains (Falone *et al*., 2008, Akdag *et al*., 2010, Eser *et al*., 2013, Akinrinmade *et al*., 2017).

Protein oxidation and lipid peroxidation are considered the primary forms of radiation-induced damage in cells and the complex interplay of many factors including low antioxidant content, elevated polyunsaturated fatty acids (PUFAs - the substrate for lipid peroxidation), high oxygen utilization, neurotransmitter auto-oxidation, etc. dictates the susceptibility of the brain to lipid peroxidation and oxidative stress (Sultana, *et al*., 2006, Makhlouf and Makhlouf, 2012, Cobley, 2018). The accumulation of potentially damaging lipid peroxidases in brain samples is measured by the levels of the metabolic tracer molecule Malondialdehyde (MDA) and an increase in MDA levels in brain tissues signifies increased oxidative stress. MDA is known to act together with and modify macromolecules to promote oxidative damage (Box and Maccubbin, 1997; Petersen and Doorn, 2004). In addition, reports show that protein oxidation resulting from oxidative stress occurs in the absence of lipid peroxidation (Shanlin *et al.*, 1997, Stadtman and Berlett, 1997). In our study, MDA levels were significantly increased compared to control following irradiation but pre-treatment with FRHT was found to be capable of reducing lipid peroxidation in rats exposed to radiation highlights the radoiprotective effects of RT. An interesting finding in this study was the unexpected significantly increased MDA levels in the RT-only treated animals, which tends to correlate with unexpected RI values and tend to suggest that FRHT does not modulate lipid peroxidases.

Superoxide dismutases (SOD) and catalase (CAT) are essential enzymes that offer defence against radiation injury (Mansour and Tawfik, 2012). Our results showed a reduction in SOD and CAT activity in rats exposed to radiation when compared to the control group. It is not unlikely that the observed reduction is due to the inhibition of SOD and CAT by ROS induced by irradiation as previously reported (Weiss *et al*., 1996, Wiseman and Halliwell, 1996 Inal & Kahraman, 2000). Other studies by Kesari *et al*., (2010) reported a reduction in

SOD and CAT levels as well as other biological alterations in brain tissues following radiation exposure. Our findings therefore generally agree with previous studies (Şener *et al*., 2003, Bhatia and Manda, 2004).

5.3. Rooibos tea prevented hippocampal neurodegeneration in rats exposed to radiation

Increased exposure to radiation even for beneficial purposes is potentially hazardous and can cause extensive organ damage and impairment (Kantor *et al*., 2008, Hladik & Tapio, 2016). Radiation-induced alterations in brain structure and function has been previously reported (Noel *et al*., 1998, Rosi *et al*., 2008). Neurons are known to be the morphologic basis of learning and memory, thus radiation exposure may cause alterations in neuronal structure which may affect such cognitive functions as learning and memory (Martin *et al*., 2000; Neves *et al*., 2008; Hladik and Tapio, 2016). The soma and dendrites of neurons constitute most of the neuronal architecture seen in histological preparations and are useful for proper synaptic transmission and the corresponding processing of cognitive and mental function (Huttenlocher, 1991, Kaufmann and Moser, 2000, Tronel *et al*., 2010).

Morphometric parameters evaluated in this study include the length and thickness of the CA-1 region of the hippocampus, both of which are accounted for by the presence of neuronal cell bodies and processes. Our findings showed that a significant reduction in the average length of the CA-1 region of the hippocampus in the X group offspring rats compared to the control while the RTX group had a rather low CA-1 length compared to the control, which indicates only a limited radioprotective effect of FRHT. Similarly, treatment with FRHT-only did not seem to significantly reverse the significant effect of radiation-induced CA-1 length reduction. This later finding appears to be consistent with other findings from our study involving animals treated with only FRHT. The thickness of the CA-1 region of the hippocampus was also found to be altered by irradiation exposure and rooibos treatment when compared to the control, albeit in an anomalous manner. A thicker CA-1 was observed in the irradiated specimens possibly due to radiation-induced dispersion of pyramidal cells which appeared to have extended the landmarks for measuring thickness in this region (see Figure 4.22). In addition, treatment with FRHT did not appear to be radioprotective in this regard for reasons not fully understood.

There are little or no studies in literature on experimentally-induced reduction in CA-1 dimensions. However, there are many reports on morphometric measurements of CA-1 pyramidal neurons in terms of neuronal density and sizes as well as alteration of their dendritic arborization using such specialized staining techniques as the Golgi-Cox staining method as well as electron microscopic techniques (Benes et., 1991, Frauenknecht *et al*., 2015, Kimura *et al*., 2016). Previous studies have reported decreased dendritic length of hippocampal neurons in mice after radiation when compared to controls (Parihar and Limoli, 2013, Parihar *et al*., 2015). In addition, a significant reduction in spine density in the basal dendrites of CA-1 pyramidal neurons one week after irradiation was reported by Chakraborti *et al*., (2012). These techniques were not included for investigation in the objectives of this study.

Caspases have for long been implicated in radiation-induced apoptosis (Hallan *et al*., 1997, Yu and Little, 1998), mainly in the regulation of cell death during normal development as well as in irradiation-induced cell death in the developing brain which demonstrates features of caspase-mediated apoptosis (Inouye, 1995, Ferrer, 1999). Caspase inhibition was reported to be essential for the interruption of apoptosis in embryonic neurons and SH-SY5Y cells after radiation (Johnson *et al.*, 1998). In agreement with these findings, our study showed a significantly high caspase-3 activity in the X group signifying increased apoptosis which appeared to have been attenuated to a limited extent by pre-treatment with FRHT as seen in the reduced number of Caspase-positive cells in the RTX group. The RT-only group had a relatively low but insignificantly low caspase-3 positive cell count comparable with the control group, indicating substantial benefit in reducing apoptosis in the CA-1 neurons.

Similarly, GFAP is an essential protein of intermediate glial filaments in astrocytes and is utilised in evaluating damage responses in astrocytes (Cikriklar *et al*., 2016). Reports indicate that increased GFAP expression is a principal feature in conditions of CNS and astrocytic damage (Hausmann *et al*., 2000, Yu *et al*., 2004). Our findings indicate that GFAP-immunostaining is increased in specimens from irradiated (X group) when compared with the control group, whereas in the RTX and RT groups, the number of GFAP-positive cells was significantly reduced when compared to the X and control groups respectively.

Taken together, our findings indicate that RT is a potent nutraceutical agent for protecting against the damaging effects of radiation-induced neuronal apoptosis and gliosis.

5.4. Morphological studies of the hippocampus

Variations in hippocampal morphology and structural alterations have been reported in previous studies that mostly used volumetric and shape analysis techniques to determine a correlation with other measures of depression other conditions that induce stress to the brain, including irradiation exposure (Solowij *et al*., 2013, Gold, *et al*., 2014). Dispersion of hippocampal pyramidal cell layer has been previously reported (Roper *et al*., 1995, Jiang *et al*., 2016). In our study, the observed thicker CA-1 in the irradiation group was attributed to dispersion and migration (Figure 4.22). In our study, irradiation was also considered to account for the relatively higher number of brown-stained, shrunken and damaged Caspase-3 positive neurons as previously confirmed by (Salford *et al*., 2003, Faridi & Khan, 2013). Another morphological observation was the altered structure of the CA-2 genu and the dentate gyrus. Amaral, *et al.*, (2007) reported that the level of the horizontal section through the rat hippocampal formation determines the shape of the hippocampus in a photomicrograph. It thus appears that most of the hippocampal sections reported in many studies were taken from more dorsally situated transverse sections. A more ventral section will have a curved or U-shaped dentate gyrus instead of the common V-shaped one as observed in this study and could therefore not have been due to irradiation exposure.

5.5. Limitations of the study

Efforts were made to investigate additional immunohistochemical markers in the brains of experimental rats, evaluate more neurobehavioural parameters, quantify neurotransmitter levels, pyramidal cell numbers and hippocampal neuronal morphology to provide more information on radiation-induced impairment of cognitive function and the underlying structural alterations. However, such objectives were not achieved mainly due to funding challenges.

5.6. Conclusions and future recommendations

Our results provide the first evidence of cognitive and the histomorphologic radioprotection conferred by rooibos tea in Wistar rats possibly mediated by the bioactive compounds present in rooibos tea acting through multimodal mechanisms, including antioxidant capacity, modulation of apoptosis and gliosis. These results tend to suggest that rooibos tea consumption could be generally beneficial for protection against accidental environmental

and other forms of radiation exposure to the brain or other nervous tissues. Future studies will incorporate the following aspects:

- Studies on the known bioactive compounds in rooibos tea, especially Aspalathin, to investigate their neuroprotective activity against radiation.
- Evaluation of inflammatory biomarkers and microglia immunohistochemistry to determine the role of inflammation in radiation exposure.
- Utilization of western blots to evaluate expression levels of apoptotic and inflammatory proteins.
- Assessment of additional neurobehavioural tests to corroborate findings on cognitive and motor dysfunction.
- Evaluation of radioprotective effects of rooibos tea and aspalathin in peripheral blood, vital organs and vascular endothelium to correlate these with findings from our study.

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