AN IN VITRO AND IN VIVO HEPATOTOXICITY STUDY OF AN ASPALATHIN-RICH GREEN ROOIBOS EXTRACT

By

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ABSTRACT

Hepatotoxicity is an undesirable condition of the liver, often occurring due to the interaction of drugs. Although different methods have been employed in the diagnosis of drug-induced hepatotoxicity, none have accurately proven the hepatotoxic effect of drug-drug interactions or drug-herb interactions. Flavonoids of aspalathin-rich green Rooibos extract (GRT) present many therapeutic properties that reduce the risk of developing cardiovascular diseases. However, there is a gap in literature regarding the efficacy or safety of this particular extract on the liver. Furthermore, Rooibos has been implicated in the development of hepatotoxicity which might have been triggered by drug-Rooibos interaction. This study therefore purposes to establish whether consumption of an aspalathin-rich green Rooibos extract (GRT) has any toxic effects on the liver using both an *in vitro* human liver cell line (C3A) model, as well as a sub-chronic Sprague- Dawley rat model.

Cultured C3A cells were treated with different concentrations of GRT ranging from untreated control, 0.1, 1, 10, 100 and 1000 µg/mL and incubated for 1 hour, 24 hours or 48 hours. Thereafter, cell viability was evaluated using the mitochondrial activity assay 3- [4, 5-dimethylthiazol-2-yl]-2,5-diphnyltetrazolium bromide (MTT) and adenosine triphosphate (ATP) assay. The impact of the GRT extract on enzyme release and membrane integrity was assessed using the adenylate kinase enzyme (AK). For the *in vivo* study, sub-chronic Sprague Dawley rats were used to investigate the toxicity of different concentrations of GRT extract. The rats were randomly divided into five groups with ten rats per group. Three of these groups were treated daily with different concentrations of GRT extract [10, 100 and 300 mg/kg body weight (bw)] for a period of 90 days, while the other groups were an untreated control and a carbon tetrachloride (CCl_4) treated group for induction of hepatotoxicity. Treatments were administered using cubes made of jelly and gelatin for the duration of the study. At 24 hours before termination, the positive control group was treated with 1 mL/kg CCl_4 in 1:1 ratio dilution with olive oil to induce liver injury. The body weights, food and water intakes were measured weekly during the period of the treatment. At termination, blood samples were collected for clinical biochemical tests [alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), Urea, total protein, total bilirubin, albumin uric acid and creatinine] and liver tissue collected for histology and protein expression (NF-kB, Bax, Caspase 3 and CYP3A4).

The *in vitro* results revealed that lower concentrations of GRT significantly sustained cell viability in C3A which was associated with the increase in cell proliferation after 1 hour, 24 hours and 48 hours incubation compared to the control. A hepatotoxicity effect was only observed when the cells were treated with higher concentration of GRT extract (1000 µg/mL). The results of the *in vivo* study showed that the GRT extract had no effect on the body weights, food intake and water intake. Increased levels of liver enzymes, AST at the highest dose (300 mg/kg) and ALT in the CCl_4 treated group were demonstrated.

Total protein was unaffected by the GRT while the serum albumin was slightly increased. Histopathological changes showed diffuse marked hepatocellular damage caused by CCl_4. There were no histopathological changes observed with 10 and 100 mg/kg while the 300 mg/kg displayed micro-and macrovesicular changes. There was a concentration-dependent inhibition of NF-KB and no significant effect was observed on the expression of caspase 3, Bax and CYP3A4.

Taken together, these results suggest that lower concentrations of GRT extract have a hepatoprotective effect *in vivo*. Supporting these findings, *in vitro* results showed that low concentrations of GRT extract exhibited a hepatoprotective effect on the human hepatocellular carcinoma cells. However, there should be vigilance in the consumption of Rooibos tea as increased consumption may impair hepatocytes.

Keywords: hepatotoxicity, Rooibos, cytotoxicity, hepatocyte, anti-oxidant, and anti-inflammation.

DECLARATION

I, **Ntandoyenkosi Nokuthula Buthelezi**, declare that this work is the output of my hard work and dedication. The secondary information used in this document have been cited accordingly and are in line with academic conventions. I further substantiate to the best of my knowledge that the thesis submitted is genuine and have never been produced or presented for examination, either entirely or partly for a degree at the University of Zululand or any other institution.

Signature (Candidate): …….

Date: .....

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LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATCC	American type culture collection
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Bax	Bcl-2 associated X protein
Bcl-2	B cell CLL/Lymphoma 2
BW	Body weight
SOD	Superoxide dismutase
CVD	Cardiovascular disease
DMSO	Dimethyl sulfoxide
EMEM	Eagle's minimal essential medium
I-kB	Inhibition of NF-kB
MTT	2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaOH	Sodium hydroxide
PBS	Phosphate buffered saline
ROS	Reactive oxygen species
rpm	Revolutions per minute
STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
WHO	World Health Organization
APS	Ammonium persulfate
CYP_{450}	Cytochrome P_{450} enzymes
CYP3A4	Cytochrome P_{450}, subtype 3A4
CYP_{2E1}	Cytochrome P_{450}, subtype 2E1
NAFLD	Non-alcoholic fatty liver disease
NF-kB	Nuclear factor-kappa B
PI	Propidium iodide
PVDF	Polyvinylidene difluoride

TEMED	Tetramethylethylenediamine tetraacetic acid
RNS	Reactive nitrogen species
RT	Room temperature
TNF	Tumor necrosis factor
SEM	Standard error of the mean
GRT	Green Rooibos extract
GLUT 4	Glucose transporter type 4
AMPK	Adenosine monophosphate-activated protein kinase
CO_2	Carbon dioxide
GSH	Glutathione
CCl_4	Carbon tetrachloride
CAT	Catalase
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
WB	Western blotting
LDL	Low density lipoprotein
FCCP	Trifluoromethoxy carbonylcyanide phenylhydrazone
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
HPLC	High performance liquid chromatography
ADP	Adenosine diphosphate
AMPK	Adenosine monophosphate activated protein kinase

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LIST OF MEASUREMENT

µL	Microliter
mL	Milliliter
L	Liter
Ng	Nanogram
µg	Microgram
mg	Milligram
g	Gram
kg	Kilogram
nM	Nanomolar
µM	Micromolar
mM	Millimolar
M	Molar
mm	Millimeters
µm	Micrometer
min	Minute
sec	Second
H	Hour
°C	Degree Celsius
× g	Centrifugal gravity
%	Percentage
kDa	Kilo Dalton

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CHAPTER 1

1 INTRODUCTION AND BACKGROUND TO THE STUDY

1.1 Overview

The liver is the primary organ responsible for detoxification of harmful chemical substances introduced into the body. As such, the liver receives blood that is rich in absorbed nutrients and substances from the digestive tract (Tso and McGill, 2003; Hall, 2015). However, some chemical substances absorbed by the digestive system are toxic to liver cells (hepatotoxic), resulting in temporary, transient or permanent liver damage. Persistent ingestion of chemical substances such as chronic medication (prescription drugs), daily supplements and certain foods increase the potential for hepatotoxicity (Farrel, 1997; Riley and Bhatti, 2001; Vuppalanchi, 2017). Conversely, certain chemical substances can protect the liver cells (hepatoprotective effects) against potentially harmful substances (Madrigal-Santillán *et al.,* 2014; Nasri *et al.,* 2015). In light of this, we have reviewed some medicinal plants that have shown activities against hepatotoxicity. *In vivo*, anti-oxidant effects of these medicinal plants have been demonstrated to protect against CCl_4 induced hepatotoxicity (Ahsan *et al.,* 2009).

Medicinal plants have long been a primary resource for health restoration in developing countries (Kala *et al.,* 2006). Lack of health care facilities has forced the rural dwellers to often rely on medicinal plants to cure minor infections and to help in the management of diseases such as cardiovascular diseases, diabetes, hypertension and digestive tract ailments (Mbewu and Mbanya, 2006; Chigora *et al.,* 2007). Moreover, studies have shown that extracts of some of these plants are effective in protecting against hepatotoxicity with its effectiveness depending largely on the component of the plant harvested, preparation procedure and method of administration (Tarkang *et al.,* 2014; Mat-Rahim *et al.,* 2017).

One such plant, *Balanites aegyptiaca* Delile, is an indigenous tree in Africa and parts of the Middle East, classified as either *Zygophyllaceae* or *Balanitaceae*. It is a semi-deciduous tree, cherished for its edible leaves, flowers, seed oil and fruits used in the preparation of foods and herbal medicines (Hall and Walker, 1991). Various researchers have demonstrated that *Balanites aegyptiaca* Delile (*Zygophyllaceae* or *Balanitaceae*) has a long and established history of treating several ailments. Medicinal properties attributed to *B. aegyptiaca* include anthelmintic (Gnoula *et al.,* 2007; Koko *et al.,* 2008), anti-inflammatory (Gaur *et al.,* 2008), anti-diabetic (Mansour *et al.,* 2002) and hepatoprotective (Chothani and Vaghasiya, 2011) properties, adding to its historic usage for treating ailments.

A study by Mohamed and colleagues (1999) documented that administration of aqueous bark extract of *B. aegyptiaca* to biliary duct-ligated rats, demonstrated a significant dose-dependent decrease in serum bilirubin levels. Further chronic and sub-chronic investigations established safety of the aqueous extract. A study by Ali *et al.,* (2001) confirmed that the aqueous bark extract of this tree had hepatoprotective effects in mice against paracetamol (0.6 g/kg) induced hepatotoxicity.

Plants of the genus *Leucas* (Lamiaceae) include over 80 species that are widely distributed all over Africa, India and Asia (Agyare *et al*., 2016). These plants have been utilized by traditional healers to remedy many health challenges, such as colds, diarrhea and inflammation, implying that *Leucas spp.* have great potential for discovery of lead compounds and new drugs (Das *et al.,* 2012). *Leucas lavandulaefolia* chloroform extract, taken from the flower by defatting with petroleum jelly, was discovered to have a hepatoprotective activity in D (+) galactosamine-intoxicated rat model (Chandrashekar *et al.,* 2007; Chouhan and Singh, 2011). Aqueous and methanol leaf extracts of *Leucas hirta* demonstrated a hepatoprotective activity against CCl_4 induced liver damage in rats (Manjunatha *et al.,* 2005). A methanol extract of *Leucas hirta* has been shown to protect the liver against CCl_4-induced toxicity compared to the aqueous extract. *Leucas hirta* significantly reduced the level of serum markers and significantly increased the total protein, as well as preventing necrosis and accumulation of macrocytic infiltrates. Additionally, Mangathayaru *et al.,* (2005) demonstrated significant hepatoprotective activities of *Leucas aspera* methanol extract against CCl_4 induced liver damage in adult swiss albino mice.

The herb *Tinospora cordifolia* is also known as “Guduchi” and belongs to the family, Menispermaceae. It is a large, deciduous climbing shrub, found at higher altitudes, and is genetically diverse (Rana *et al.,* 2012). It belongs to a group of medicinal plants known for their application in the treatment of several maladies in the traditional ayurvedic literature (Panchabhai *et al.,* 2008; Sengupta *et al.,* 2011).

In that context, researchers have shown great interest in this plant in recent times because of its reported properties, such as anti-oxidant, anti-allergic, anti-inflammatory, anti-leprotic, immunomodulatory, anti-neoplastic and hepatoprotective activities. Additionally, stems of this plant have been demonstrated to ameliorate CCl_4-induced acute liver damage in rats (Bishayi *et al.,* 2002).

This medicinal plant is advocated to treat jaundice, to decrease hepatosplenomegaly and to aid the flow of bile in the presence of hindering lesions (Geungerich and McDonalds, 2007). Furthermore, studies have documented that this plant decreases fibrosis in CCl_4-induced hepatotoxicity in rats and that extracts from this plant can protect against lead-induced hepatic damage (Dahanukar *et al.,* 2000). This plant has the ability of scavenging lead-induced free radicals (Sharma and Pandey, 2010).

Wedelia calendulacea and *Eclipta alba,* belonging to the family Asteraceae, present hepatoprotective potential. The ethanolic extract preparation of most Asteraceae family plants has demonstrated hepatoprotective effect against CCl_4-induced hepatotoxicity. Treating rats with 0.2 mL/100 g, 25 mg/100 g and 50 mg/100 g concentrations of *Wadelia calendulacea* extract for 10 days after CCl_4 administration reduced ALT, AST and ALP enzymes, markers of liver damage. The extract of *Wadelia calendulacea* also increases the serum total protein and bilirubin (Murugaian *et al.,* 2008; Saleem *et al.,* 2010).

Aspalathus linearis, commonly known as Rooibos, is endemic to the Western and Northern Cape regions of South Africa. Green Rooibos contains about 4-12% of aspalathin, a dihydrochalcone glycoside unique to *Aspalathus linearis* (Erlwanger and Ibrahim, 2017). Rooibos extracts demonstrated chemoprotective, anticarcinogenic and anti-allergic effects in rats exposed to the cancer initiator, diethylnitrosamine (Standley *et al.,* 2001; Marnewick, 2010).

Administration of Rooibos extract reduces levels of lipid peroxides, oxidative stress and ameliorates brain damage in streptozotocin-induced diabetic rats (Inanami *et al.,* 1995; Uličná *et al.,* 2006). Enhancement of anti-oxidant enzymes, such as superoxide dismutase, catalase, and the regulation of glutathione are amongst the suggested mechanisms for the protective activity of Rooibos extract (Hong *et al.,* 2014). The GRT extract has been shown to have a hypo-uricemic effect, significantly suppressing increased plasma uric acid with 20.4 µg/mL and 4.5 µg/mL doses of Rooibos extract (Kondo *et al.,* 2013).

In a study of Van der Merwe *et al.,* (2015), it was discovered that an aspalathin-rich green Rooibos extract increased food intake while body weight and organ weights of the rats remained stable. However, alkaline phosphatase activity was significantly increased after 90 days and significant increases in glutathione reductase activity was also discovered after 28 days.

Furthermore, the anti-oxidant enzyme activity of the liver showed no adverse changes when 29.5 mg/kg BW/day, equivalent to 4.8 mg/kg BW/day human dose, was used repeatedly for 90 days. The results from their study showed reduced glutathione content and significant differences in the expression of the oxidative stress related genes. The authors suggested that the aspalathin-rich green Rooibos extract causes biliary obstruction (J. D. Van Der Merwe *et al*. 2015). Hepatocyte failure or liver cell dysfunction creates a platform for the occurrence of biliary dysfunction (White *et al.,* 1963).

A study by Uličná *et al.,* (2008) evaluated the effect of Rooibos tea on hepatic biochemical parameters such as ALT, AST, bilirubin, glucose concentration and albumin during rat liver regeneration following intoxication with CCl_4. The rats were post-treated with Rooibos tea and carbon tetrachloride (RT+CCl_4 31.71 ± 4.97 mL/24 h/rat) for 42 days. The post treatment exhibited a significant decrease in the plasma levels of ALT, AST and total bilirubin when compared to the water administered control group (33.7 ±6.16 ml/24 h/rat; CCl_4+H_2O 29.68 ± 3.82 ml/24 h/rat). After the regeneration period of 42 days, the Rooibos tea was able to reinstate the biochemical parameters to healthy levels. This study levels, suggesting that Rooibos tea presents a preventive and medicinal potential for liver disease therapy (Uličná *et al.,* 2008).

In a study by Ajuwon *et al*., 2014, lipopolysaccharide-induced liver injury rat model, liver and kidney function tests evaluating the parameters that indicate anti-oxidant content and activity, such as glutathione and markers of lipid peroxidation were assessed. The consumption of Rooibos tea significantly reduced lipid peroxidation markers while total glutathione reduced significantly (Ajuwon *et al.,* 2014).

1.2 Hepatotoxicity

Drug-, herb- or herb-drug induced hepatotoxicity is a major health challenge. The problem is further confounded by the growing use of over-the-counter herbal remedies which are unregulated (Calitz *et al*. 2015). Many herbal products are not standardized, and safe and effective dosages have not been established. Herbal medicine use is common among rural dwellers and those living in remote areas due to lack of health care facilities and limited access to Western culture medicine (Scott, 2010). Traditional healers lack the understanding of biochemical pathways, genetics and molecular biology that are the major characteristics for understanding pathogenesis and management of non-communicable diseases (Kumar, 2007; Nolan *et al.,* 2011).

Traditional healers have no documented proof of the characteristics of the plants; they use their spiritual instinct to select the plant for use and to measure the dose for administration (Ngarivhume *et al*. 2015). Often patient record keeping is neglected or absent and therefore adverse reactions or effects are not properly recorded or reported.

Herbal-induced liver damage caused by a single or combination of substances, disturbs the normal functioning of the liver (Deshpande, 2002). Hepatotoxicity is the most common form of toxicity due to the exposure of the liver to harmful substances during detoxification. It commonly occurs due to adverse drug reactions and also with herbal supplements (Navarro and Senior, 2006). Induction of oxidative stress and lipid peroxidation are the common pathways for chemical-induced hepatotoxicity, often leading to hepatitis and cirrhosis (Bhakuni *et al.,* 2016). Mitochondrial dysfunction is an essential mechanism in chemical compounds-induced hepatotoxicity. Late stage damage to the mitochondria causes oxidative stress and vesicular steatosis (Jaeschke *et al*. 2011).

An indigenous South African plant *Callilepsis laureola,* commonly known as Impila, has been associated with renal failure and acute liver toxicity (Steenkamp and Stewart, 2005). The cytotoxic effect of *C. laureola* in human hepatocellular HepG2 cells was shown to be mediated through depletion of cellular glutathione with resultant hepatotoxicity (Bunchorntavakul and Reddy 2013).

1.3 Rooibos tea

Rooibos tea is a commonly known hot beverage highly consumed by a huge number of people, globally, for its great aroma and sweet flavour. The tea is produced from *Aspalathus linearis,* a South African endemic plant (McKay and Blumberg, 2007). The rapid increase of Rooibos consumption with time has raised consumers concerns on the significance of food and beverages in the prevention, management and treatment of health threats and diseases (Joubert *et al.,* 2013). Steenkamp *et al.* (2004) established that increased levels of superoxide and hydroxyl radical scavenging ability of the GRT extract exceeded that of other South African commercially-available herbal teas, roselle (*Hibiscus Sabdariffa* L) and honey bush tea (*Cyclopia intermedia*) (Steenkamp *et al.,* 2004).

Rooibos tea has shown to possess cardiovascular effects due to its ability to reduce activity of angiotensin-converting enzyme (ACE) (Persson *et al*., 2010). The angiotensin-converting enzyme inhibition is essential in preventing and treating high blood pressure and heart related complications (Chen *et al.,* 2013).

The inhibition of the ACE prevents the activity and continued production of angiotensin II from interfering with the cardiovascular system. Increased production of angiotensin II has been shown to narrow blood vessels, thus releasing aldosterone, causing increase in blood pressure levels (Duprez, 2006; Cohn *et al.,* 2017).

The liver is a large organ in the body. It contains enzymes that detoxifies foreign substances and execute other important metabolic functions (Meyer, 1996; Hall, 2015). In metabolism of drugs, Phase I metabolism which includes processes of reduction, hydrolysis and oxidation take place in the liver. After phase I metabolism the drug might sustain chemical activity , therefore requiring Phase II which involves conjugation (Gibson and Skett, 2001).

The liver metabolizes both useful and harmful substances and stores nutrients as well as other vital substances. After phase II metabolism the drug is therefore water-soluble and easily excreted through the kidneys (Hall, 2015).

The hepatocytes assist in the transformation of certain lipid-dissolving substances before excretion. Several signaling pathways are involved in significant differences of glucose uptake during starvation and nourished periods. This is adjusted through restoration of glucose by glycogenolysis or gluconeogenesis (Saltiel and Kahn, 2001).

Cytochrome P_{450} enzyme is the major enzyme found in the liver and it is responsible for biotransformation of herbs and drugs. However, the evidence to whether Rooibos tea affects crucial cytochrome P_{450} metabolizing enzymes is lacking. Therefore, the efficacy of Rooibos has to be established and caution must be taken in patients taking Rooibos with drugs and medicinal plants that are metabolized by cytochrome P_{450} (Zanger, 2013).

1.4 Beneficial Health Effects of Rooibos tea

Rooibos tea's beneficial effects are recapped through its anti-oxidant, antidiabetic, antimutagenic and anti-inflammatory activities (Kawano *et al.,* 2009; Marnewick *et al.,* 2005). Nevertheless, records of health benefits of the tea have been summarized including treating cardiovascular diseases, headaches and cancer (Cabrera *et al.,* 2006; Dludla *et al.,* 2014), insomnia, eczema (Lall and Kishore, 2014), allergies and bone weakness (McKay and Blumberg, 2002; da Silva, 2013) amongst others. Additionally, Rooibos tea contains many anti-oxidants that could protect against harmful effect of free radicals. However, high concentrations of aspalathin, a major polyphenol and anti-oxidant of Rooibos have been established in unfermented green Rooibos (Bramati *et al.,* 2003).

Rooibos tea has been shown to promote relaxation and strengthen the functioning of the immune system. Rooibos tea can be ingested at any desired time, it contains no caffeine, and this present an advantage to the functioning of the central nervous system (CNS) providing protection against flu, heart related illnesses and viruses (Marnewick, 2009).

The escalation of reactive oxygen species (ROS) and free radicals is directly proportional to the resulting oxidative stress and damage. Oxidative stress-related complications include damage to nerve cells which may cause neurodegenerative disease (Willcox *et al.,* 2004; Luo and Shi, 2005; Drechsel and Patel, 2008). As the production of ROS increases, the oxygen is used up by the brain. The endogenous defense metabolism acting against reactive oxygen species is activated to inhibit the oxidative damage initiated by ROS (Hong *et al.,* 2014). Rooibos tea has the potential of blocking oxidizing capabilities, inhibiting lipid peroxidation and scavenging free radicals.

Aspalathin has been found to have a hypoglycemic effect in rats and in humans. It has been established to prevent type 2 diabetes by increasing the content of GLUT4 in skeletal muscle and promoting translocation of GLUT4 from cytosol to cell membrane, thereby improving glucose uptake and enhancing insulin sensitivity (Kawano *et al.,* 2009; Son *et al.,* 2013; Cheng *et al.,* 2014). Other benefits of Rooibos relate to its positive effect in alleviating skin problems as well as enhancing hair growth (Cooper, 2012). *In vitro* and *in vivo* studies confirm that green Rooibos extract enhances plasma lipid profile and reduces oxidative damage in diabetic monkeys (Orlando *et al*. 2017). Recent findings demonstrated that aspalathin elevates cellular glucose uptake (Son *et al.,* 2013). The activated protein kinase (AMPK) pathway has been associated with promotion of GLUT 4 translocation from cytosol to the plasma membrane (Son *et al.,* 2013*;* Mazibuko *et al.,* 2014*).* The higher aspalathin content in green Rooibos tea presents a more dominant anti-oxidant activity compared to fermented red tea (Chen *et al.,* 2012)

The health benefits of green Rooibos tea are attributed to the high contents of polyphenols and flavonoids in unfermented Rooibos in fewer servings compared to the fermented Rooibos tea (Joubert *et al.,* 2008; Joubert and de Beer, 2012). However, the efficacy and safety of ingesting high doses of the phytochemicals has not been clearly explained or verified. The present study was aimed to establish whether consumption of higher concentrations of GRT extract has any toxic effects with specific reference to the potential of hepatotoxicity using human liver cells (C3A cells) and a sub-chronic Sprague- Dawley rat toxicity model.

1.5 Problem Statement

To date most studies on Rooibos have been performed using *in vitro* models and *in vivo* models, mainly limited to rodents. Apart from one human study, which was performed on human subjects vulnerable to developing cardiovascular diseases (Marnewick *et al.,* 2011), there is no anecdotal evidence that suggests that consumption of Rooibos has any undesirable side effects in humans. However, two case studies have been published that associate the occurrence of hepatotoxicity with Rooibos consumption (Sinisalo *et al.,* 2010; Engels *et al*., 2013).

In the first case study published by Sinisalo *et al.,* (2010), a 42-year old woman presented with the occurrence of hepatic enzymes ALP, gamma-glutamyl ransferase (GGT) and ALT, detected in plasma and was treated for a low- grade β-cell malignancy. All criteria were within normal ranges. However, the ultrasound revealed moderate steatosis in the liver. The woman confessed to have not taken excessive alcohol or any other medications but have been drinking Rooibos excessively for the past two weeks prior to the examinations. She was instructed to refrain from consuming Rooibos and discontinue her antibiotics (co-trimozole). Within a week, the patient's liver enzymes had returned to normal and she started her treatment of prophylactic co-trimozole.

The authors of the study concluded that the "association of Rooibos with hepatotoxicity" could be due to unintended contamination by harmful substances initially not meant to be present in Rooibos. However, traces of pyrrolizidine alkaloids have recently been found in export consignments of Rooibos to Germany (Van Wyk *et al*., 2017).

A poster presentation in San Francisco 15-18 June (2013) at the 95th Endocrinology Society conference was the second case study, and this described a 52-year old hyperlipidemia male patient on atorvastatin. The patient displayed pruritus, jaundice and malaise. Increased liver enzymes suggested cholestasis and liver injury. Upon interrogation, the patient reported that he had been consuming increased amounts of Rooibos for the past three months.

The authors then suggested that Rooibos could induce hypercholesterolemia, cholestasis and hepatocellular injury at increased consumption. Thus, this study will establish if the consumption of high amounts of green Rooibos has any toxic effects in human liver cells (C3A cells) and in the liver of a sub-chronic Sprague-Dawley rat toxicity model.

1.5.1 Diseases emanating from hepatotoxicity

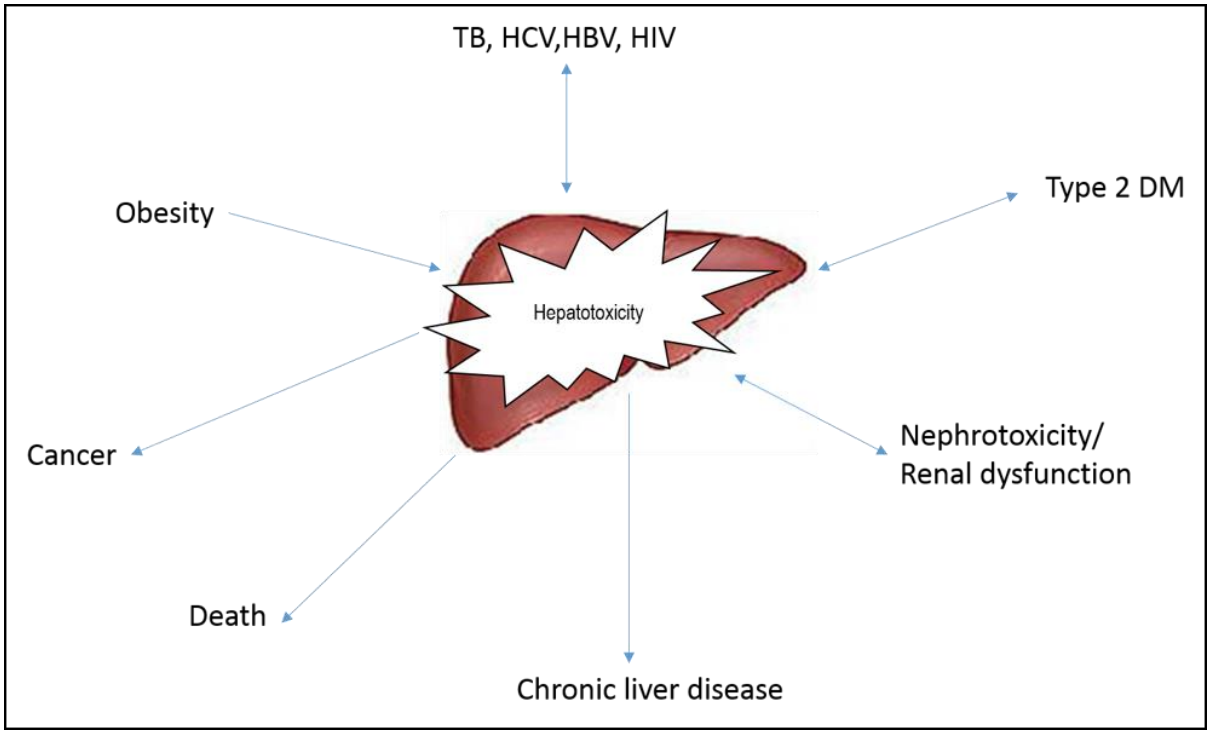

Figure 1.5-1: Disease cause and effect in relation to hepatotoxicity, modified from (Singh *et al.,* 2016)

Although liver injury is commonly thought to be drug- induced, dietary supplements, interactions with medication and diseases can have hepatotoxic consequences representing symptoms that are like drug induced hepatotoxicity (Figure 1.5.1). In addition there is a close relationship between liver disease such as non-alcoholic fatty liver disease with cardiovascular disease and metabolic disorders (Targher and Arcaro, 2007). Oxidative stress and mitochondria are involved in the development of cardiovascular disease (Dorn *et al.,* 2015).

1.6 Aim and Objectives of the study

1.6.1 Aims

1. To assess *in vitro* cytotoxicity of a green Rooibos aspalathin-rich extract in human liver cell line (C3A).
2. To assess *in vivo* toxicity of a green Rooibos aspalathin-rich extract in a Sprague-Dawley rat model.

1.6.2 Objectives

1. To investigate the effects of the green Rooibos aspalathin-rich extract on human liver cell line (C3A) viability using MTT and ATP assay.
2. To ascertain whether an aspalathin-rich green Rooibos extract is toxic in a sub-chronic toxicity Sprague- Dawley rat model or not.

CHAPTER 2

2 LITERATURE REVIEW

2.1 Introduction

Immortalized cell lines and primary isolated liver cells are currently the most widely used *in vitro* models for liver toxicity (Saldatow *et al.,* 2013). Cell culture models are believed to explain toxicity pathways, cellular response networks and mechanisms of action, and have produced substantial data (Nelson *et al.,* 2017). In the liver, the most abundant cell type and primary functional unit are the hepatocytes, which perform many tasks, including the xenobiotics metabolism (Bandele *et al.,* 2012). The HepG2/C3A, which is a sub-clone of the human HepG2, is a preferred cell line because of its retention of the biological characteristics of the adult liver (Kelly and Darlington 1989). The other reason that makes these cells favourable for a toxicity study is their ability to shift from fetal to phenotype during the sub-constituent log growth phase. Upon reaching confluence and expressing functional cytochrome P_{450} enzymes (Bandele *et al.,* 2012). This makes the HepG/C3A a suitable model for hepatotoxicity study.

In vivo studies are commonly used to discover effects of compounds in the whole living organism or targeting specific organs (Ting *et al.,* 2015). These models are used to examine any toxicity, develop new nutraceuticals and are necessary in the safety evaluation of the compound before administration to humans. The *in vitro* and *in vivo* models in hepatotoxicity are compulsory for correct diagnosis and evidence-based actions such as grounds to withdraw the use of certain drugs. They provide information on dosage limits that are not harmful to humans (Ekor, 2014; Elzagalaai and Rieder 2015; Raschi and De Fonti 2015).

The liver is an essential organ of the body and without it, or with lack of immediate transplantation, there is no life. The liver receives toxins in many forms and processes them through several reactions (oxidation, hydrolysis, reduction and conjugation) (Gu and Manautou, 2012). These reactions reduce the effect of the harmful substance. These substances for example free radicals, drugs, and chemical toxins in excess have a potential to cause tissue damage (Lobo *et al.,* 2010). The liver enzymes, most importantly the cytochrome P450 enzyme and glutathione are responsible for processing of toxins.

Most of the liver enzymes are located in the cytosol or the mitochondria of the cells and any increase or decrease of the plasma liver enzymes from the normal levels may indicate liver disease. Stage progressive diseases of the liver have become one of the contributing factors in reduced life expectancy (William, 2006).

The liver and kidneys work together to facilitate excretion of drugs and xenobiotics from the body (Sun *et al., 2006)*. The effectiveness of a drug is associated with the action site to which it binds, bioavailability and interaction with other drugs or herbal supplements (Delafuente, 2003). The drug dose, absorption, distribution, metabolism and excretion of the drug are factors contributing to the enzyme activity and tissue function (Zhou *et al.,* 2007). Over 2000 cases of acute liver disease occur yearly in the United States, and out of the 2000 cases, a 1000 of them are drug induced. The leading impactful drug is acetaminophen, responsible for 39% of the drug induced cases (Mehta, 2016). In South Africa data on liver disease is sparse however the 2010 global burden of disease study show that the incidence of liver cirrhosis double between 1980 and 2010 (Spearman *et al*. 2015).

2.2 Liver physiology

2.2.1 Metabolic functions of the Liver

The liver is a crucial component of the digestive system; it performs many functions in normal, healthy conditions. This vital organ is located below the diaphragm in the upper right part of the abdomen (Sibulesky, 2013). The hepatic portal vein provides the liver with blood rich in nutrients obtained from the small intestine, while the hepatic artery delivers oxygenated blood to the liver. The liver has a larger right lobe, a smaller left lobe and segments of many lobules. These lobules connect to the duct forming the hepatic duct, which is imperative for the transportation of bile by liver cells to the small intestine (Muriel, 2017). The liver cells produce bile, a fluid imperative in digestion and absorption of fats in the small intestine. Besides these functions, the liver is important for removing body waste and other harmful substances, metabolic processing (like the conversion of glucose to glycogen to provide energy during starvation) and helping the body fight infections. Increased hepatocyte proliferation enhances the regenerative capacity of the liver (Huch and Dolle, 2016, as cited in Muriel, 2017).

2.2.2 Detoxification by the liver enzymes

The liver transforms and metabolizes substances and chemicals to increase water solubility and thus promoting easy excretion. The liver further filters the debris that circulates in the system, such as dead cells, drugs and other microorganisms from the blood stream (Baker *et al.,* 2016). The sinusoidal system consists of Kupffer cells that are responsible for the biotransformation of xenobiotics (Losser and Payen, 1996; Larson and Hauswald, 2014). Apart from the metabolism of drugs, the liver fulfills the function of lipid and carbohydrate metabolism (Chiang, 2014), however, when the liver fails to function accordingly, liver diseases are manifested.

Metabolic organs are at high risk of exposure to poisonous substances. Hepatic enzymes remove species and xenobiotics that threaten to harm the organisms. Glutathione-S-transferases are catalytic enzymes that promote the conjugation of glutathione and protects against the organophosphorus insecticides (Townsend and Tew, 2003). Glutathione-s-Transferase has extensive binding properties that promote detoxification (Sheehan *et al.,* 2001).

Biotransformation of compounds requires action of the xenobiotic metabolizer, the liver, which reduces the biochemical activity of compounds (Malsh *et al.,* 2008). Deficiency in glutathione causes a serious threat to the functioning of the liver and results in liver injury. Changes in glutathione-S- transferase enzyme expression, therefore indicates presence of toxins (Strange *et al.,* 2001).

2.2.2.1 The cytochrome P_{450} enzyme

The cytochrome P_{450} enzymes (CYPs) are the enzymes of the body's defense system, dominant in the liver. They protect the body from toxic substances by detoxifying drugs and herbal supplements and synthesizing essential molecules (Guengerich, 2005). CYPs are membrane proteins containing heme and they play a major role in drug metabolism as they indicate and transform a range of toxins and reduce the chemical activity of the toxic substances (Zanger and Schwab, 2013). CYP_{450} enzymes are categorized by their family code CYP and the expression of these CYPs is influenced by different mechanisms. The oxidative biotransformation of drugs and other substances exposes the liver to various diseases complications (Pelkonen *et al.,* 2008; Coleman, 2010).

Literature reveals that the CYP_{450} system is a major marker in interaction occurrences with possibility for toxicity or adverse side effects. CYP3A4, a CYP_{450} isoform, is highly expressed in the liver and the small intestine. Induction of CYP3A4 enzyme reduced the concentration and efficacy of the drug, resulting in drug resistance. Inhibition of this enzyme causes metabolic imbalance and enhances drug concentration in the blood and tissue, inducing toxicity (Mann, 2006; Matsuda *et al.,* 2007).

2.3 Hepatotoxicity and liver diseases

Liver disease is caused by injury to the liver and interferes with its biochemical functions. Non-alcoholic fatty liver disease (NAFLD) is a liver condition that occurs because of unhealthy behaviour. NAFLD is identified by traces of hepatic steatosis in non-alcohol consuming individuals. Patients suffering from diabetes mellitus and obesity are at risk of developing NAFLD. Management of NAFLD requires medication for liver disease or for metabolic disturbances (Chalasani *et al*. 2012). Hepatitis is inflammation of the liver that can cause tissue scarring known as fibrosis. Cirrhosis and hepatocellular carcinoma gradually occurs as severe conditions of hepatitis (WHO 2016). Cirrhosis is a liver damage condition resulting from severe hepatitis and other liver complications such as steatosis and alcoholic fatty liver disease (Suk *et al.,* 2014). Cirrhosis is an irreversible stage of liver disease and has limited treatment options. Cirrhosis also includes reactions to medication, repeated occurrences of heart failure and obesity. Hepatocellular Carcinoma (HCC) is cancer of the liver cells (hepatocytes). Hepatitis and cirrhosis are pre-conditions of hepatocellular carcinoma.

The liver synthesizes plasma proteins and failure to perform these functions, unique to the liver, suggest liver damage. A study by Treinen-Moslen (2001) shows that evidence of liver damage or dysfunction requires an appreciable amount of cell death. Cell survival rate (>50%) and the consequence of impaired hepatocyte function suggests the function of the liver is affected or altered. Excessive bleeding suggests that the liver is unable to synthesize blood clotting factor proteins (Treinen-moslen, 2001). Furthermore, occurrence of hepatotoxicity depends on the population of cells, concentration of the toxicant and duration of exposure (Hardman, 2006).

2.3.1 Toxicity types and mechanisms of toxicity

Toxicity is an extent to which a toxic substance harms the living system, this may affect the functioning of the system and severe cases of toxicity result to death. For a damaged organ to be restored to good health, moderate cases of liver injury are curable by commercially available medications while severe cases may demand organ transplant (Astashkina *et al.,* 2012). The toxicity contributing factors include the genetic make-up and health of the recipient, the routine of administration of the toxin (which can be by means of inhaling, ingestion or injection), the number of times the substance is taken, the duration of exposure and the phase of the toxin. Toxicity may occur because of unusual metabolism due to alterations in Phase I oxidation and Phase II conjugation metabolism (Philips *et al.,* 2001). Other causes of toxicity include substances from personal care products, food poisoning, automobiles, cleaning detergents, over-the-counter medications and herbal remedies, etc. (Swierzewski, 2008). The type of toxicity is determined by the part of the body affected by the substance (i.e. when the liver is affected the damage is termed hepatotoxicity, the nervous system is termed neurotoxicity and when it affects the cells, it is referred to as cytotoxicity) (Sharma *et al.,* 2014). There are two main categories used to describe the extent of toxicity, the effect caused instantly and the degree of exposure that shows effect at repeated doses. These are acute and chronic toxicity respectively.

2.3.2 Risk factors of hepatotoxicity / Liver diseases

The liver is a very complex body organ and it is essential for survival, therefore, any exposure to a form of liver disease increases the chances of death. The chances of surviving diseases depend on the proper functioning of the liver in metabolizing the drug aimed at treating that disease (Bernal *et al*. 2010). When the liver fails to function, though not severe enough to cause death, other diseases take advantage, and this implies that liver dysfunction exposes the body to harm and reduces life expectancy (Blasco-Algora *et al*. 2015). A study by O'Shea *et al.,* (2010) shows that other causes of liver disease include, not only age and excessive abuse of alcohol, but our lifestyle affects the function of the liver e.g. processed food and additives cause a build-up of fat in the liver.

2.3.2.1 Regeneration of reactive species

According to Kim *et al.* (2014), air pollution also induces liver inflammation and steatosis. Liver damage may occur because of the accumulation of toxins at a higher rate than the rate the liver can clear the toxins. This accumulation causes complications such as fatty liver, oxidative stress, cell death, inflammation, hepatocellular carcinoma and leads to the final shut down of the liver and death (Bhakuni *et al.,* 2015).

Oxidative stress is the imbalance between produced reactive species and the body's ability to rid the system of the reactive substances (Betteridge, 2000). However, anti-oxidants are introduced to reduce the activity of free radicals, reducing oxidative stress and having curative benefits in liver disease (Li *et al.,* 2015). Hesperidin is a flavone of citrus fruit and provides a protective effect against oxidative stress (Hepel and Andreescu, 2015). Inflammation also plays a part in the development of hepatotoxicity. It increases the reactivity of tissues to toxic substances. Inflammatory cells release reactive oxygen species, therefore attacking and damaging tissues (Deng *et al*. 2009).

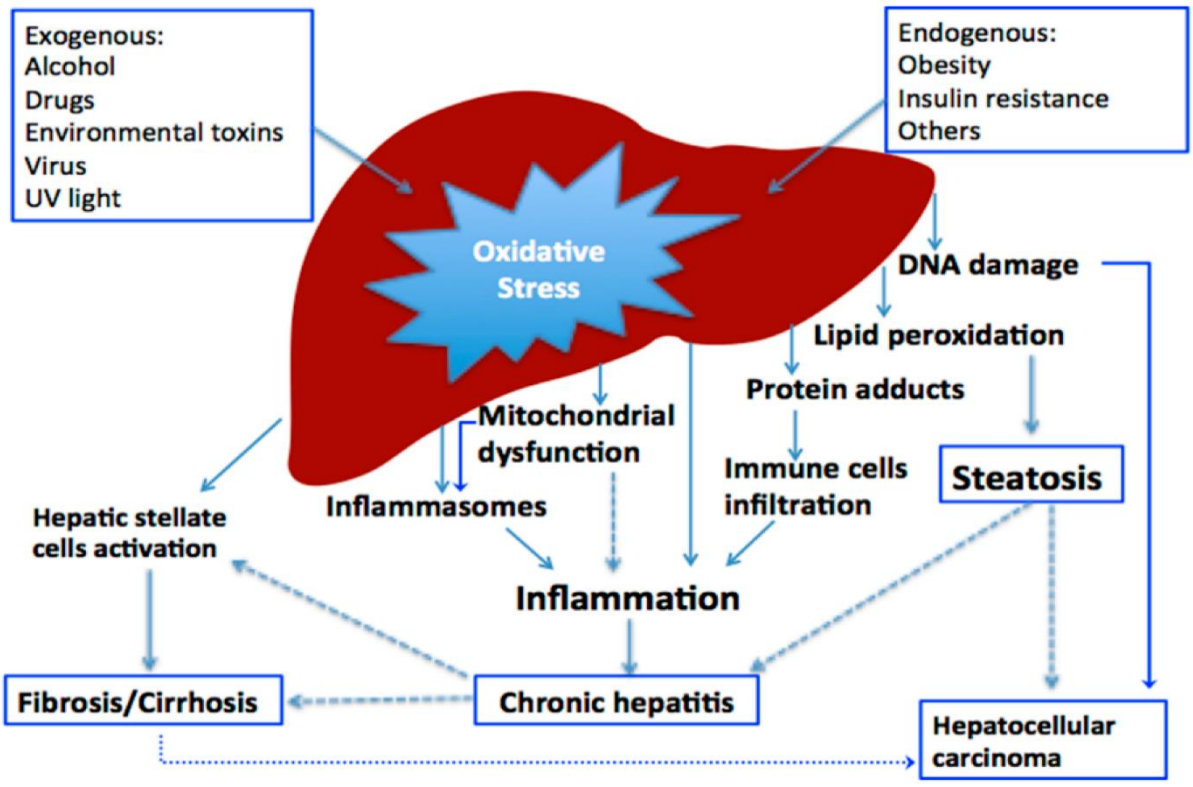

Figure 2.3-1: A schematic representation of the cause and effect of oxidative stress in the liver. (Modified from Li *et al.,* 2015)

Toxins cause a rise in the production of free radicals resulting in oxidative damage (Ercal *et al.,* 2001). Excessive reactive oxygen species (ROS) disturbs the homeostasis, subsequently leading to oxidative stress. The oxidative stress triggers liver diseases and other severe and degenerative diseases (Lindblom *et al*. 2015). It also induces hepatic damage by triggering irreversible alterations of DNA content, lipids and proteins and more importantly, regulating pathways that control normal biological activities. Since these biological pathways control cell apoptosis, genes transcription, hepatic stellate cell activation and protein expression, oxidative stress is regarded as one of the pathological mechanisms that leads to initiation and development of several liver diseases like non-alcoholic steatohepatitis, chronic viral hepatitis and alcoholic liver diseases (James and Day, 1998; Buzzetti *et al.,* 2016). The increase in content of ROS or other free radicals, results in cell death. Necrosis and apoptosis are the two modes of cell death that are reported as the response to chemical, drug and herb toxicity (Treinen-moslen, 2001).

2.3.3 Drugs and chemicals associated with liver damage

Drug induced hepatotoxicity propagation is determined by the occurrence of damage to the liver, caused by a daily dose of a certain drug (usually known as dose-dependent toxicity), metabolism properties or the drug-drug and/or drug-substance interaction (Pandit *et al.,* 2011).

Carbon tetrachloride (CCl_4), also known as tetra-chloromethane, is a cleaning agent and is also used in fire extinguishers. CCl_4 has been evaluated for toxicity in the liver and studies show that exposure to this chemical increases the production of free radicals which disturb the nervous system and cause necrotic cell death and injury to the liver and the kidneys (Weber *et al.,* 2003; Dong *et al.,* 2013). Literature reveals that CCl_4 induces lipid peroxidation and reduces the activity of the anti-oxidant enzymes causing overproduction of free radicals and oxidative damage to the liver cells. Oxidative damaging of cells causes the mitochondrial enzymes of the liver (i.e. adenosine triphosphates and adenylate kinase) to leak into the blood stream. Induction of hepatotoxicity by CCl_4 causes accumulation of fat in the liver. Metabolism of CCl_4 by the CYP_{450} converts CCl_4 to microphage inflammatory protein 1- alpha (CCl_3) and CCl_3OO. This bio-activation process of reactive oxygen species leads to lipid peroxidation and injury to the liver (Treinen-Moslen, 2001).

Studies showed induced CYP2E1 is a common mechanism for CCl_4, ethanol, acetaminophen and other toxic substances (Jaeschke *et al.,* 2002; Kesič and Kumari 2016). Chronic exposure to substances such as aflatoxin, a poisonous, carcinogenic Aspargillus toxin reduces the enzymatic anti-oxidant activity. About 4.6-28.2% of hepatocellular carcinoma cases have been associated with aflatoxin (Liu and Wu, 2010). Aflatoxin induces oxidative stress which causes changes in liver enzymes, creatinine, triglycerides, glucose, total protein and albumin (Karabacak, 2015). Black tea anti-oxidants ameliorated the effect caused by aflatoxin on cells and tissues of rats (Alm-Eldeen *et al*. 2015).

2.3.4 Hepatotoxicity of drug and herb interactions

Drug interactions have been studied and viewed as one of the leading causes of toxicity. Most importantly, the dose and variety of medications administered increase the chance of interactions that are toxic (van Leeuwen *et al.,* 2015; Kane *et al.,* 2017). Drug-drug interactions are known to affect the mode of action of drugs by either decreasing or increasing the activity of the drug and resulting in undocumented or unexpected reactions for each particular drug consumed (De Boer *et al*., 2015; Boullata 2005). Apart from drug and drug interactions, drug-beverage interactions may also result in toxicity (Ogu and Maxa, 2000). The mechanism of interaction of Rooibos occurs through the effect of Rooibos flavonoids on the P-glycoprotein or the CYP3A4, an iso-enzyme of the CYP_{450}. The flavonoids of Rooibos tea inhibit the drug transporter protein and decrease the initial metabolism of some agents (US, Medicinal Plants, 2011).

Inhibition and induction are by far the most common mechanism through which drug-herb interaction occurs (Plant, 2007). Both drugs and medicinal plants have beneficial effects and side effects. The effectiveness depends on preparation procedure, dose administered, concurrent use with any drug or medicinal plant, health status of the drug recipient and other factors (Brown and Brown 2004; Kibiti and Afolayan 2015). Professionals in toxicity studies often study the effect of drugs on target organs and on target cell types while failing to study the effect on the whole system. The concurrent use of herbs and allopathic drugs modulates the activity of metabolizing enzymes, influencing the overall outcome of the administered drugs and herbs (Gouws *et al*. 2012).

2.3.5 Cell culture models of liver toxicity

Cell culture models have been the preferably used models for determining potential toxicities from drugs and other chemicals. The most performed experimental practices are tissue slices, immortalized cell lines and hepatocyte and stem cell cultures (Soldatow *et al*. 2013). The cell culture models are usually called *in vitro* models and are used to detect a variety of complications. These models are used to detect the functionality, viability and expression in the liver and are accurate at investigating complications at cellular levels.

Cell culture models proved to be useful in many studies to assess the underlying causes of liver toxicity and depict what is happening in living organisms. However, there have limitations, which include availability, inconsistent growth, limited survival and high risk of contamination (Guillouzo *et al.,* 2007). The study of Guillouzo *et al.* (2007) further explained that in drug and chemical metabolism and toxicity studies, the primary hepatocyte remains the most efficient model. Cytotoxicity, specifically with C3A cell line, has contributed useful insight into the understanding of the pathogenesis of liver disease. Cell growth, membrane and metabolism integrity or cytosol enzyme release, and other functions specific to the cell line, are usually conducted for measuring toxicity (Ekwall *et al.,* 1990).

In vitro models increase the rate of success of animal studies and clinical trials since they provide cell-based toxicity of the compound and this can aid the selection of the concentrations to be used for *in vivo* studies. Cytoprotective effect and metabolic activity/metabolic pathway genotoxicity studies of the liver are usually tested with the cell lines of liver origin such as HepG2/C3A. The HepG2/C3A is a widely used cell line for detecting toxicity (Baudoin *et al*. 2011).

2.3.6 Animal models of liver toxicity

In vivo models are animal experiments used to investigate the problems or benefits of a test material in organisms resembling human to gain information that can be used to identify the dose and reactivity of the material and issue warning where necessary. Successful evaluation of *in vivo* studies increases the confidence to carry the study further to clinical trials (Van de Staay *et al.,* 2009). Bhakuni *et al.,* (2016) proposed that to derive new therapeutic drugs, it is essential to fully understand the main mechanism of action in animals, to allow for translation to the human.

The focus of their study was to investigate different animal models and the manner of action in which the hepatotoxicity is produced.

Ex vivo models, such as bio-artificial livers, are also used in cases where the liver disease has reached the critical stage and consumable remedies and have completely failed (Andria *et al.,* 2010).

2.3.7 Diagnostic biomarkers of hepatotoxicity

Biomarkers are measurable factors or indicators of biological, pathological and pharmacological processes under normal living conditions, and in this case the influence of toxins used for therapeutic interventions (Table 2.3.1).

2.3.7.1 Bile acids and intracellular proteins as biomarkers for hepatotoxicity

Hepatocyte apoptosis is associated with cholestasis (Hua *et al*. 2017). Cholestasis is the result of reduction in bile flow from the liver, which may be caused by the failure of the liver to produce or to transport bile (Chiang, 2017; Li *et al.,* 2017). Accumulation of hydrophobic bile acids in the liver serves as indication of impaired hepatocyte or damaged liver. Toxicity of bile acids can be produced by endogenous toxicants introduced into the system and occurrences of liver injury, liver dysfunction, cirrhosis and loss of life are common. Bile acids provide a sound model for detecting the mechanism of cell death. In the liver cells, elevated toxic bile acids activate the translocation of intracellular proteins such as Fas to the cell membrane, thereby initiating apoptosis (Jaeschke *et al*. 2002).

The adenosine triphosphate assay (ATP assay) is a bioluminescence assay used for quantifying the content of ATP. ATP produced is related to the number of cells that are viable or metabolically active (Morciano *et al*. 2017).

Table 2.3-1: Clinical biomarkers of hepatotoxicity (Ramaiah, 2007, as cited in Van De Merwe, 2012)

	Function		
Biochemical Test	**Hepatocellular**	**Hepatobilliary**	**Kidney**
*Alanine aminotrasferase (ALT)	X		
*Aspartate aminotransferase (AST)	X		
Sorbitol deghydrogenase (SDH)	X		
Glutamate dehydrogenase (GLDH)	X		
*Alkaline phosphatase (ALP)		X	
Gamma glutamyltransferase (GGT)		X	
5'-Nucleotidase (5'NT)		X	
*Total bilirubin (TBIL)		X	
Total bile acids (TBA)		X	
Ammonia			
*Albumin (ALB)			
*Total protein (TP)			X
*Creatinine (CREAT)			X
*Blood urea nitrogen (BUN)			

2.3.7.2 Anti-oxidant enzymes, anti-inflammatory response (NF-kB signaling) and apoptotic response

Anti-oxidant enzymes are free radical scavenging enzymes used to measure the effect of oxidative stress on the functioning of the anti-oxidant defense system (Amacher *et al.,* 2010). Decrease in the anti-oxidant enzyme concentration signifies oxidative stress related damage (J. D. Van Der Merwe *et al*. 2015).

Glutathione (GSH) and glutathione peroxidase (GPx) are anti-oxidant proteins synthesized in the cytosol and they are the signaling regulators that protect the cells from free radicals. Depletion of glutathione enhances the exposure of mitochondria to ROS and intra-cellular depletion of GSH induces mitochondrial damage (Yuan and Kaplowitz 2009).

The transported glutathione into the mitochondria from the cytosol is of paramount importance for cell sustainability, survival and cell functioning. Significant to diagnosing hepatotoxicity is the capability of glutathione to determine the intracellular cell death stimulus.

Superoxide dismutase (SOD) and catalase (CAT) are other anti-oxidant defense system enzymes induced by the elevation of oxidative stress. CAT and increased levels of intracellular glutathione, aids in the prevention and control of apoptosis initiated by ROS.

In the liver increased oxidative stress induces inflammation. Inflammation causes increased deposition of the extracellular matrix that transforms the tissue structure and function, leading to organ failure (Ueha *et al.,* 2012). The hepatoprotective effect of nuclear factor- KappaB (NF-κB) was discovered by (Beg *et al.,* 1995). Advances confirmed the hepatoprotective effect of NF-κB on TNF-α induced cell death (Ghosh and Karin, 2002). NF-kB is a 65 kDa protein responsible for controlling cell survival and production of cell signaling proteins. NF-kB is an important regulator of cellular response to stimuli, particularly in the inflammation and immune response (Baeuerle and Batimore, 1996). Activation of the inflammation response gene NF-kB and release of pro-inflammatory proteins, such as the TNF-α which has been implicated in the generation of excessive ROS, signifies cell damage and the intensity of inflammation which later leads to liver dysfunction (Kleniewska *et al*. 2013).

Apoptosis is a naturally occurring process of cell death. Bax, a pro-apoptotic protein triggers the release of cytochrome c from mitochondria which enhances the activation of caspases, mediators of apoptosis, representing a family of cysteine-aspartate proteases (Simon *et al.,* 2000; Chen and Wang, 2002). On the other hand tumour necrosis factor-induced oxidative damage can enhance the activation of the anti-apoptotic pathway as a result of transcriptional NF-Kb activation (Berghe *et al*. 2014; Catrysse *et al*. 2014). Apoptosis inhibition causes the accumulation of deadly inflammatory cells which is an early stage of HCC (Simon *et al.,* 2000).

2.3.8 Nephrotoxicity markers in detection of hepatotoxicity

Nephrotoxicity is an undesirable condition of the kidneys and is usually caused by poisonous chemicals and medications. Kidneys are co-joined with the liver in the maintenance of homeostasis, detoxification and excretion of xenobiotics. Literature reveals that drugs increase the incidence of nephrotoxicity by 66% with an increase in average life span (Kim and Moon, 2012). Creatinine is the degradation product of creatine, an amino acid produced and secreted in the liver (Edison *et al*. 2007).

2.3.8.1 RUCAM model

The Roussel uclaf causality assessment method is a seven-explicit category system for the analysis/ investigation of liver injury features. This method assesses the causality of drug initiated liver injury and literature confirms the validity of the model for clinical, biochemical, radiological and serological assessments of liver injury (Cavalieri and D ’agostino, 2017; Vuppalanchi, 2017). Among the categories highlighted in the RUCAM model are the non-drug causes and concomitant drugs; consideration of these factors and other contributing risk factors, such as age, in diagnosis bring about accuracy in pinpointing the cause of liver injury. It is not possible, without thorough investigation that includes all factors involved in disease onset, to diagnose based on the history.

2.4 Prevention, Indication and treatment for hepatotoxicity/ liver diseases

Liver biopsy and serum biomarkers of liver disease are some common methods used for diagnosis of liver disease. Medicines and surgical approaches have been used for treating liver diseases (Wong and Frenette, 2011). Prevention of minor diseases that lead to liver cirrhosis, such as hepatitis, is by immunization against viruses and maintaining all other conditions that exposes the liver to the damage. Cirrhosis is incurable, treatment only prevents further damage to the liver and managing the disease includes several doctor visits, medication and change of lifestyle.

Drugs for treating cirrhosis are acetaminophen, naproxen and ibuprofen, but these medications have undesirable side effects, which include more harm to the liver or bleeding depending on the patient’s response to the medication (MedicineNet, 2014).

2.4.1 Medicinal plants

For thousands of years, plants have been used as medicines for treating diseases and these medicines initially were in raw state such as powders, poultices, teas, tinctures and other herbal formulations (Balunas and Kinghorn, 2005). These plants are administered either orally, rectally, by bathing or intramuscular injection and nasally, which can be through snuffing, steaming or smoking. Some of the plant derived medicines are still included as part of the common remedy for several maladies. This provides insight into a therapeutic model and fast-tracking the drug discovery process; this is termed reverse pharmacology (Martin *et al.,* 2012). The compendium of medicinal plants is global and the utilization of these plants in industrialized societies has been due to the extraction and development of various drugs and chemotherapeutics from these plants and from traditionally used rural herbal remedies (Hoareau and DaSilva, 1999).

With all this we can say medicinal plants are a vital component of pharmaceutical companies' research development. Due to lack of scientific insights to explain the mechanism of actions, the use of medicinal plants in some countries is frequently associated with witchcraft and superstition (Gurib-Fakim, 2006). However, since us scientist have a better knowledge of how the body works, we are in a better position to comprehend the healing potentials of these plants. Medicinal plants are made up varieties of chemical compounds that may work independently, supplementary or in synergy to improve health (Gurib-Fakim, 2006). On a regular basis, more than 3.3 billion persons use medicinal plants in the less developed countries today (Davidson-Hunt, 2000). Approximately, 35,000 plant species are found in developing countries and they possess medicinal values which are valuable to human health and continued existence. In the modern pharmacopoeia, roughly 7,000 medical compounds are obtained from plants (Hefferon, 2012).

These natural medicines have a safe profile. They are gentle, cost-effective and easily accessible, which makes humans self-medicate (Abebe, 2002) rather than consulting a medical practitioner. Though these botanicals are safe, some problems are still associated with them and these include contamination, toxicity, adulteration and interaction with other drugs (Ernst, 2004).

So far, only a few researchers have worked on studying the negative sides of these plants. However these plants have been used in the remedy of various health problems such as cancer (Itharat *et al.,* 2004; Desai *et al.,* 2008), malaria (Snounou *et al.,* 1999; Ayoola *et al.,* 2008; Titanji *et al.,* 2008), schistosomiasis (Fennell *et al*. 2004; Wink and Michael 2012), hypertension (Eddouks *et al*. 2002), diabetes (Eddouks *et al.,* 2002; Sabu *et al.,* 2002), cardiovascular disorders (Sabu *et al.,* 2002; Shukla *et al.,* 2007), HIV/AIDS (Lamorde *et al*. 2010) and hepatotoxicity (Ahsan *et al.,* 2009; Osadebe *et al.,* 2012) amongst others.

Liver diseases pose a major health concern and countless attempts have been made to invert new therapeutic strategies in dealing with liver diseases, with each update proving the weakness of the medication in use. Drugs with a documented severe impact on the liver's ability and structure are restricted or discontinued (Guan and He, 2013) and that is why researchers have sought a breakthrough in medicinal plants. Over the years, experts have exhausted their potential for discoveries of active compounds; all to reduce pain, prevent and cure several diseases. The healing properties of medications in use to treat cardiovascular diseases have always presented tremendous results but more thorough investigations were carried out associating the treatment with harmful behaviors on one body part or the other. Medicinal plants have often proven to be more effective with less negative effects (Gurib-Fakim, 2006).

The availability of plants for medical treatments gives the urge for the transition. Rural dwellers use medicinal plants as a primary source of treatment and the methods they use for preparations of medication are infusion and decoctions. Medicinal plants and their method of preparation are relatively cheaper than other treatment options (Patrovska, 2012).

Green tea has a wide variety of medical benefits including the treatment of diabetes mellitus (Eddouks *et al.,* 2002; Sabu *et al.,* 2002; Kim and Kim, 2013). However, the study of Emoto and co-workers (2014) proved that one dose of green tea extract can induce acute and chronic liver damage and the authors concluded that this might be related to oxidative damage and lipid peroxidation in the liver. Rooibos tea is brewed from an *Aspalathus linearis*, an endemic plant found in Cedarburg Mountains of Cape Town.

Aspalathus linearis extract (GRT) presents unique phytochemical compositions (Figure 2.4.1) exhibiting a protective effect in many tissues (Canda *et al.,* 2014). Rooibos consists of constituents, such as magnesium, zinc and iron, which are all useful in a healthy functioning nervous system. Fermented Rooibos tea has proven to calm digestive disorders, lower blood pressure and reduce nervous tension (McKay and Blumberg, 2007). A study of green Rooibos extract carried out by Kawano *et al.,* (2009), investigates the influence of aspalathin on glucose metabolism. This was done by culturing the L6 myotubes and RIN-5F pancreatic β- cells with the results revealing aspalathin having a beneficial effect on the balance of insulin and glucagon in type II diabetes. The hypoglycemic activity of aspalathin is also associated with the increased content of GLUT4 and translocation to the plasma membrane through AMPK activation (Son *et al*. 2013).

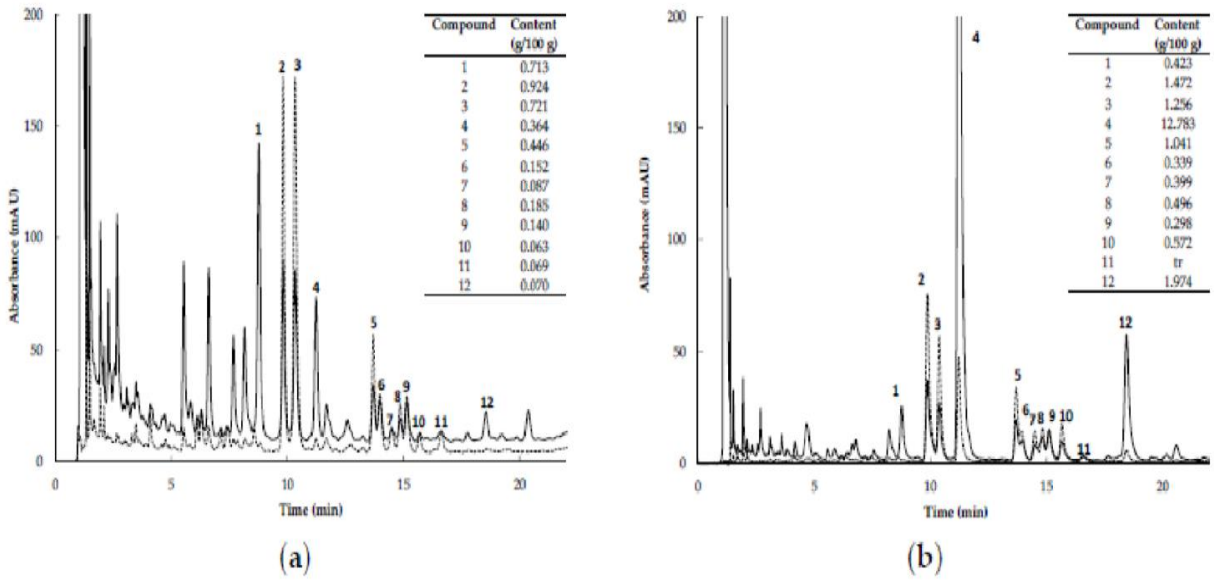

Figure 2.4-1: Chemical composition of GRT obtained using HPLC. This figure shows the (a) fermented Rooibos extract (FRE), and (b) unfermented green Rooibos extract (GRT) with compound content values presented in g/100g extract. (1, PPAG (Z-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid),2, isoorientin; 3, orientin; 4, ASP (aspalathin); 5, quercetin-3-O-robinobioside; 6, vitexin; 7, hyperoside; 8, rutin; 9, isovitexin; 10, isoquercetrin; 11, luteolin-7-O-glucoside, and 12, nothofagin). (Jourbet, 1995; Muller *et al.,* 2012; Patel *et al.,* 2016)

Rooibos is produced through fermenting (oxidation) the leaves and stems of *aspalathus linearis* to produce a red-brown tea. In comparison, unfermented green Rooibos tea, in which oxidation is kept minimal, preserves the quality of anti-oxidants in the tea (Villaño *et al.,* 2010) and maintains the flavonoids and polyphenols, making it a preferable method for the serving of flavonoid-enriched fractions (Joubert *et al.,* 2005). Bramati and colleagues (2003) studied the anti-oxidant potential of green Rooibos and found that its anti-oxidant effect is double that of fermented Rooibos.

The consumption of Rooibos has increased dramatically world-wide since the 1990s (Joubert *et al.,* 2008). This is partly due to the beneficial nature of the tea in improving health. This is evident in numerous studies conducted on the plant. Apart from its anti-oxidant properties, Rooibos also possesses anti-mutagenic and anticancer properties (Marnewick *et al.,* 2005; Sissing *et al.,* 2011), reduces inflammation (Baba *et al.,* 2009) and possesses antidiabetic effects (Kawano *et al.,* 2009; Muller *et al.,* 2012; Son *et al.,* 2013). Additionally, it has been shown to ameliorate insulin resistance, conquer related metabolic disturbances (Beltrán- Debón *et al.,* 2011; Mazibuko *et al.,* 2013), and provide cardio-protective effects (Marnewick *et al.,* 2011; Pantsi *et al.,* 2011). Rooibos also improves hepatic anti-oxidant potential through preservation of glutathione's redox state and increasing the liver's capacity of oxygen radical absorbance (Marnewick *et al.,* 2003). The liver protective potential of Rooibos has also been confirmed against experimentally induced oxidative hepatotoxicity (Ulicná *et al.,* 2003; Ajuwon *et al.,* 2013).

The hypotensive effect of Rooibos tea is by the inhibition of the Angiotensin Converting Enzyme (ACE), an important part of the Renin-Angiotensin System (RAS) that controls blood pressure levels. The ACE governs the blood pressure by regulating the volume of fluid in the body. It brings about the conversion of the decapeptide angiotensin I to the potent vasoconstrictor angiotensin II causing an increase in blood pressure by narrowing the blood vessels (Figure 2.4-2) (Zhao and Xu, 2008). This takes place because the ACE degrades bradykinin, which is responsible for dilation of the blood vessels (Hemming and Selkoe, 2005).

Angiotensin-Converting Enzyme Inhibitors: ACE inhibitors are medications aimed at inhibiting or lowering the activity of ACE, which in turn reduces the amount of angiotensin II produced, allowing blood vessels to enlarge and reduce the blood pressure (Zhao and Xu, 2008).

Captopril, benazepril, ramipril and perindopril are a few in the list of inhibitors used. Studies reveal that the currently known ACE inhibitors have some undesirable side effects such as birth defects in pregnant women, renal artery stenosis and low blood pressure and thus should be avoided.

Even more harmful situations that can be caused by these inhibitors include kidney failure, liver dysfunction and swelling of tissues (Medicine Net Review, 2016)

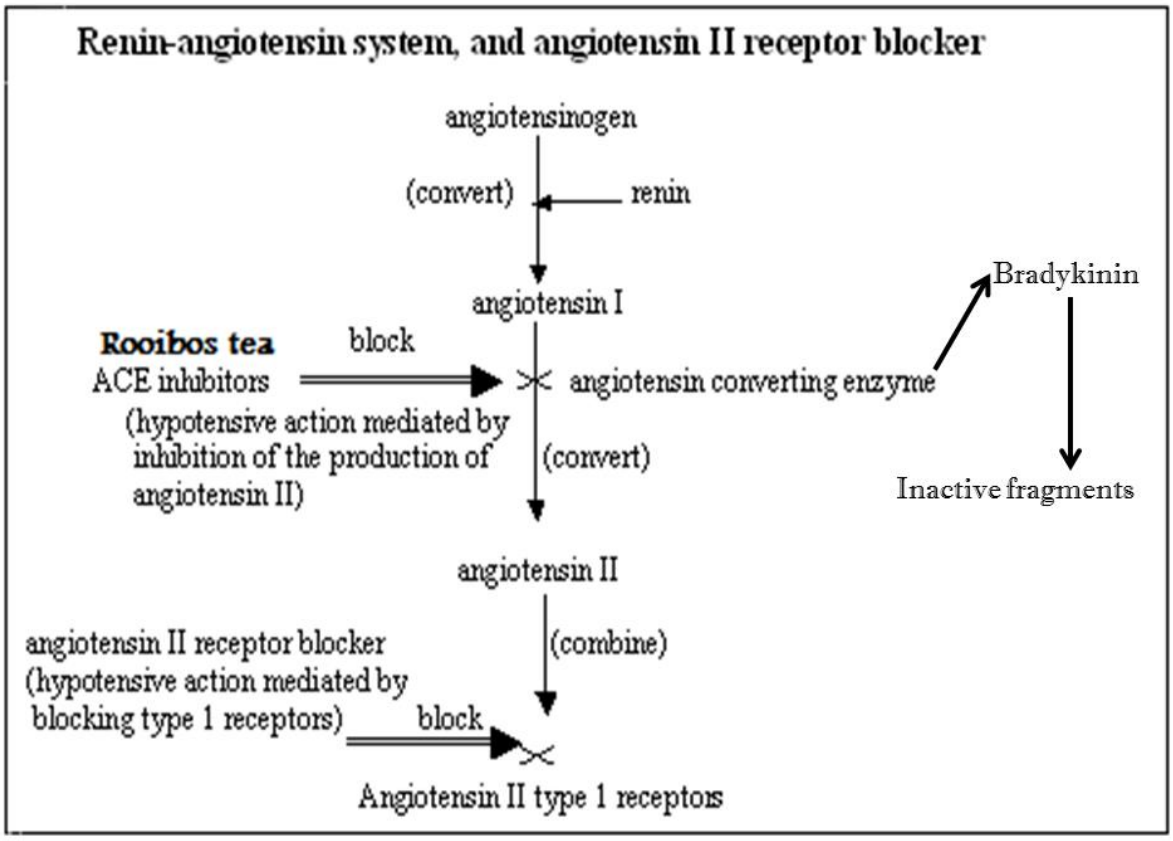

Figure 2.4-2: Diagram showing how Rooibos blocks or inhibits the Angiotensin Converting Enzyme**.**

Figure taken from https://www.astellas.com/en/corporate/news/yamanouchi/020124.htmL (Retrieved 20June 2016).

CHAPTER 3

3 MATERIALS AND METHODS

3.1 Introduction

This chapter gives full details of materials and methods used for this study. All the reagents, chemicals, kits and equipment used with the relevant companies' details are listed in Appendix B. Appendix B also gives additional information on procedures followed for preparation of reagents and treatment stocks. Both *in vitro* (C3A cells) and *in vivo* (Sprague Dawley) models were used for the study to investigate the effect of Afriplex GLP green rooibos extract (GRT) on the liver.

Early detection of toxicity from an *in vitro* model provides necessary guidelines for diagnosis and early diagnosis avoids the progression of damage, surgery (transplants) and premature deaths. *In vitro* determination of toxicity avoids exposure of laboratory animals or the use of human subjects in phase one clinical trials to potentially harmful substances.

3.2 Source and preparation of the plant material

A pharmaceutical grade GRT was obtained from Afriplex (Pty) Ltd. Afriplex is an authorized company that provides readymade extracts and they followed good manufacturing practices (GMP pharmaceutical grade) in the preparation of the extracts.

3.3 Phenolic make-up of the Rooibos extract (GRT)

The GRT extract used in this study contained ca. 12.8 % of aspalathin as per GRT composition given by Patel *et al.,* (2016).

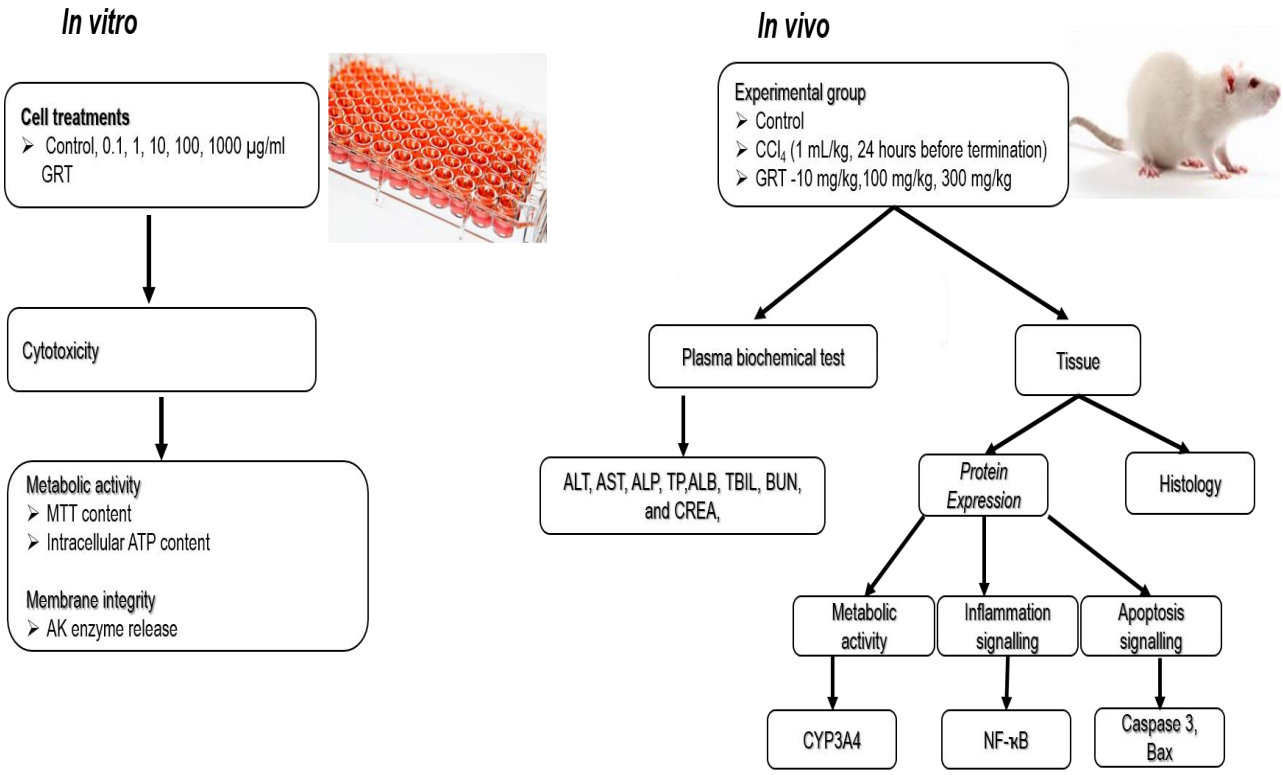

Figure 3.3-1: A depiction of the methods undertaken to investigate potential hepatotoxicity of GRT.

3.4 Part 1: *In vitro* cytotoxicity testing

3.4.1 Selection of the cell line

Human hepatocyte cell line (C3A) was used for this study. The cell line was obtained from the American Type Culture Collection [ATCC CRL-10741], at passage 9 and the passage number of the cells was kept below 20 passages within the exponential phase at all times to ensure genetic stability, appreciable viability and consistency between experimental repeats. HepG2/C3A liver cell line is a clonal derivative of HepG2 hepatocellular carcinoma cell line suitable for predicting hepatotoxicity *in vivo* (Guillouz, 1998; Prot *et al.,* 2011).

3.4.2 Thawing of the C3A Cells

The cryo-preserved cells were kept in nitrogen storage and thawed in a 37°C circulating water bath according to a standard cell culture protocol from the ATCC. Thereafter, thawed cells were transferred into a 15 mL tube containing 4 mL of Eagle's Minimum Essential Medium (EMEM) containing sodium pyruvate and Non-Essential Amino Acids NEAA (Cat no: BE12-662F, Lonza), supplemented with 10 % Fetal Bovine Serum (FBS) (Highveld Biological, Lyndhurst, South Africa), 1 % of L-Glutamine and 1 % of penicillin and streptomycin at 37°C.

The cells were centrifuged at 200 x g for 5 minutes, the supernatant was discarded, and the cell pellet was resuspended in fresh pre-warmed EMEM medium. Thereafter, 10 µL trypan blue stain was used to assess cell viability and cell number (see subsection 3.4.4). Following cell counting, 2.25 x10^6 cells/mL (30 000 cells/cm^2) were transferred to a 75 cm^2 cell culture flask with 17 mL of pre-warmed cell growth medium and incubated at 37°C under TC standard conditions (5 % CO_2 and 95 % humidified air). Cells were refreshed every two days with pre-warmed medium until confluency of 80 % was reached.

All cell culture activities were performed in a class 2 biological safety cabinet as per BRIP tissue culture SOP.

3.4.3 Human liver C3A cell subculture

Upon reaching 80 % confluency, pre-warmed Dulbecco's Phosphate Buffered Saline (DPBS) was used to wash the cells, [Cat no. BE17-513F, Lonza]. Thereafter, 2 mL of trypsin was added to dissociate adherent cells and incubated for 12 minutes at 37°C and 5 % CO_2. After incubation, cells were then visualized under a Nikon Eclipse Ti inverted microscope to ascertain if cells have detached.

The trypsinization activity was stopped by suspending cells with equal amount of pre-warmed EMEM with sodium pyruvate and Non-Essential Amino Acids NEAA (Cat no: BE12-662F, Lonza), and supplemented with 10 % Fetal Bovine Serum (FBS) [Highveld Biological, Lyndhurst, South Africa], 1 % of L-Glutamine, and 1 % of penicillin and streptomycin and clumping of cells was avoided by simply pipetting up and down several times.

The cell suspension was centrifuged at 200 x g for 5 minutes. Thereafter, the supernatant was removed, and the cell pellet was re-suspended in 10 mL pre-warmed EMEM with sodium pyruvate and Non-Essential Amino Acids NEAA (Cat no: BE12-662F, Lonza), supplemented with 10 % Fetal Bovine Serum (FBS) (Highveld Biological, Lyndhurst, South Africa), 1 % of L-Glutamine, and 1 % of penicillin and streptomycin. Cell density and viability were determined as described in section 3.4.4.

3.4.4 Cell Counting

The Countess Automated cell counter (Invitrogen, Carlsbad, CA, USA) was used to count the number of cells per mL of media. Total cell count was carried out by mixing 10 µL of cell and 10 µL of a 0.4 % (w/v) trypan blue solution. Thereafter, the mixture of the cell suspension/trypan blue (10 µL) was transferred to an automatic cell counting chamber before the chamber was inserted in the Countess Automated cell counter to determine the number of viable cells. The percentage equal to or greater than 80 % was considered for freezing, seeding and maintaining cells.

3.4.5 Seeding the C3A cells into multi-well plates

Cell counting information was used to determine the number of cells to be seeded at the required seeding density of 11000 cells/ per well (55 000 cells/ mL) in a 96 well plate. The 36 outer wells of a 96 well plate was filled with Dulbecco's Phosphate Buffer Saline (DPBS) to prevent evaporation and preserve experimental volumes for treated experimental cells. Thereafter, cells in a 96 well plate were incubated at 37 °C and 5 % CO_2.

3.4.6 Treatment conditions

3.4.6.1 Preparation of the GRT extract

The stock solution of 100 mg/mL was prepared by weighing the powdered Afriplex GRT extract into a 2 mL Eppendorf tube. The extract was diluted to a required concentration by adding sterile tissue culture water (Cat no. BE17-724Q, Lonza). The stock solution was mixed with 8 mM KRBH glucose medium to make a 1000 µg/mL concentration, then filter sterilized with a 0.22 µM pore size (Milex-GP Polyether-sulfone (PES) on a syringe filter.

A log dilution of the GRT extract was prepared from the working stock (100 µg/mL, 10 µg/mL, 1 µg/mL, 0.1 µg/mL and 0.01 µg/mL) in 8 mM HEPES-buffered Krebs Ringer Bicarbonate (KBRH) glucose medium which was also used as the vehicle control.

3.4.6.2 Treating the C3A cells

Following the preparation of the extracts, the cells were treated with pre-warmed GRT at varying concentrations mentioned above for 1 hour, 24 hours and 48 hours as depicted in table 3.4-1. Each treatment condition had 10 replicates.

Table 3.4-1: The 96-well template and depiction of how treatments were arranged

PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
PBS	CN	CN	CN	CN	CN	CN	CN	CN	CN	CN	PBS
PBS	T1	T1	T1	T1	T1	T1	T1	T1	T1	T1	PBS
PBS	T2	T2	T2	T2	T2	T2	T2	T2	T2	T2	PBS
PBS	T3	T3	T3	T3	T3	T3	T3	T3	T3	T3	PBS
PBS	T4	T4	T4	T4	T4	T4	T4	T4	T4	T4	PBS
PBS	T5	T5	T5	T5	T5	T5	T5	T5	T5	T5	PBS
PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

CN-Control; **T1**- GRT - 0.1 µg/mL; **T2**- GRT- 1 µg/mL; **T3**- GRT- 10 µg/mL; **T4**- GRT- 100 µg/mL; **T5**- GRT- 1000 µg/mL

3.4.7 Assessment of cytotoxicity

3.4.7.1 MTT Assay

The MTT (3-(4, 5-Dimethyl-2-thiazolyl) 2, 5-diphenyl-2H-tetrazolium bromide) assay is a cell viability testing method for evaluating mitochondrial dehydrogenase activity of the cells. The MTT yellow soluble dye is reduced to a non-soluble purple formazan product by mitochondrial dehydrogenase activity (Weyermann *et al.,* 2005).

The MTT stock solution (2 mg/mL) was made by dissolving the dye in phosphate buffered saline (CN 3295, Highveld Biological, Lyndhurst, South Africa). The resulting yellow solution was wrapped in foil to protect from light. The medium was removed from treated cells, washed with 100 µL of pre-warmed (DPBS) and 100 µL of MTT solution was added to each well of a 96-well plate treated wells and incubated at 37°C for 30 minutes. Observation (under an inverted light microscope) of the purple formazan crystal indicates mitochondrial dehydrogenases of viable cells or the presence of the NADPH-dependent oxydoreductase enzyme, which are only found in functional mitochondria. The purple formazan crystals were then dissolved in 200 µL of DMSO and 25 µL of Sorenson's Glycine Buffer (0.1 M Glycine, 0.1 M NaCl) pH 10.5. Thereafter, absorbance was measured at a wavelength of 570 nm in a BioTek® ELX 800 plate reader equipped with Gen 5® software.

3.4.7.2 The Adenosine Tri-Phosphatase (ATP) Assay

The adenosine tri-phosphatase (ATP) assay was performed to confirm MTT cell viability. The Adenosine 5' triphosphate concentrations were determined using the Vialight® plus assay following the manufacturer's instructions. The 96-well plate, with cells treated with the GRT extract, was removed from the incubator and allowed to cool at room temperature for at least 5 minutes.The medium was aspirated from the treated cells and 65 µL of Vialight® plus cell lysis reagent and 65 µL of DPBS was added to each well. The plate was kept at room temperature in the dark for 10 minutes. The cell lysate (100 µL) was transferred into a 96-well white opaque plate, 100 µL of the Vialight® plus assay buffer was added and the plate incubated for 2 minutes. Luminescence was measured in a BioTek® ELX 800 plate reader equipped

with Gen 5® software. The plate reader was programed to take a 1-second integrated reading.

3.4.7.3 Adenylate kinase assay

The adenylate kinase is the mitochondrial enzyme involved in ATP production that forms part of the metabolic monitoring network and is essential in cellular energy homeostasis. During extreme physical challenges and metabolic stress, the adenylate kinase serves as a stress susceptible protein (Dzeja and Terzic, 2009). The bioluminescent cytotoxicity assay was used to approximate the toxicity from the quantity of the adenylate kinase released into the medium as the cells are losing their mitochondrial integrity and function due to the exposure of a cytotoxic test compound.

The adenylate kinase detection reagent was reconstituted by adding 20 mL of the assay buffer and was allowed to equilibrate for 15 minutes, according to the manufactures instructions. The reagents were all brought to room temperature before the experiment was initiated. The supernatant (20 µL) were removed and transferred to a white luminescence compatible 96- well plate. Thereafter, 100 µL of the adenylate kinase detection reagent was added to each well. The plate was incubated at room temperature for 5 minutes and luminescence was read in a BioTek® ELX 800 plate reader equipped with Gen 5® software.

3.4.8 Analysis of Data

The data was analyzed using Excel and percentage viability was calculated related to the control value set at 100 %. The GraphPad prism software was used to present the data in graphical format with mean and standard deviation values from three independent experiments. The statistical analysis of data was performed by subjecting the data to one-way Analysis of Variance (ANOVA) with a Dunnett's *post hoc* test. Values were regarded statistically significant if $p<0.05$.

3.5 Part 2: Sub-chronic toxicity study

3.5.1 Animal model, housing and environmental conditions

Nineteen-day-old male and female Sprague-Dawley rats weighing (78.1 ± 17.8 g), bred at the Animal Unit of the University of Zululand were selected and acclimatized to the environment for three weeks before proceeding with the study. Fifty rats were randomized into five experimental groups consisting of males (n = 5 rats/group) and females (n=5 rats/group) to make the total of (n= 10 rats/group), caged 2 or 3 rats in a cage with corn cob bedding. The rats received water and food (Massa rodent ripe, 25 kg) *ad libitum.* The environmental conditions were kept at 23ºC - 25ºC (Relative Humidity: ~50 %), 12 hours' light/dark cycle and 15-20 air changes per hour. The study was performed according to the approved protocol of the University of Zululand ethics committee (Reg. no. UZREC171110-030) [Appendix A].

3.5.2 *GRT* extract dosage

Based on general human consumption, 1 cup of "bush tea" approximately equates to 3 mg dry extract/kg body mass per day. Based on pre-clinical data (rodent and monkeys) the effective doses for GRT were 30-90 mg/kg body weight per day. However, due to higher metabolic rates in rats [a warm-blooded animal's metabolism is a function of body surface in contrast to the body mass as per van Miert, 1989], equivalent doses for humans extrapolated from rats (10 times higher) and monkeys 3 times higher. The doses selected for this study were 10 mg/kg, 100 mg/kg, and 300 mg/kg body weight per day. A solution of jelly and gelatin was used as a vehicle for the extract administration. Briefly, a solution of jelly (27 g) and gelatin (3 g) in 100 mL boiling water was prepared every day, with or without extract (at the dosage concentrations indicated), for the duration of the study. The appropriate amount of jelly was added into an ice cube tray using the body weight of each rat to determine the correct volume and dosage. The tray was placed at 4ºC until the jelly sets.

The experimental groups (n= 50) consisted of the vehicle control, three GRT treatment groups (10, 100, and 300 mg/kg BW) treated daily for 90 days and a positive control receiving a single CCl_4 injection (1 ml/kg in 1:1 ratio with olive oil) 24 hours prior to termination to induce acute liver toxicity.

3.5.3 Observation and examination of rats

Daily cage side monitoring of the rat's well-being was performed as recommended by the US Food and Drug Administration, Centre for Food Safety and Applied Nutrition, Office of Food Additive Safety Redbook (2000)– Toxicological Principles for the Safety Assessment of Food Ingredients. The rats were examined for any visible signs of toxicity. These signs included: fur and skin condition; eyes and mucous membranes (secretions and excretions); autonomic activity (e.g. breathing rate, vomiting, piloerection, pupil size and urination); response to handling; the presence of chronic changes in gait; posture or tonic movements; stereotypic behavior (e.g. excessive or lack of grooming, repetitive circling); or bizarre behaviour (e.g. self-mutilation). Body weight, water and food intake was measured weekly for all the groups throughout the period of the study.

3.5.4 Termination and tissue collection

Toxicological parameters were taken including biochemical parameters consisting of liver function tests and hematology (Lancet Laboratories, Empangeni). After three months (90 days) the rats were fasted overnight and anaesthetized by intraperitoneal injection of sodium pentobarbital (Euthanasia - Bayer Pty. Ltd., Animal Health Division, Isando, South Africa - Reg no. 1968/011192/07) at a dose of 15 mg/kg body weight. Blood was collected from the abdominal vena cava by laparotomy and the rat sacrificed by exsanguination. The liver samples were harvested, washed in 0.9 % saline solution at room temperature, weighed and fixed in 10 % phosphate buffered formalin for histology and some frozen at -80 °C for protein expression analysis. The samples fixed in formalin were sent to the histopathology laboratory (Vetdiagnostix, Pietermaritzburg) for histological assessment.

3.5.5 Biochemical assessment for toxicity

Biochemical tests were performed to assess hepatotoxicity and kidney toxicity by Lancet Laboratories. These tests included the measurement of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein (TP), albumin (ALB), and total bilirubin (TBIL). Kidney function was assessed biochemically by testing for serum levels of urea, uric acid and creatinine (CREA).

3.5.6 Histological preparation of tissue samples

Histological assessment was carried out by a registered veterinary pathologist Dr. Rick Last at the Vetdiagnostix Pathological Laboratories.

3.5.7 Histopathological assessment

A veterinary pathologist Dr. Rick Last at the Vetdiagnostix Pathological Laboratories examined histological segments of the post mortem tissue for any toxic reflections.

3.5.8 Western blotting (WB) analysis

3.5.8.1 Protein extraction and determination of protein concentration

The kidney and liver samples frozen in liquid nitrogen were cut and weighed (approximately 100 mg) into Eppendorf tubes. The samples were homogenized by adding a steel bead-ball and tissue lysis buffer (1 mL/100 mg tissue), then mounted onto a tissue lyser set at 25 Hertz for 60 seconds, cooled in ice for 60 seconds and this was repeated 5 times. The lysate was centrifuged (3000 x g for 5 minutes at 4 °C) and the supernatants transferred to new 1.5 mL Eppendorf tubes. Thereafter, protein concentration was determined using RC DC™ protein assay kit (Bio-Rad) following the manufacturer's instructions. Briefly, 5 µL of the commercially-available standards (Bio-Rad bovine serum albumin at suggested concentrations (0. 125, 0.25, 0.5, 0.75, 1, 1.5, and 2 mg/mL) and samples were added to a clear 96-well plate. Thereafter, 25 µL of reagent A, which is the mixture of RC Reagent C (20 µL) and RC reagent (1 mL) and 200 µL of reagent B was added to each well with either standards or samples. The absorbance was then read at 630 nm using Bio-Tek® ELx800 plate reader equipped with Gen5® software.

3.5.8.2 Gel electrophoresis

Samples with pre-determined protein concentrations, 30 µg or 40 µg in sample buffer (SB), were denatured at 95°C for 5 minutes before loading onto a 12 % SDS Mini-Protean® TGX™ Gel for Western blotting analysis, with the Precision Plus Protein™ WesternC™ Protein Standard (Bio-Rad) as a marker. The gel was placed in a Bio-Rad tetra cell tank filled with commercially available SDS running buffer, ran at constant voltage of 150 Volts and was monitored for the complete appearance of bands of the standard protein (Table 7.2.6). Coomassie brilliant blue stain was used to stain the gel overnight for protein profiling, destained with commercial destaining solution and visualized by Bio-Rad ChemiDoc for image analysis.

Table 3.5-1: WB Antibodies with their corresponding dilution factor

Antibody	Company	Catalog Number	MW (kDa)	1 °Ab dilution	2 °Ab	2 °Ab dilution	%Gel	Amount load (µg)
β-Actin	SCB	SC-47778	42	1: 1000	Anti-Mouse IgG, HRP-linked	1: 4000	12%	30, 40
NF-κB	CST	#3034	65	1: 2000	Anti-Rabbit IgG, HRP-linked	1:4000	12%	30
Caspase -3	CST	#9662	17,19,35	1: 2000	Anti-Rabbit IgG, HRP-linked	1: 4000	12%	30
Bax	SCB	SC-493	20	1: 1000	Anti-Rabbit IgG, HRP-linked	1: 4000	12%	30
CYP3A4	CST	#13384	50	1: 1000	Anti-Rabbit IgG, HRP-linked	1: 4000	12%	40

SCB- Santa Cruz Biotechnology, **CST**-Cell Signaling Technology, NF-kb- Nuclear- factor kappa enhancer of activator of β cells.

3.5.8.3 Transfer of gel to PVDF membrane and Western blot analysis

A semi-dry transfer of the SDS-PAGE into polyvinylidene fluoride (PVDF) membranes was performed. The membranes were submerged in Ponceau S stain for 5 minutes to confirm protein transfer and then destained with 1× Tris-buffered saline (TBS) with 0.1 % Tween 20 (TBST). Thereafter, PVDF membranes were blocked with 5% non-fat milk (Clover, South Africa) in 1 x TBST in a shaker for 90 minutes. The membranes were then incubated with the primary antibody in a cold room (at 4°C) on a shaker overnight. The following day, the labelled membranes were washed with 1x TBST (3x) for 10 minutes and incubated with the secondary antibody in 2.5% non-fat milk in TBST for 90 minutes in a shaker. Immunoblotting was performed to study the expression of nuclear factor -Kappa enhancer of β cells (NF-kB), caspase 3, Bax, and CYP3A4 in the liver and kidney samples. Beta actin, was included as an endogenous control. Relative protein expression was calculated against beta actin. The LumiGLO Chemiluminescent Substrate Kit was used for chemiluminescent detection of the labelled protein using Bio-Rad ChemiDoc. Restore Plus Western Blot Stripping Buffer (Thermo scientific, 46430) was used to strip the membranes for relabeling with different antibodies.

3.5.9 Analysis of data

Group numbers for the sub-chronic toxicity study (n= 5 male and females per experimental group) are based on the guidelines of the US Food and drug Administration, Centre for Food Safety and Applied Nutrition, Office of Food Additive Safety Redbook (2000). Statistical differences between groups and controls were analyzed using one-way ANOVA, followed by a Dunnett and Bonferroni multiple comparison *post hoc* test if $p<0.05$. Results were considered statistical different if p-value was $p<0.05$.

CHAPTER 4

4 RESULTS

4.1 Introduction

This chapter presents the results of the study aimed at assessing the potential cytotoxicity of GRT *in vitro* and *in vivo.* The chapter is sectioned into two parts, presenting the *in vitro* (Part1) and the *in vivo* (Part 2) findings. In part 1, we examined cell viability by assessing mitochondrial activity using MTT and ATP assays and the membrane integrity using adenylate kinase assay. In part 2, the study investigated the effect of GRT extract on the liver and kidney using the sub-chronic Sprague-Dawley rat model. Additional information on the results obtained in the study was presented in tables [Appendix C].

4.2 Part 1: The cytotoxic effect of GRT extract

This section presents the *in vitro* results of the study. C3A cells were exposed to a concentration range of 0.1- 1000 µg/mL GRT extract.
Three independent experiments, each with at least three technical replicates were done for each assay to ensure reliability, quality and the repeatability of the results produced in this study.

Cell viability was determined by estimating the production of formazan in the MTT assay to determine the relative changes of mitochondrial dehydrogenase activity. Incubating the C3A cells for 1 hour with GRT did not result in any changes using the MTT assay (Figure 4.2.1). However, a significant decrease ($p<0.05$) in the ATP cellular content was observed with 1000 µg/mL GRT (Figure 4.2.2). Incubation with the extract for 24 hours resulted in significant decreases in cell viability at the highest concentration (1000 µg/mL) in both MTT and ATP assays. After 48 hours incubation GRT not only significantly reduced cell viability at 1000 µg/mL, but at 100 µg/mL as well. We noticed a time and dose dependent reduction in cell viability, following the exposure to different concentrations of GRT. The dose that reduced cell viability of ≤50 % of the cells was 100 µg/mL of GRT.

In addition to the intracellular assays, the release of the adenylate kinase enzyme into the media was also assessed as an early marker of cell death. The release of the enzyme (AK) was tested in this study; luminescence is a relative indication of AK enzyme release into the extracellular media suggestive of cell death.

As expected a time dependent increase in the enzyme release was demonstrated, however, we were not able to confirm a concentration dependent increase in AK.

4.2.1 The effect of GRT on C3A liver cells tested with MTT assay

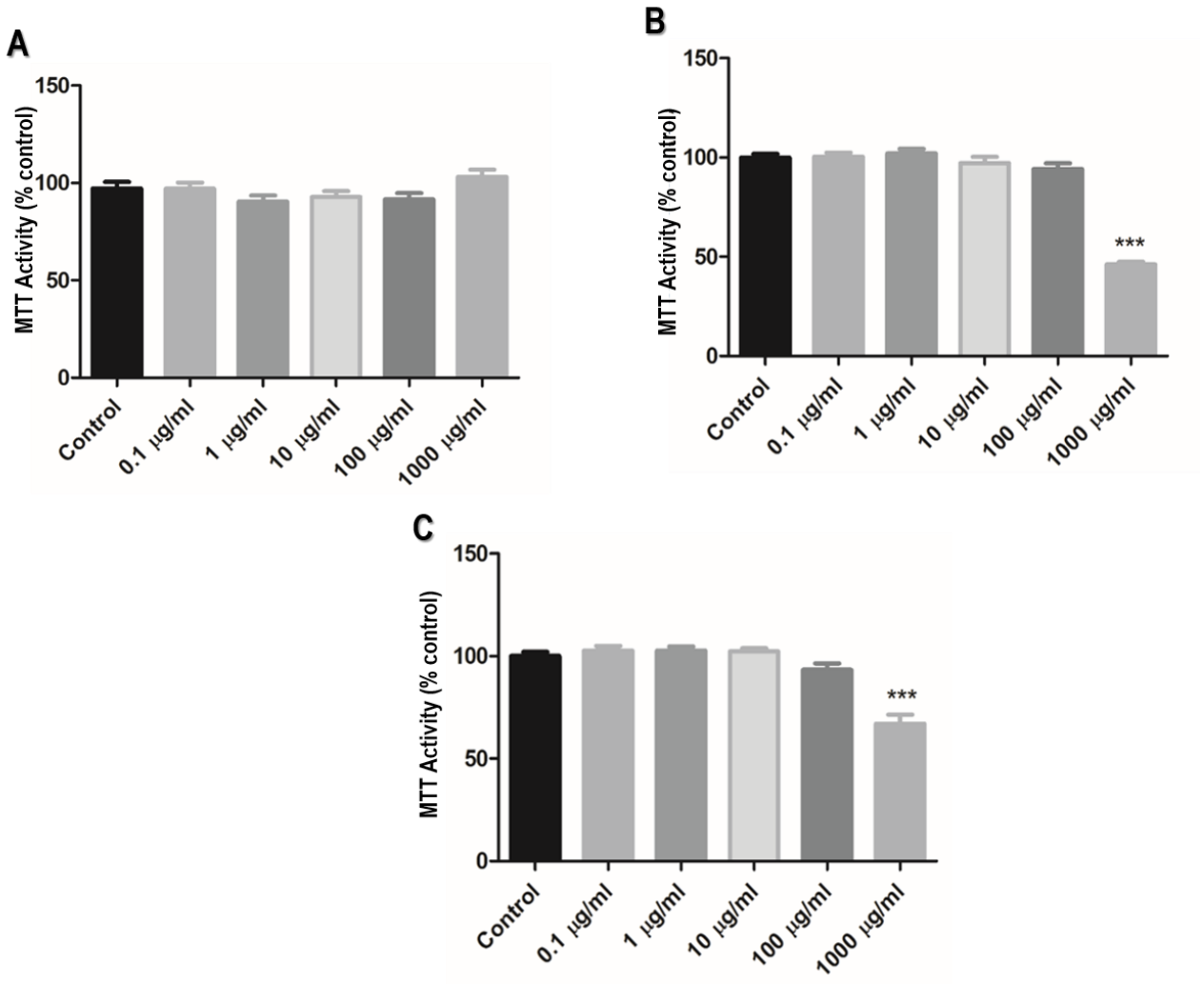

Figure 4.2-1: The effect of GRT on cell viability on C3A cells using MTT assay. The effect of GRT on C3A cells after 1 hour incubation **(A),** after 24 hours **(B),** and after 48 hours incubation **(C)** is demonstrated. The data is presented as mean ± SD of three independent experiments, each with ten replicates as technical repeats, normalized to the control set at 100%. *** *p-value ≤ 0.0001,* ANOVA Dunnett's post-hoc test.

MTT activity confirmed that the GRT extract had no adverse effect on the viability of the C3A cells up to a concentration of 1000 µg/mL which reduced cell viability by less than 50% (p-<0.05) compared to the control group.

4.2.2 The effect of GRT on C3A cell viability tested with ATP assay

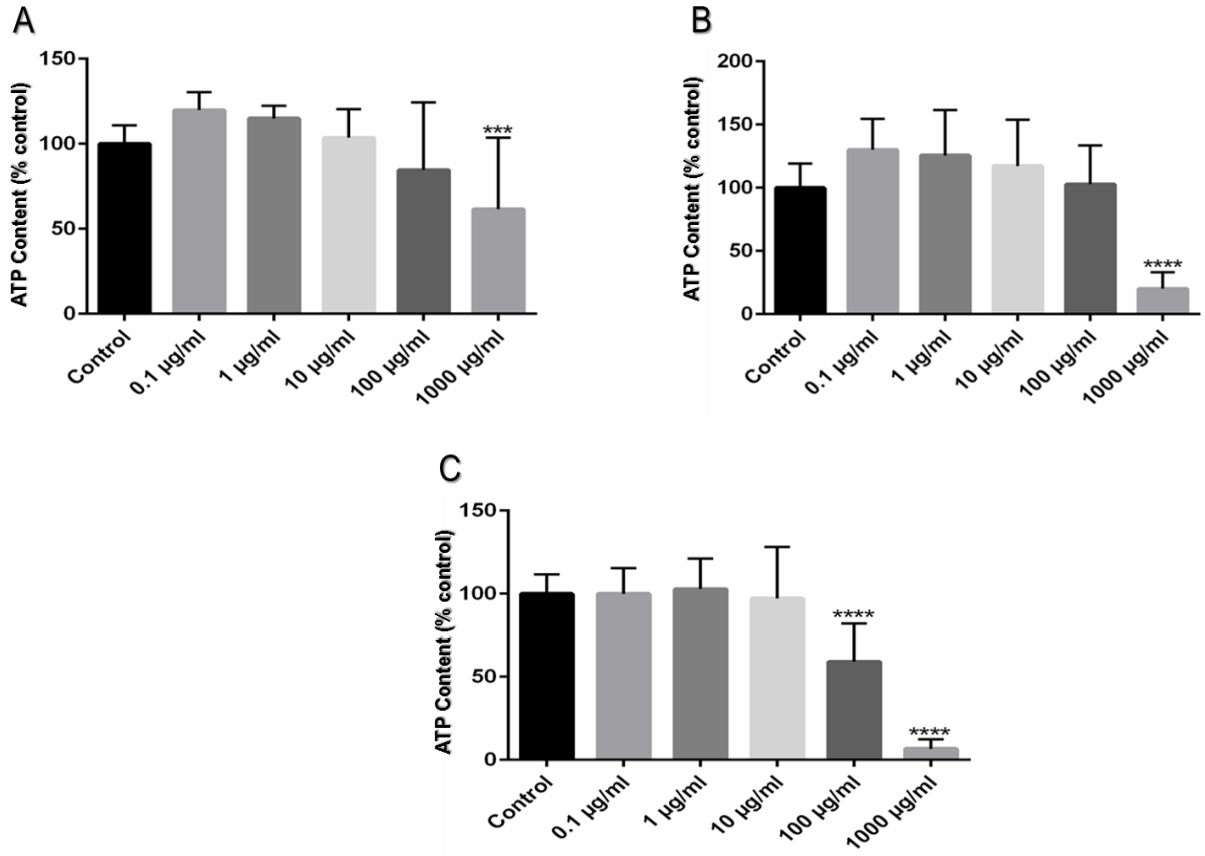

Figure 4.2-2: The effect of GRT on cell viability on C3A cells using an ATP cellular assay. The effect of GRT on C3A cells after 1 hour incubation (A), after 24 hours (B), and after 48 hours' incubation (C) is demonstrated. The data is presented as mean ± SD of three independent experiments, each with ten replicates as technical repeats, normalized to the control set at 100%. *** p-value ≤ 0.001, ****-*p-value* ≤ *0.0001,* ANOVA Dunnett's post-hoc test.

Cellular ATP content analysis confirmed that 1000 µg/mL GRT was toxic to the C3A cells at all incubation periods. In addition to the 1000 µg/mL concentration, 100 µg/mL was also shown to reduce cellular ATP content by >50% after 24 and 48 hours.

4.2.3 The effect of GRT on membrane integrity evaluated using AK assay

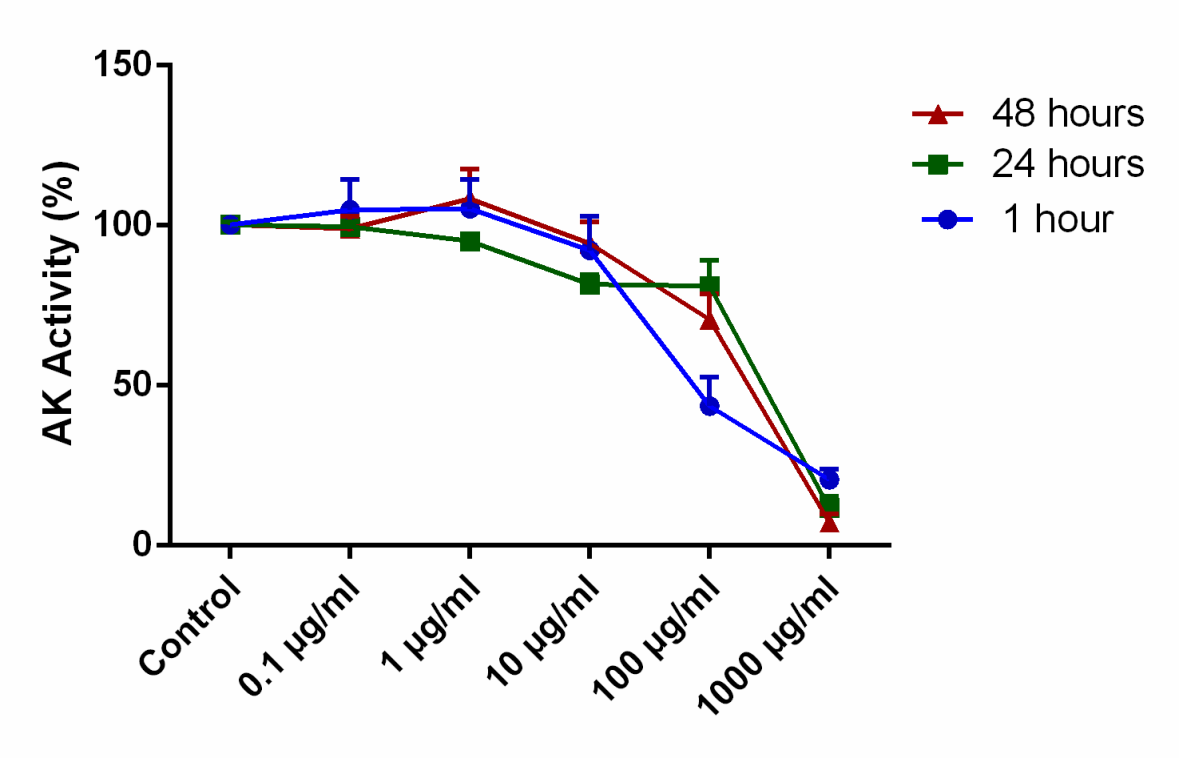

Figure 4.2-3: Adenylate kinase release by C3A cells from the cells into the media following 1, 24 and 48 hours exposure to GRT extract. Three independent experiments were carried out with at least five replicates for each adenylate kinase experiment. No statistical significance.

The exposure of C3A to GRT treatment showed a decrease in the release of adenylate kinase into the media at concentrations above 10 µg/mL (Figure 4.2.3).

4.3 Part 2: The effect of GRT on Sub-chronic rat model

Following the daily ingestion of the GRT extract at doses of 10, 100 and 300 mg/kg BW, there were no changes in the behavior of the rats, signs of visible stress or any other symptoms related to toxicity observed during the 90 days duration of the study. All the rats survived the treatment for the period of the study.

The results showed no significant differences in body weight (Figure 4.3.1), food and water intake (Table 4.3.1) and in liver weights (Figure 4.3.2) in all groups. Increased levels of liver test enzymes ALT, AST and ALP (Figure 4.3.3) confirmed the hepatotoxicity of CCl_4 and mild dose dependent changes with GRT treated groups.

The GRT extract had no significant effect on the levels of total protein and total bilirubin, although there was a significant increase in albumin for the group treated with 100 mg/kg (Figure 4.3.4). The results showed a concentration dependent decrease in the levels of uric acid and creatinine. No changes were demonstrated for urea (Figure 4.3.5).

The histopathology of the liver showed mild hepatocellular hydrophic, micro and macro vesicular vacuolar degeneration in all the groups except the CCl_4 treated group which showed severe hepatocellular macro vesicular degeneration accompanied by single cell and cluster necrosis (Figure 4.3.6 and 4.3.7).

The effect of GRT on the expression of inflammation and apoptotic response proteins was further assessed. The 300 mg/kg GRT-treated induced a significant decrease in the activation of NF-kB, while treatment with 10 mg/kg demonstrated increase in the relative expression of NF-kB in the liver (Figure 4.3.8).

As represented in (Figures 4.3.9 to 4.311), no significant changes in the expression levels of Bax, caspase 3 or CYP3A4 were demonstrated.

4.3.1 Daily observation of the behavior and appearance of the rats

The rats showed no visible symptoms of stress and disease during the study period. In all the treated groups, the rats had no abnormal activity without observable changes in their behavioral pattern.

4.3.1.1 Morbidity and mortality

None of the rats displayed any signs of sickness within the population of this study, and none of the rats were treated for any disease or disorder, neither did any deaths occur during the study.

4.3.2 Body weights

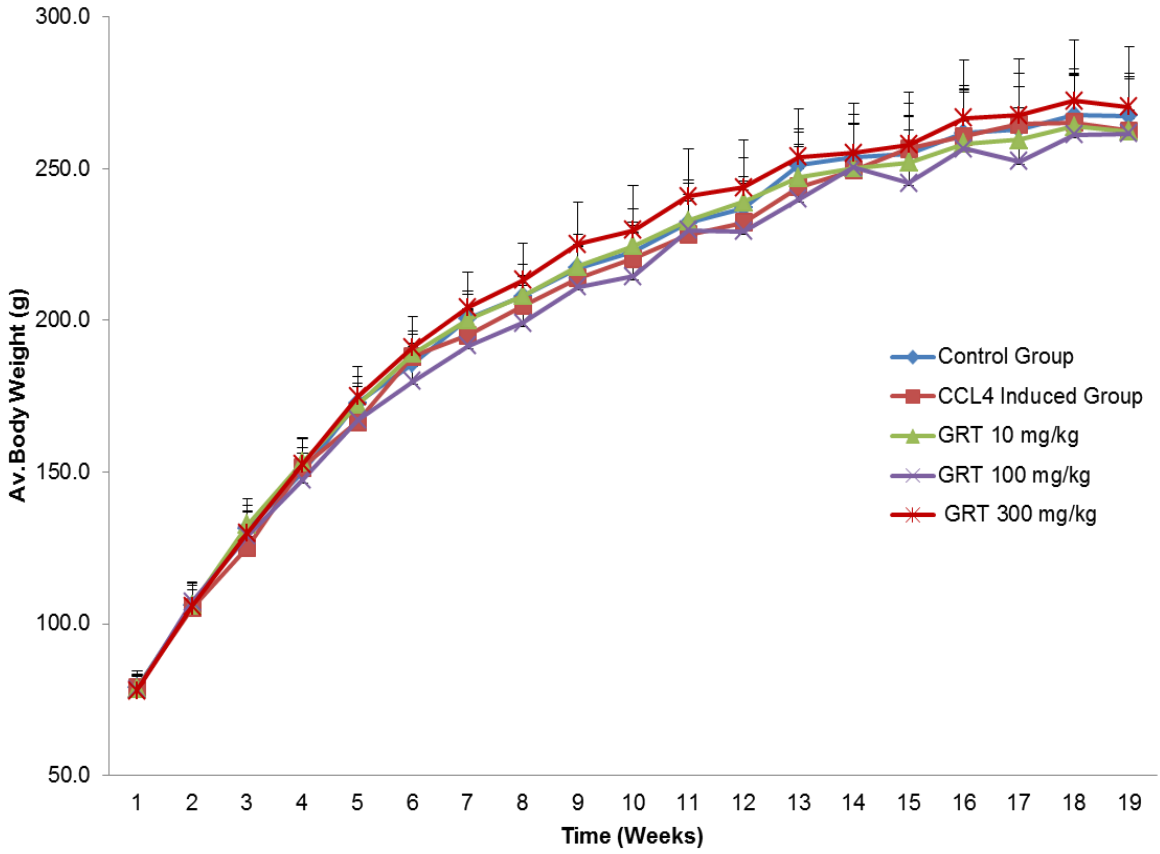

Figure 4.3-1: The effect of GRT extract on body weight of Sprague-Dawley rats. The body weights were monitored weekly and recorded as mean ± SEM, n=10 rats/group.

The results presented in (Figure 4.3.1) confirmed that the treatments had no significant effect on body weigh over the 90 days' treatment.

4.3.3 Water and food intake

Table 4.3-1: The effect of GRT on food and water intake, relative liver weight

	Control	CCL4	10 mg/kg	100 mg/kg	300 mg/kg
Month 1					
Food Intake (g)	422.7 ± 65.0	470.9 ± 36.0	530.8 ± 22.8	462.4 ± 32.8	592.2 ± 40.1
Water Intake (mL)	369.9 ± 54.55	426.9 ± 31.47	437.3 ± 45.81	449.7 ± 34.06	454.4 ± 51.32
Month 2					
Food Intake (g)	335.8 ± 52.4	368.7 ± 32.3	439.1 ± 27.9	387.9 ± 28.4	473.1 ± 23.7
Water Intake (mL)	380.9 ± 59.71	402.8 ± 30.94	405.9 ± 36.39	425.6 ± 40.13	446.6 ± 51.29
Month 3					
Food Intake (g)	359.4 ±57.4	371.8 ± 34.6	450.0 ± 27.0	392.4 ± 32.3	485.9 ± 23.2
Water Intake (mL)	391.3 ± 58.21	403.1 ± 33.39	404.7 ± 42.76	430.6 ± 38.53	425.6 ± 44.74
Month 4					
Food Intake (g)	349.5 ± 55.7	367.5 ± 33.6	447.0 ± 32.8	388.0 ± 34.7	424.6 ± 41.8
Water Intake (mL)	386.9 ± 67.20	415.0 ± 32.44	416.9 ± 49.78	443.1 ± 46.16	443.8 ± 48.15
Relative liver weight	3.9 ± 0.11	4.7 ± 0.21	4.3 ± 0.35	4.0 ± 0.13	4.1 ± 0.32

Values presented as mean ±SEM

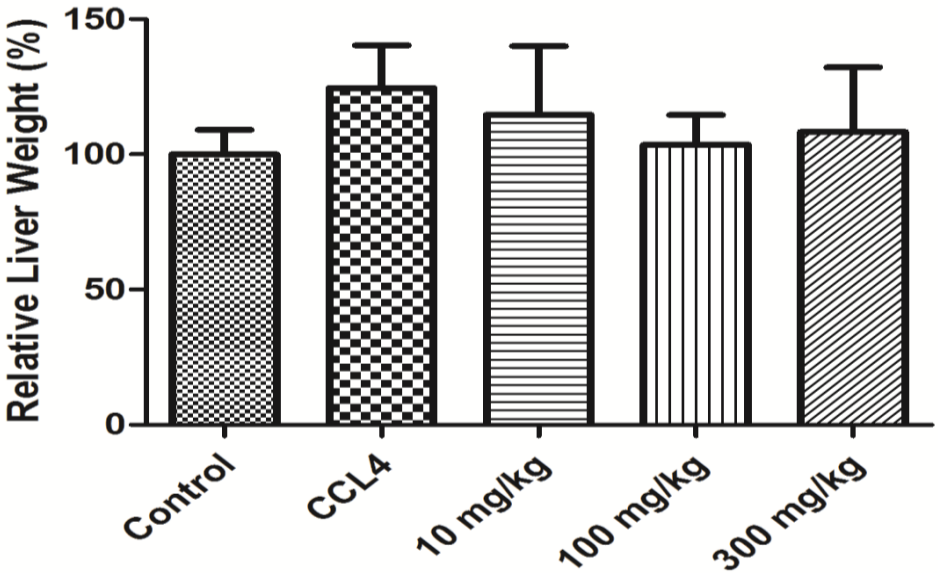

Figure 4.3-2: Relative liver weight calculated by dividing the body weight of the rat with the liver weight expressed relative to the control set at 100%, presented as Mean ± SD. (n=10 rats/group)

4.3.4 Serum biochemical parameters

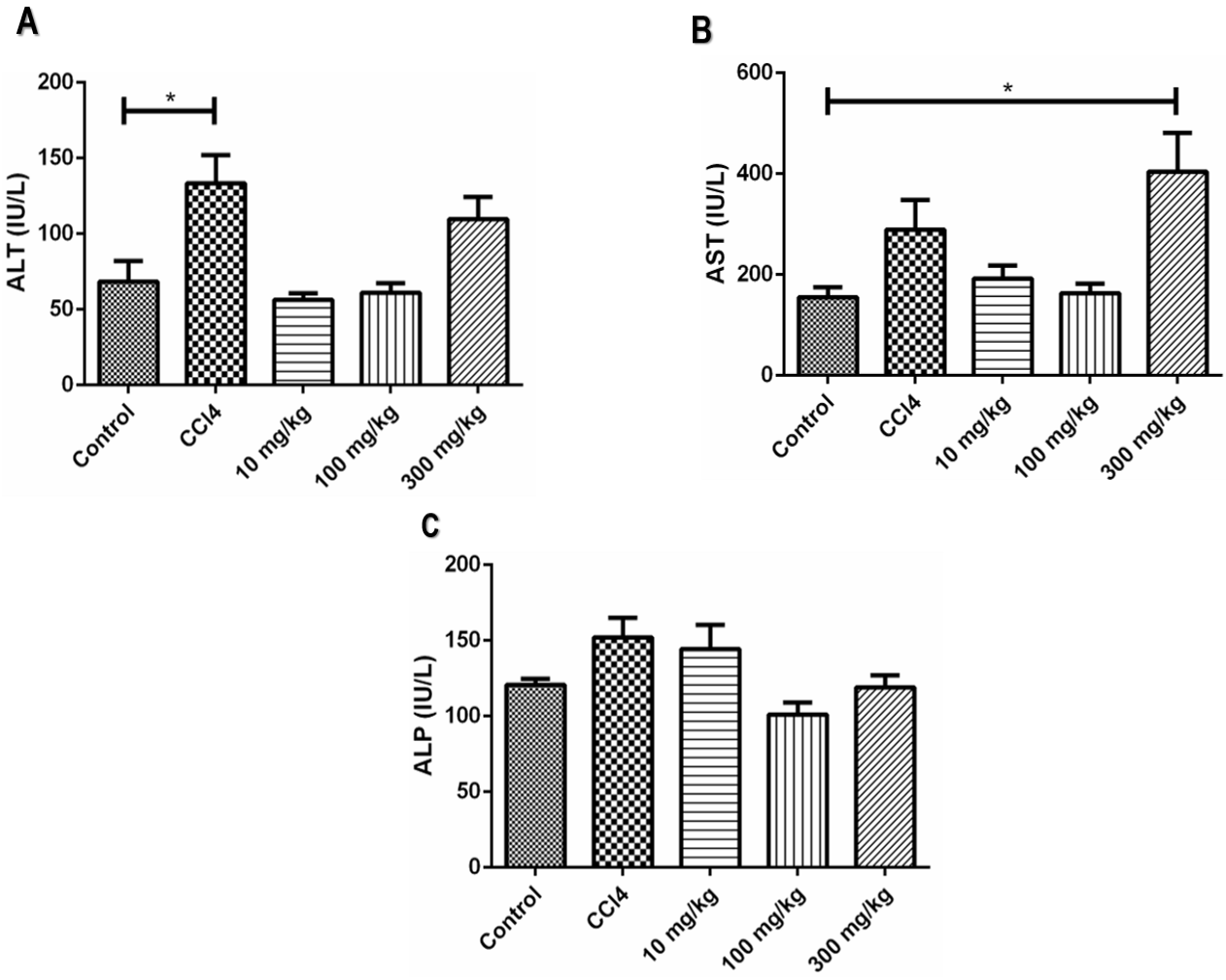

Figure 4.3-3: Liver function enzymes alanine aminotransferase **(A)**, aspartate aminotransferase (**B),** and alkaline phosphatase (**C)** levels for the untreated control group and treated with CCl_4 (1 mL/kg; positive control) and GRT (10, 100, 300 mg/kg) treated groups (n=10 rats/group). Results represents the mean ± SD. Statistical analysis One-way ANOVA with Dunnett's *post hoc* test, *p < 0.05 versus the vehicle control group.

Serum blood chemistry results showed that 300 mg/kg BW increased aspartate aminotransferase by >100%. No other significant changes were observed for GRT on alanine aminotransferase or alkaline phosphatase. CCl_4 induced a significant increase in alanine aminotransferase.

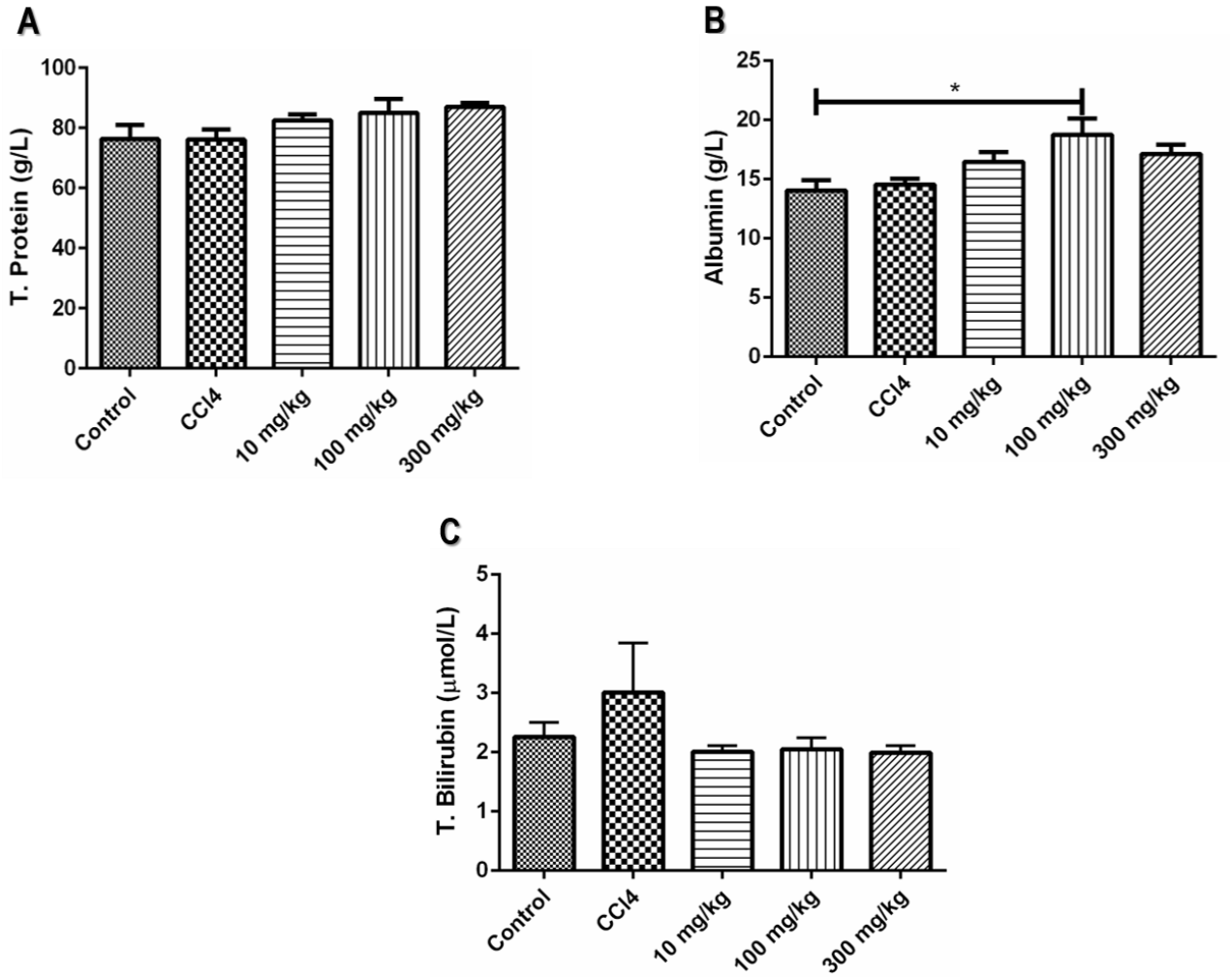

Figure 4.3-4: Serum protein. Total protein **(A)**, albumin (**B)** and total bilirubin (**C)** levels for the control group, the CCl_4 (1 mL/kg; positive control) and GRT (10, 100, 300 mg/kg) treated groups, (n=10 rats/group) are shown. Results represents the mean ± SD (n=10 rats/group). Statistical analysis One-way ANOVA with Dunnett *post hoc* test, *$p < 0.05$ versus the control group.

There were no differences in total protein, albumin or total bilirubin of the GRT treated groups when compared to the control group. The administration of CCl_4 at 1 mL/kg, 24 hours before termination increased the activity of the hepatic enzyme ALT. CCl_4 (1 mL/kg) is the minimal dose used for toxicity evaluations and 24 hours being the lowest duration of exposure. Studies have shown that at least 10 mg/kg dose of CCl_4 induces hepatic injury with repeated exposure (Bruckner *et al.,* 1986).

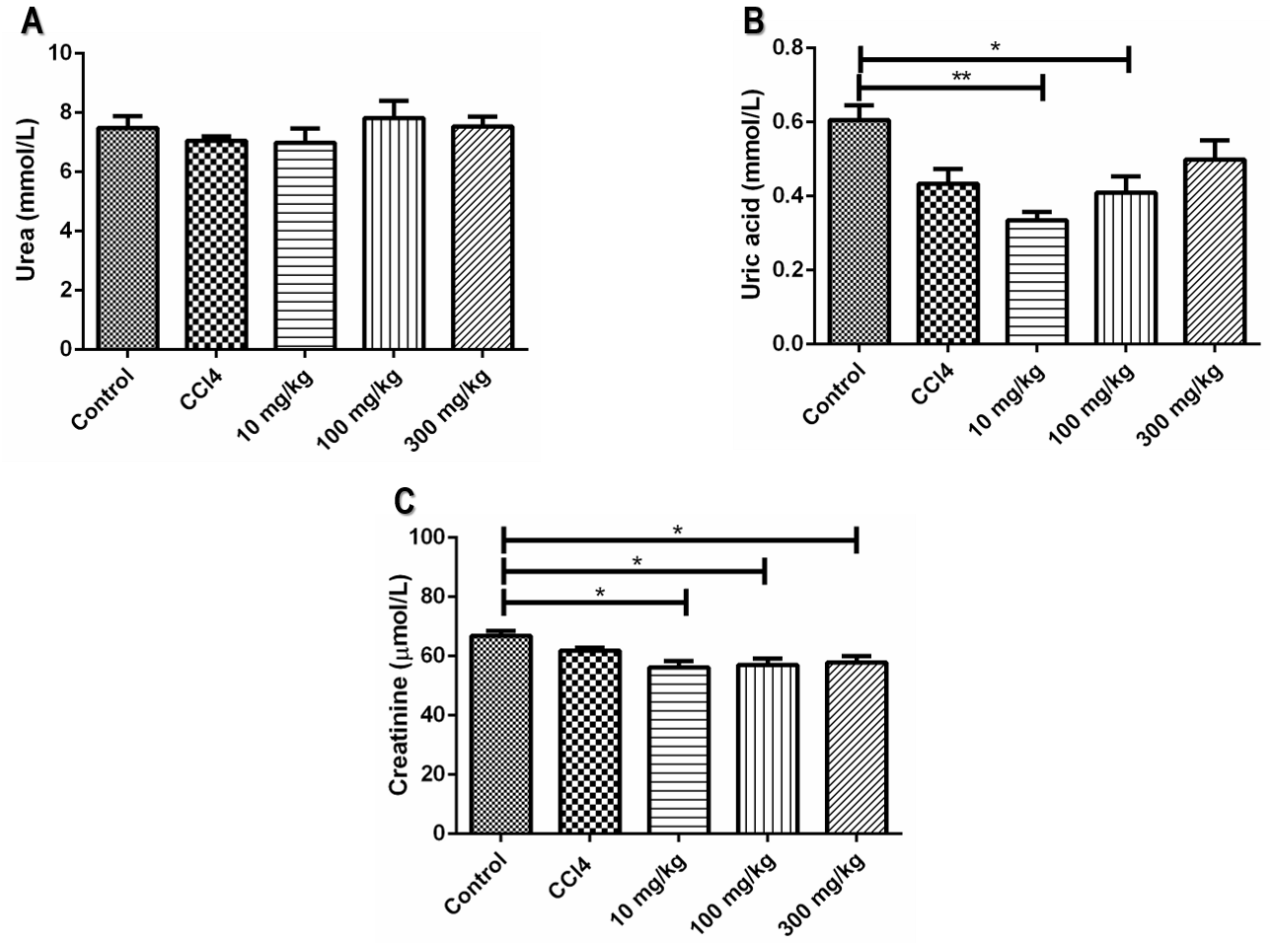

Figure 4.3-5: Urea **(A)**, uric acid (**B)** and creatinine (**C)** levels for the untreated control, CCl_4 (1 mL/kg; positive control) and GRT (10, 100, 300 mg/kg) treated groups (n=10 rats/group). Results represents the mean ± SD (n=10/group). Statistical analysis One-way ANOVA with Dunnett's *post hoc* test, *p < 0.05 versus the vehicle control group.

GRT had no effect on serum urea concentration. However, at doses of 10 and 100mg/kg BW GRT significantly reduced serum uric acid concentration. GRT at a dose of 10 mg/kg BW reduced creatinine albeit a small change in physiological terms.

4.3.5 Histopathology

Histological evaluation of the liver, performed by veterinary pathologist, revealed several minor histopathological abnormalities which were not limited to the treatment groups (Table: 6.2-20).

4.3.5.1 The histopathology of the liver

Histopathological changes in the liver included moderate micro- and macro-vesicular degeneration in female and males rats of the control groups. These changes were expected as inherent to this rat model (Figures 4.3.6 A and 4.3.7 A).

The positive control groups (CCl_4 treated) showed the occurrence of severe micro- and macro-vesicular degeneration including periportal brown pigment within the hepatocytes as well as the Kupffer cells in the females. The micro- and macro-vesicular degeneration was also severe in males of the positive control groups; however, no pigment was observed (Figures 4.3.6 B and 4.3.7 B).

In the 10 and 100 mg/kg GRT treated groups, the histological features were similar to the control, but with the occurrence of intracytoplasmic pigment (iron/lipofuscin) which was present in the hepatocytes and in the sinusoidal microphages of the females, but not observed in the males (Figures 4.3.6 C and D, 4.3.7 C and D).

In the 300 mg/kg GRT treated groups, for the females there was a noticeable accumulation of the brown pigment with proliferation of the Kupffer cells and pigment engorgement of sinusoidal microphages. The males also presented with proliferation of Kupffer cells but without the pigments observed in the females (Figures 4.3.6 E and 4.3.7 E).

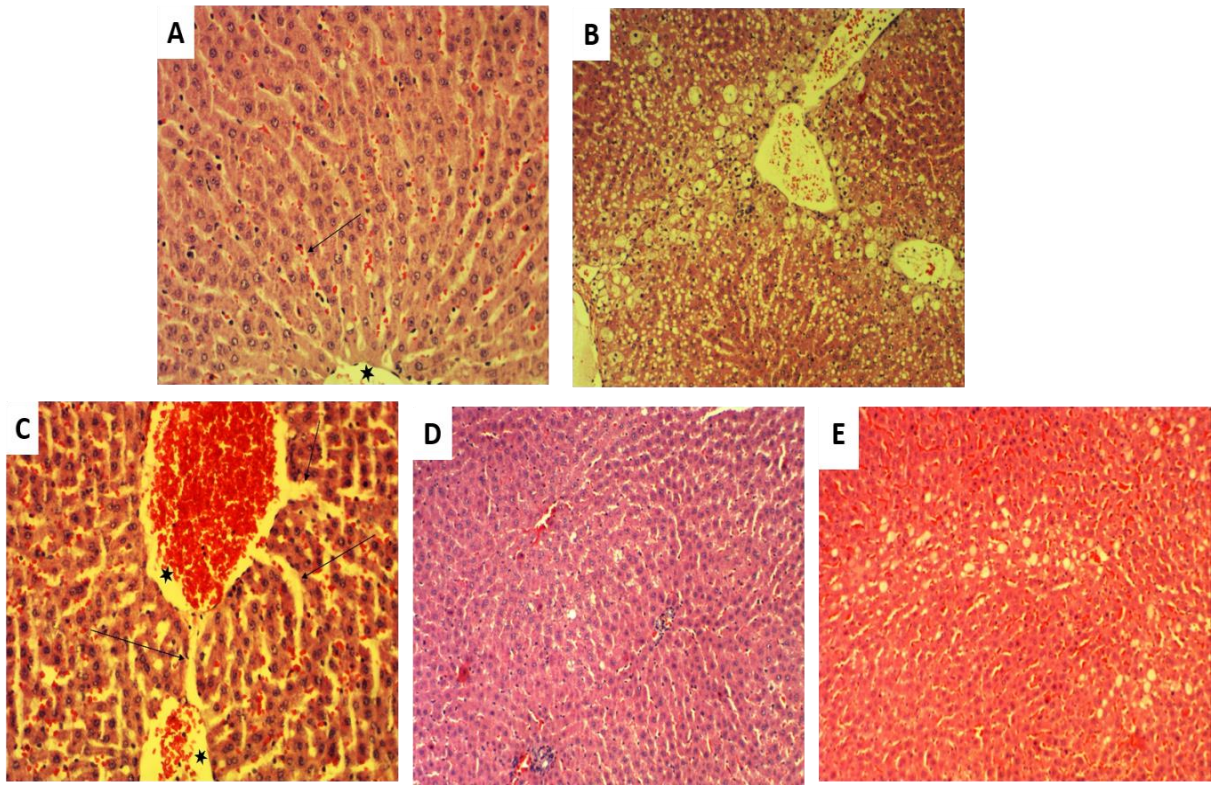

Figure 4.3 6: Representative liver hematoxylin and eosin (H&E) sections from different in vivo treatment groups. Normal liver tissue demonstrating typical liver morphology with hepatocytes and sinusoids (arrows) and central veins (star) **(A)**. CCl_4 treated positive control **(B)** demonstrate severe and diffuse hepatocellular micro- and macro-vesicular degeneration with pyknotic nuclei. The 10 mg/kg treated group **(C)** showed central vein (star) and sinusoids (arrows) without micro—and macro-vesicular degeneration. The 100 mg/kg treated group **(D)** showing minor hepatocellular micro-vesicular degeneration changes present. The 300 mg/kg treated group **(E)** shows a focal area of hepatocellular micro-vesicular degeneration. H & E x 200 magnification.

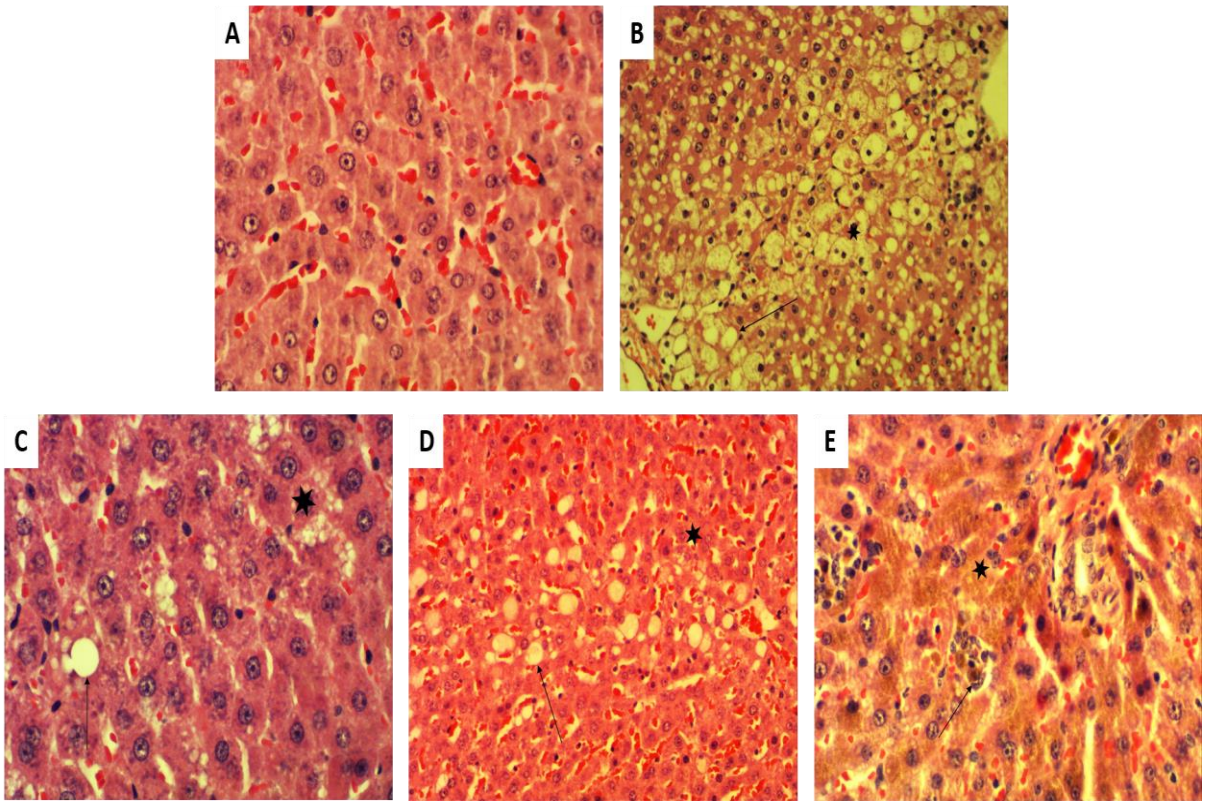

Figure 4.3-7*: Representative liver hematoxylin and eosin (H&E) sections from different in vivo treatment groups*. Normal liver tissue **(A).** CCl_4 treated positive control **(B)** demonstrating severe diffuse micro- and macrovesicular lipid vacuolization (arrow) and pyknotic nuclei (star). The 10 mg/kg treated group **(C)** showing normal hepatic architecture with occasional lipid vacuolization (arrow) and pyknotic nuclei (star). The 100 mg/kg treated group **(D)** showing focal areas of micro-vesicular lipid vacuolization (arrow) and occasional pyknotic nuclei (star), and the 300 mg/kg treated group **(E)** showing the presence of brown pigment accumulation, Kupffer cell engorgement of pigment (arrow) and a few pyknotic nuclei (star).

4.3.6 The effect of GRT on protein expression in the liver and kidney

NF-kB, caspase 3, Bax and CYP3A4 expression in the liver and the kidneys were assessed by Western blot. Statistical analysis revealed that treatment with GRT had no effect on the relative expression levels of caspase 3 (Figure 4.3.9) and Bax (Figure 4.3.10) in the liver. The relative expression of the NF-kB was reduced by treatment with 300 mg/kg GRT extract (Figure 4.3.8). CYP3A4 expression (Figure 4.3.11) in the liver was not altered by GRT treatment. In the kidney, Western blots only detected NF-kB which was unchanged by the treatment, Bax, Caspase 3 and CYP3A4 were undetectable by Western blot (data not shown).

4.3.6.1 The effect of GRT on protein expression of the liver

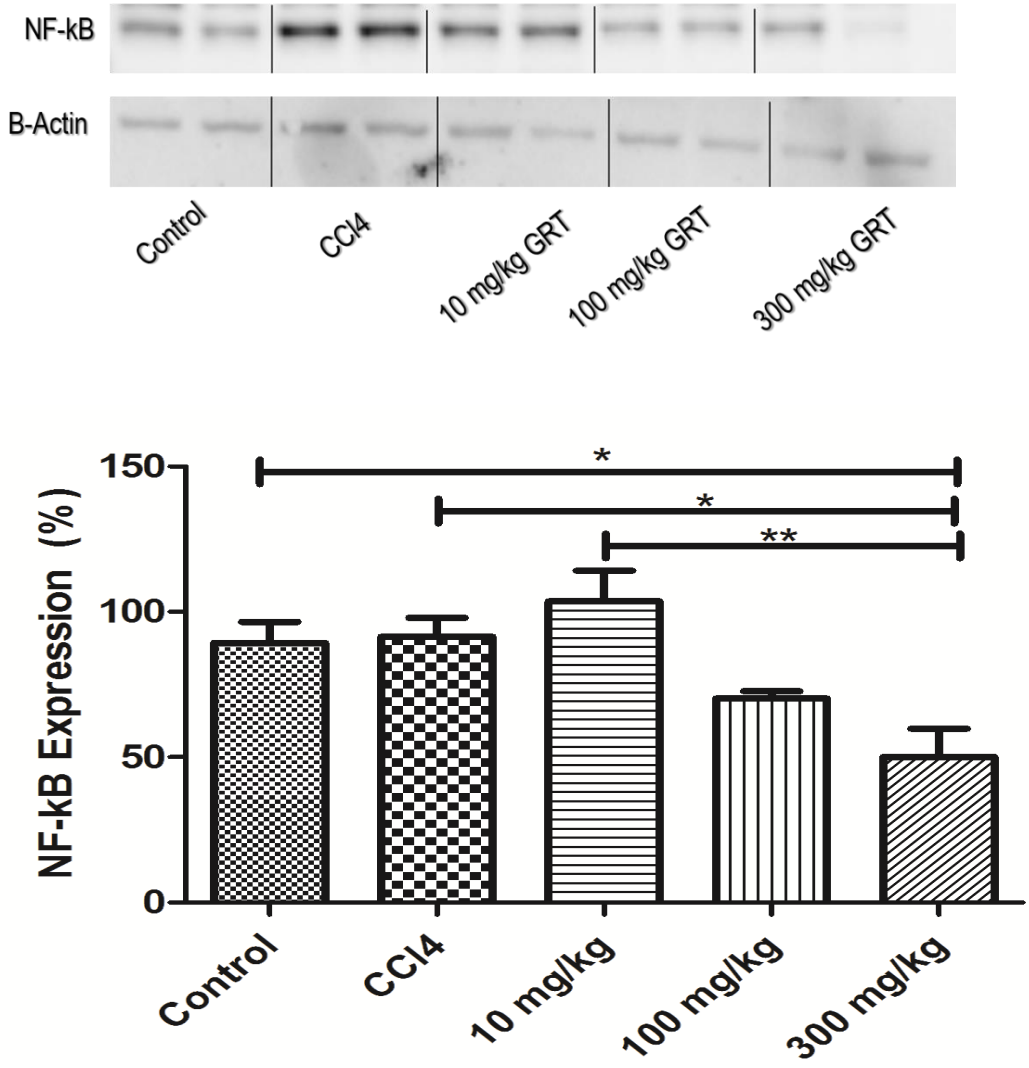

Figure 4.3-8: Relative expression of nuclear factor-kB, assessed by Western blot in liver of male rats (n=5 /group), NF-kB p65 (1:2000) primary antibody was used on liver samples of untreated, treated with CCl_4 (1 mL/kg; positive control) or GRT (10, 100, 300 mg/kg) rats. Results represents the mean ± SD in three independent experiments relative to the house-keeping gene, B-Actin. Statistical analysis One-way ANOVA with Dunnett's *post hoc* test, *p < 0.05, ** *p< 0.001* versus the control group.

4.3.6.2 The effect of GRT on caspase 3 expression of the liver

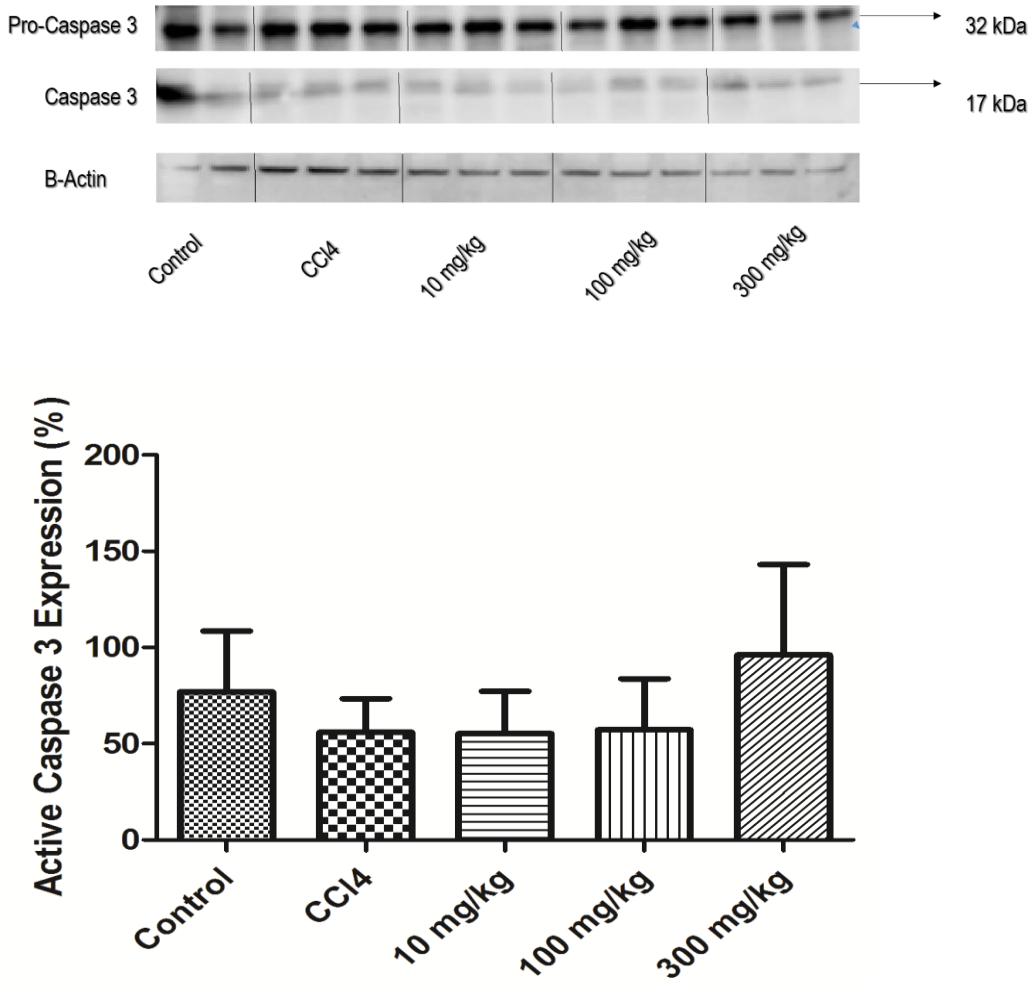

Figure 4.3-9: Relative expression of nuclear Caspase 3, assessed by Western blot in liver of male rats (n=5 /group), Caspase 3 expression on liver samples of untreated, treated with CCl_4 (1 mL/kg; positive control) or GRT (10, 100, 300 mg/kg) rats are demonstrated. Results represents the mean ± SD in three independent experiments relative to the house-keeping gene, B-Actin. Statistical analysis One-way ANOVA with Dunnett's *post hoc* test, *p <0.05.*

4.3.6.3 The effect of GRT on Bax expression of the liver

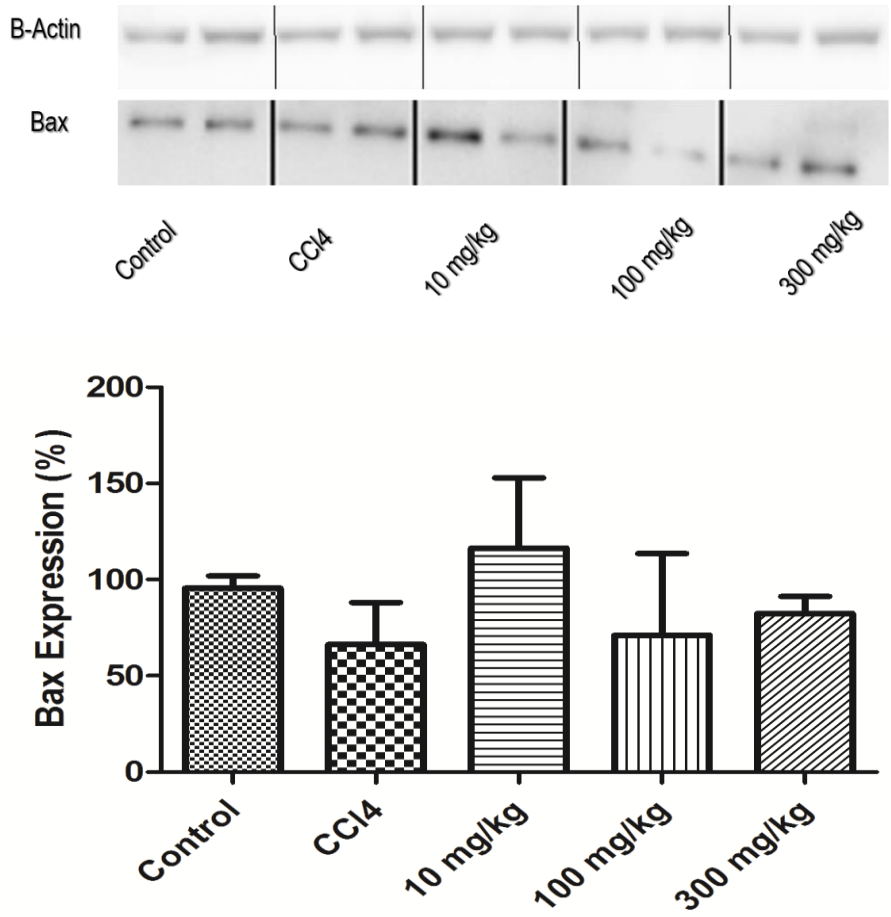

Figure 4.3-10: Relative expression of Bax, assessed by Western blot in liver of male rats (n=5 /group), Bax expression of liver samples of untreated, treated with CCl_4 (1 mL/kg; positive control) or GRT (10, 100, 300 mg/kg) rats are demonstrated. Results represents the mean ± SD in three independent experiments relative to the house-keeping gene, B-Actin. Statistical analysis One-way analysis of variance, Dunnett's *post hoc* test, $p < 0.05$.

4.3.6.4 The effect of GRT on CYP3A4 enzyme expression of the liver

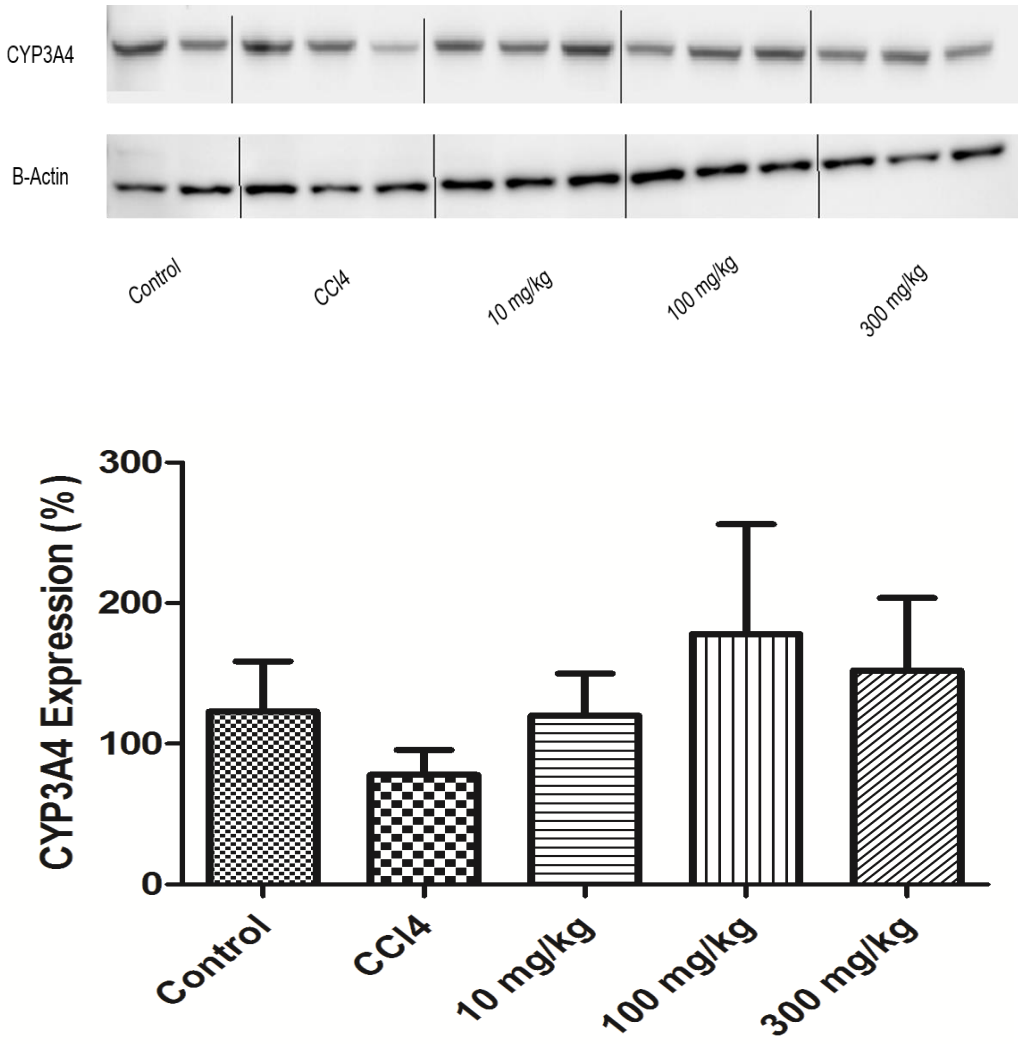

Figure 4.3-61: Relative expression of CYP3A4, assessed by Western blot in liver of male rats (n=5 /group), CYP3A4 expression on liver samples of untreated, treated with CCl_4 (1 mL/kg; positive control) or GRT (10, 100, 300 mg/kg) rats are demonstrated. Results represents the mean ± SD in three independent experiments relative to the house-keeping gene, B-Actin. Statistical analysis One-way ANOVA with Dunnett's *post hoc* test, *p <0.05.*

CHAPTER 5

5 DISCUSSION

5.1 Rooibos health benefits

Aspalathus linearis, commonly referred to as Rooibos, is an indigenous fynbos plant used to produce Rooibos tea. Aspalathin, a *C*-glucosyl dihydrochalcone is uniquely present in *Aspalathus* spp., of which *A. linearis* is the only one of the species that is commercially cultivated (Bramati *et al.,* 2002). A small human study using rooibos tea (6 cups/day) by participants at increased cardiovascular risk, showed a significant decreased serum low density lipoprotein (LDL) and triglycerides, and lowered plasma markers of lipid peroxidation (Marnewick *et al.,* 2010). Further *in vitro* and *in vivo* studies have demonstrated that the beneficial metabolic and cardioprotective effects can, in part be attributed to the aspalathin content of Rooibos (Muller *et al.,* 2017). Subsequently, there is a growing demand for Rooibos products, with increased aspalathin content as health products that could help protect against various metabolic ailments including reducing cardiovascular risk (Chang *et al*. 2013). A method to produce a standardized aspalathin-rich green Rooibos extract containing 12.8 % aspalathin was developed jointly by the Agricultural Research Council (ARC) and the SAMRC. Afriplex, a South African company specializing in the development and manufacturing of botanical extracts and complementary medicines, produced a pharmaceutical grade standardized aspalathin-rich green Rooibos extract (Afriplex GRT) containing 12.8 % aspalathin, under license from the SAMRC for the health product market.

5.2 Safety of Rooibos

Although the safety of Rooibos as a herbal tea with health benefits relies mainly on anecdotal evidence, more substantive safety evidence is required for enriched extracts meant to deliver higher concentrations of bioactive compounds such as aspalathin content. Recently, two case studies have associated hepatotoxicity with Rooibos, although causality was not proved.

In a recent paper by Patel *et al.* (2016), GRT was shown to concentration- and time-dependently inhibit CYP3A4 activity, the cytochrome P450 isoenzyme enzyme that is responsible for the metabolism of atorvastatin, suggesting the potential adverse herb-drug interaction with drugs such as atorvastatin.

Although adverse reactions to Rooibos are rare, the use of aspalathin-rich green Rooibos extracts, such as GRT, will require safety and toxicity studies to establish maximum tolerable dose (the highest dose with acceptable side effects) is established in animals and humans.

5.3 *In vitro* cytotoxicity

Cell viability is commonly detected by testing the reductase enzyme using the MTT assay or the ATPase enzyme in the ATP assay, by utilizing the luciferase that reflects the luminescence or by testing the release of the adenylate kinase that signals the death of cells through apoptosis due to the compound. The cell viability tests are evaluation tests of metabolic functions of the cells or the activity of the enzymes (Riss *et al.,* 2013). Cell viability reduction is caused by cell death as a response to toxic chemicals. The type of cell death depends on the duration of exposure and the dose administered. Necrosis occurs as a result of acute cytotoxicity while apoptotic cell death is a sub-chronic to chronic response of cells to toxic stimuli (Lamasters *et al.,* 1998).

In this study, the MTT assay was performed to estimate the number of viable cells. A constant number of cells were maintained based on the MTT results and this was interpreted as an advantage for replacement therapies.

The results of the *in vitro* cytotoxicity study revealed that treatment with GRT extract concentrations below 10 µg/mL (0.1 µg/mL, 1 µg/mL, and 10 µg/mL) had no effect on the cell viability of C3A liver cells. However, the ATP assay showed a time and concentration dependent reduction in the cell viability as seen in the results. Interestingly the ATP assay showed that not only the 1000 µg/mL of the GRT reduced cell viability, but also the 100 µg/mL after 48 hours incubation providing a more sensitive result compared to the MTT assay, supporting the observations by Wang *et al.* (2010) that emphasized the sensitivity of the ATP assay over other cell viability testing methods.

In combination with intracellular cell viability assays, it is often useful to use an extracellular marker of cell viability that allows the continued use of the cells for further or the continuation of experiments. Although there are several options for measuring loss of cell membrane integrity, the release of essential metabolic enzymes into media such as lactate dehydrogenase and adenylate kinase (AK) indicates loss of membrane integrity. Adenylate Kinase (AK) is an essential enzyme for cellular energy production and is present in all eukaryotic cells. It facilitates the conversion of ADP to ATP and AMP. Theoretically when cells die, AK is released into the culture medium and the release is proportional to the number of cells dying. Interestingly, as could be expected, detectable AK in the media increased over time, except for the cells treated with 1000 µg/mL. Contrary to expectations, AK concentrations decreased after 24 and 48 hours respectively, whilst at the 48 hour timepoint decreases from the controls were also noted for the 100 µg/mL concentration. The technical reason for this is hard to explain. Firstly, GRT could be interfering directly with AK in a concentration dependent manner as is the case for Trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP). It could also be that as this assay more effectively detects cellular necrosis, cells dying by apoptosis do not release AK (Miret *et al.,* 2006). However, it is interesting that the AK release into the media mirrors the intracellular ATP concentrations, suggesting that the extract could mechanistically be interacting with AK and intracellular ATP production.

It would be interesting to assess the ADP: ATP ratio to confirm this. Mechanistically, Rooibos extracts and aspalathin have been shown to increase the expression and activation of AMPK, the cellular energy sensing enzyme. Therefore, an ATP to ADP shift could also tie in very nicely with the activation of AMPK (Muller *et al.,* 2012; Mazibuko *et al.,* 2015; Muller *et al.,* 2017).

5.4 Liver enzymes and hepatotoxicity

Aspalathin undergoes liver metabolism and is excreted in urine (Kruez *et al.,* 2008). Metabolism in the liver involves the activity of the cytochrome P450 enzymes. GRT is an exogenous substance or non-nutrient xenobiotic, therefore the human system aims to eliminate it (Van der Merwe *et al.,* 2015; Holst and Williamson, 2008).

An aspalathin-enriched green Rooibos extract (GRE), with 18% aspalathin content (Muller *et al.,* 2012), at a high average dosage of ca. 1900 mg/kg body weight (BW) significantly reduced cholesterol and iron levels, whilst increasing ALP in the serum when compared to the control treated rats. At the given dose for 90 days GRE adversely affected the glutathione (GSH) redox pathway suggestive of biliary dysfunction (Van de Merwe *et al.,* 2015). Unfortunately, this was not confirmed with histology. On the other hand, Uličná *et al.,* (2003, 2008) showed that drinking Rooibos tea (average 32 mL per day) protected rats against CCl_4-induced liver damage and promoted regeneration of the CCl_4-damaged livers. In this study a dose escalation design of 10, 100 and 300 mg/kg was used. The starting dose chosen was based on a likely human equivalent (HED) consumption of 2 – 3 cups of Rooibos, whilst the higher doses of 100 and 300 mg/kg BW represent daily HEDs of 1130 and 3400 mg per day, whilst the very high dose of 1900 mg/kg BW used by Van der Merwe *et al.* (2015) equates to 21600 mg daily.

In the current sub-chronic 90-day study, increased levels of the liver enzyme AST were shown in the GRT treated rats at the highest dose (300 mg/kg), and for ALT in the CCl_4 (1 mL/kg) treated positive control groups. Increased levels of ALT were also observed for the 300 mg/kg BW GRT rats, but the difference was not significantly different to the vehicle controls. ALT, present in hepatocytes, and AST, present in liver parenchymal cells, are liver enzymes generally considered useful markers of acute liver toxicity. Whilst, in the Van de Merwe *et al.,* (2015) study, using a very high dose of GRE (1900 mg/kg BW) demonstrated that ALP was significantly increased but not AST or ALT. The differences related to this study and the study of Van der Merwe *et al.* (2015) are interesting as it appears that dose determines either hepatocellular predominant enzyme pattern (elevated ALT) with increased AST: ALT ratio (2.26 vs. 3.7) as is seen in this study or a cholestatic predominant pattern (increased ALP) (Giannini *et al.,* 2005). The difference is difficult to explain at a functional level as the liver acinus, the functional unit of the liver, receives blood from the portal area (portal vein and hepatic artery) where the blood, rich in nutrients and toxins, starts to percolate through the liver sinusoids to the central vein.

The acinus is divided into 3 zones, of which the first zone (zone 1) is adjacent to the portal area and receives oxygenated blood, rich in nutrients, and with high metabolic activity. This zone is also the most resilient to injury, whilst blood supplied to zone 3, has low oxygen, increased mitochondria and is more susceptible to toxin injury. As 80% of the AST activity is found in the mitochondria, AST predominates zone 3 and as ALT activity is cytoplasmic, it predominates zone 1. Hepatotoxic compounds, such as alcohol and some herbs, such as senna, germander, gentian, chaparal leaf and kava, tend to increase ALT (Giboney, 2005), while toxins such as CCl_4 that cause lipid peroxidative injury classically affects zone 3. In terms of severity in the case of AST and ALT, a 5-fold increase is regarded as mild, 5-10-fold as moderate, and > 10-fold as severe (Giannini *et al.,* 2005). The slight increases in the ALT (1.6-fold from control) and AST (2.6-fold from control) without any increase in total bilirubin suggests that GRT at a dose of 300 mg/kg BW, causes slight hepatocellular damage at least from a biochemical perspective. In this regard, the late Hyman Joseph Zimmerman, an expert in drug-induced liver injury (DILI) published the so-called Hy's law (Reuben, 2004) based on the following three components:

- Drug-induced hepatocellular injury generally results in 3-fold or greater elevations above the ULN of ALT or AST than the placebo.
- Among subjects showing such aminotransferase elevations, serum total bilirubin should be >2-times the upper limit of normal without findings of cholestasis (ALP < 2-times the upper limit of normal).
- The absence of any other reason to explain the combination of increased aminotransferase and serum total bilirubin.

Further, as the liver is responsible for the synthesis of almost all the plasma proteins, and approximately 90% of plasma albumin, the most abundant plasma protein (determining total protein and albumin plasma levels), it provides a good indication of liver function (Schreiber, 1978). Total protein was unaffected by the GRT (Figure 4.3.5 A), whilst GRT appeared to increase serum albumin slightly but significantly.

Albumin is an anti-oxidant protein synthesized in the liver usually associated with increase in ROS and serves as an indication of inflammation in hypoalbuminemia (Danielski *et al.,* 2003). Conversely, reduction in serum albumin could indicate liver disease. Slight changes in levels of albumin however, as was seen in this study, could be related to environmental factors such as dehydration, which the authors of this study suspect might have led to the obtained results. Increased levels of hepatic enzymes and reduced cell viability indicate leakage of cellular proteins and loss of membrane integrity (Sharma *et al.,* 2016).

Green tea (*Camellia sinensis)* extracts, marketed as bodybuilding, weight loss and immune supplements, have been implicated with hepatotoxicity. There was a significant association between the concentration of catechins and the dose of green tea extract consumed, and liver injury causality score, severity, or pattern of liver injury. Products sold as weight loss supplements generally have high catechin levels, with doses 0.7 to 3 g per day causing hepatic injury (Sarma *et al.,* 2008). The catechins and their gallic acid esters have been implicated in oxidative stress injury and typically hepatocellular injury (Navarro *et al.,* 2013).

5.5 Liver histopathology and protein expression

Histopathological changes to the livers showed diffuse marked hepatocellular damage caused by CCl_4. Liver sections studied showed extensive micro- and macrovesicular degeneration of hepatocytes with pyknotic (apoptotic) nuclei. In comparison to the control, no obvious hepatotoxic histopathology was noted in the male rats treated with 10 and 100 mg/kg BW GRT, respectively. Rats treated with 300 mg/kg BW GRT presented with focal areas of micro- and macrovesicular changes, associated with occasional pyknotic nuclei. An interesting observation in the female rats was the presence of an unidentified brown intracytoplasmic pigment frequently present in all the GRT treated female rats (10, 100 and 300 mg/kg BW), which increased in intensity with the dosage, presenting with the engorgement of sinusoidal macrophages/Kupffer cells in the 300 mg/kg BW group. Although the pigment was not identified, it is most likely to be haemosiderin (iron pigment) which gets to be deposited when there is tissue damage and is associated with some of diseases.

Accumulation of iron pigment with experimental animal models has been associated with the presence of iron pigment in the liver and Kupffer cells with increased oxidative stress, activation of nuclear factor-kappa (NF-κB), increased expression of proinflammatory cytokine and TNF-α, thereby potentially exacerbating hepatocellular damage (Harrison-Findik, 2007). In the liver, increased production of ROS leads to pathogenesis of oxidative stress, associated with inflammation, DNA damage, lipid peroxidation and mitochondrial dysfunction. The NF-kB signaling pathway is a target mechanism for hepatoprotective agents (Jaeschke *et al.,* 2002; Giacco, 2011; Cichoż-Lach and Michalak, 2014).

Although the involvement of NF-kB activation in hepatocyte apoptosis is not fully understood, the inhibition of NF-kB has been associated with the induction of caspase 8, playing an anti-apoptotic role and evading inflammation (Tak and Firestein 2001; Wang 1998). Interestingly, in the 300 mg/kg BW GRT group the protein expression of NF-kB was significantly reduced, whilst the expression of caspase 3 and Bax was unchanged, suggesting the reduction of NF-kB could be a functional mechanism of preventing cell death and improving cell viability after GRT treatment.CYP3A4, is one of the most abundant of the cytochrome P450 isoforms (accounting for about 40%) and is involved in the metabolism and biotransformation of about 80% of conventional drugs and xenobiotics, including phytochemicals such as flavonoids (Pasiasi *et al.,* 2001; Ferguson and Tyndale, 2011; Singh *et al.,* 2011). In the present study, the effect of the GRT extract on the expression of CYP3A4 was investigated. Patel *et al.* (2016) found that GRT significantly dose and time-dependently inhibited CYP3A4 activity. This suggests that GRT could influence the pharmacokinetics of various drugs including statins (Pichard *et al.,* 2002, Patel *et al.,* 2016). However, in this study GRT did not affect the expression of CYP3A4, which might suggest that GRT has minimal to no toxic effect on the liver.

CHAPTER 6

6 CONCLUSION AND LIMITATIONS

6.1 Conclusion

The current study indicates that an aspalathin-rich green Rooibos extract (GRT) has a concentration- and time-dependent cytotoxic effect on C3A liver cells. However, at concentration up to 10 µg/mL, no cytotoxicity was observed. In the 3 month subchronic *in vivo study* in the rats that received 300 mg/kg of GRT BW daily, mild hepatocellular toxicity was observed in terms of slightly raised liver enzymes (AST and ALT). Although this is below levels specified by Hy's law for drug induced liver injury (DILI), damage was confirmed by focal areas of micro-vesicular lipid accumulation and, in the female rats, deposition of pigment. No signs of toxicity could be demonstrated in rats receiving 10 and 100 mg/kg BW daily. This suggests that in terms of HED for a 70 kg human a dose of 3405 mg per day could theoretically be regarded as the maximum tolerated dose, although this must be confirmed in different animal species to fulfil the FDA guidelines for toxicity testing where more than one species should be used. These findings confirm that despite the general perception that natural products are safer than conventional drugs, adverse effects can occur especially in products that are enriched with bioactive compounds by extraction. The need for toxicity studies are therefore essential.

6.2 Limitations

There were variations in the assay results which could be as a result of variations in the cellular metabolism or the errors of pipetting that affect the density of cells seeded per well.

Time and resources limited the progress of the study and the results produced. Problems were encountered to obtain sufficient number of rats for sub-chronic study and the preparation of the extract. Once these problems were overcome the project progressed smoothly. Another problem encountered was obtaining the histopathology results from the veterinary pathologist.

6.3 Future work suggestions

- Further histological investigation and quantification of the type of pigment found in the female rats and image analysis to quantify the extent of lipid accumulation will further add to the estimation of the liver damage.

- Suggestion from this study for future work would be to ascertain the hepatotoxicity of the interaction of GRT with drugs and other commonly used diet supplements that are associated with idiopathic hepatotoxicity.
- A pathway-based investigation of the effect of GRT that target either oxidative stress or mitochondrial mechanism of action of hepatotoxicity.
- A study aimed at designing a hepatoprotective model for aspalathin-rich green Rooibos extract.

6.4 Output from the study

6.4.1 Scientific presentations

1. **Buthelezi N.N**, Kappo A.P, Muller C.J.F, Mosa R.A, and Gabuza K.B. An *in vitro* investigation of hepatotoxic effect of an Aspalathin-rich green Rooibos extract. Oral Presentation, Biomedical Research and Innovation Platform, Research Symposium, Nelspruit, 2017.
2. **Buthelezi N.N**, Kappo A.P, Muller C.J.F, Mosa R.A, and Gabuza K.B. An *in vitro* investigation of hepatotoxic effect of an Aspalathin-rich green Rooibos extract. Oral Presentation. Faculty Postgraduate Presentations, Department of Biochemistry and Microbiology, University of Zululand, 2017.
3. **Buthelezi N.N**, Kappo A.P, Muller C.J.F, Mosa R.A, and Gabuza K.B. An *in vitro and in vivo* hepatotoxicity study of an Aspalathin-rich green Rooibos extract. Oral Presentation, Biomedical Research and Innovation Platform, Research Symposium, Cape Town

6.4.2 Projected output

1. **Buthelezi N.N,** Kappo A.P, Muller C.J.F, Aruleba R.T, Mosa R.A, and Gabuza K.B. **Review article titled:** Therapeutic potentials of medicinal plants in the treatment of CCl_4-induced hepatotoxicity. Phytomedicine.
2. **Buthelezi N.N**, Kappo A.P, Muller C.J.F, Khanyile S.P, Mosa R.A, and Gabuza K.B. **Experimental paper titled:** An *in vitro and in vivo* hepatotoxicity study of an Aspalathin-rich green Rooibos extract. Journal of hepatology.

CHAPTER 7

7 APPENDICES

7.1 Appendix A

7.1.1 Ethical clearance (UZREC 171110-030)

UNIVERSITY OF ZULULAND
RESEARCH ETHICS COMMITTEE
(Reg No: UZREC 171110-030)

RESEARCH & INNOVATION
Website: http://www.unizulu.ac.za
Private Bag X1001
KwaDlangezwa 3886
Tel: 035 902 6837
Fax: 035 902 6222
Email: Mangcie5@unizulu.ac.za

ETHICAL CLEARANCE CERTIFICATE

Certificate Number	UZREC 171110-030 PGM 2017/388						
Project Title	An in vivo and in vitro hepatotoxicity study of an Aspalathin-enriched green Rooibos extract						
Principal Researcher/ Investigator	NN Dutheleni						
Supervisor and Co-supervisor	Prof. A.P Kappo & Dr. K.B Gabuza		Prof. C.J.F Muller & Dr. R.A Mosa				
Department	Biochemistry						
Faculty	Science & Agriculture						
Type of Risk	Medium risk – laboratory research						
Nature of Project	Honours/4th Year		Master's	x	Doctoral		Departmental

The University of Zululand's Research Ethics Committee (UZREC) hereby gives ethical approval in respect of the undertakings contained in the above mentioned project. The Researcher may therefore commence with data collection as from the date of this Certificate, using the certificate number indicated above.

Special conditions:

(1) This certificate is valid for 2 years from the date of issue.
(2) Principal researcher must provide an annual report to the UZREC in the prescribed format [due date-01 July 2018]
(3) Principal researcher must submit a report at the end of project in respect of ethical compliance.
(4) The UZREC must be informed immediately of any material change in the conditions or undertakings mentioned in the documents that were presented to the meeting.

The UZREC wishes the researcher well in conducting research.

Professor Gideon De Wet
Chairperson: University Research Ethics Committee
Deputy Vice-Chancellor: Research & Innovation
18 July 2017

CHAIRPERSON
UNIVERSITY OF ZULULAND RESEARCH ETHICS COMMITTEE (UZREC)
REG NO: UZREC 171110-30
25 -07- 2017
STAMP
RESEARCH & INNOVATION OFFICE

7.2 Appendix B

Table 7.2.1: Reagents, chemicals and apparatus used in the study

Material	Supplier	Catalogue no
75 cm^2 Flasks	Greiner Bio-one, Frickenhausen, Germany	658975
Absorbance reader	Bio-Tek	ELx800
Anti-Rabbit IgG (Horseradish peroxidase labelled secondary antibody)	sc-2012	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Aspirator	Sigma	
Benchtop Centrifuge (Orto Alresa Digicen20)	Thermo Fisher Scientific, Waltham, MA, USA	SL16R
Beta actin (Actβ) antibody	Sc-47778	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Bio-Rad Protein DC assay kit	500-0201	Biorad, Hercules, California, USA
Biosafety cabinets	Airvolution, United Scientific	-
Bovine serum albumin (BSA) fatty acid free	Capricorn Scientific	BSA-FAF-1U
Bradford reagent	Bio-Rad	500-0201

C3A cells	American Type Culture Collection	CRL-10741
Cell Culture 96-well plate (non-cell binding)	Greiner, Bio-one, Frickenhausen, Germany	655180
Cell culture tested water	Lonza	BE17-724Q
Cell lysis Buffer	FNN0011	Life Technologies Corporation, Carlsbad, CA, USA
Centrifuge tubes	Greiner Bio-one, Frickenhausen, Germany	
CO_2 Incubator	RS BioTek	
Coomassie blue stain	161-0437	Bio-Rad, Hercules, CA, USA
Countess Cell counter and counting chambers	Invitrogen	
Cryo-tubes	Greiner Bio-one, Frickenhausen, Germany	430659
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D4540
Dulbecco's phosphate buffered saline (DPBS)	Lonza	BE17-513F
Eagle's modified essential medium (EMEM)	Lonza	BE12-662F

ELX 800 plate reader	BioTek Instruments Inc., Winooski, V	
Eppindorf tubes	Sigma- Aldrich, St. Louis MO, USA	30123301
Ethanol	2875	Sigma-Aldrich, St Louis, MO, USA
Ethanol 96.4% without denaturing agents	Sigma- Aldrich	2875
Ethyl alcohol	Sigma-Aldrich	E7023-500mL
Fetal Bovine Serum	GIBCO, Invitogen	10499-044
Filter Pads	23385	Sigma-Aldrich, St Louis, MO, USA
Fine balance scale	United Scientific	AR2140
Flame-Boy	Labotec	
Gen5 Software (Version 1.05)	Bio-Tek Instruments Inc., Winooski, VT, USA	
Glycine	Sigma-Aldrich	G7126
GraphPad Prism® (Version 5.04, GraphPad Software Inc)	La Jolla, USA	
HEPES	Lonza	17-737E
Inverted Light microscope	Olympus, Melville, NY, USA	CKX 41

L-glutamine	Lonza	G8540
Low fat free milk powder	2082054	Clover, JHB, SA
Luminometer	Bio-Tek	FLx800
Opaque multi-well plate		
Penicillin and streptomycin	Highveld Biological Lyndhurst, SA	CN 3351
pH meter	Lasec	702038
Pipette-Boy	Whitehead Scientific	
PolyvinylideneFluoridine Membrane (PVDF)	88585	Pierce, Rockford, IL, USA
Running buffer SDS	161-0772	Bio-Rad, Hercules, CA, USA
Scale	United Scientific	ARC120
SDS-PAGE gels	161-0993	Bio-Rad, Hercules, CA, USA
Serological pipettes	Greiner Bio-one, Frickenhausen, Germany	
Serum albumin bovine serum (BSA)	Bio-Rad	100-10SB
Sodium hydroxide (NaOH)	109140	Merck, Whitehouse Station, NJ, USA
Stainless steel beads 5mm	69989	Qiagen, Hilden, Germany
Sterile Tissue Culture water	Lonza	59900C

Syringe Filters (0.22 µm pore size Milex-GP Polyethersullfone (PES)	Merck Millipore	SLGP033RS
Syringes	Merck Millipore	R5MA55246
Tris	93352	Sigma-Aldrich, St Louis, MO, USA
Trypan blue	Sigma-Aldrich	T93595
Trypsin	Lonza	17-161F
Tween-20	58980C	Sigma-Aldrich, St Louis, MO, USA
ViaLightTM plus ATP Kit	Lonza	LT07-321
Water bath	Memment, Heilbronn, Germany	
Waterbath treatment	Sigma	#S5525
Whatman 3MMChr sheets	3030-931	Sigma-Aldrich, St Louis, MO, USA

Table 7.2.2: KRBH Media

Krebs-Ringer bicarbonate HEPES buffer (KRBH)
115 mM NaCl [M_W=58.44]
24 mM $NaHCO_3$ [M_W=84.01]
5 mM KCl [M_W=74.55]
1 mM $MgCl_2$ [M_W=95.21]
2.5 mM $CaCl_2$ [M_W=110.98]
BSA 0.1 % (w/v)

Table 7.2.3: Sorensen's Buffer

Reagent	**Final Conc.**	**Amount**
Glycine	0.1 M	0.751 g
NaCl	0.1 M	0.584 g

0.1 mM NaOH was titrated to the solution to obtain a buffer pH of 10.5.

Table 7.2.4: 10x Tris Buffer Saline

Reagent	**Final Conc.**	**MW**	**Amount**
Tris	200 mM	121.1	24.22 g
NaCl	1.37 M	58.44	80.06 g
Distilled water	-	-	1000 mL

Adjusted to pH= 7.6 using HCl

Table 7.2.5: 1x Tris buffered saline and Tween 20

Reagent	Amount
10x TBS	100 mL
Distilled water	900 mL
Tween 20	1 mL

Table 7.2.6: Shows the sequence of gel loading in 12% SDS Mini-Protean® TGX™ Gel

A

Lane	Gel 1
1	Marker
2	
3	Control
4	
5	
6	CCl4
7	
8	
9	10 mg/kg
10	
11	
12	100 mg/kg
13	
14	
15	100 mg/kg

B

Lane	Gel 2
1	Marker
2	
3	Control
4	
5	CCl4
6	
7	10 mg/kg
8	
9	100 mg/kg
10	
11	100 mg/kg

7.3 Appendix C

Table 7.3.1: The MTT content of C3A cells after 1 hour incubation

Group	Control	0.1µg/ml	1µg/ml	10µg/ml	100µg/ml	1000µg/ml
	94.28	87.55	85.52	82.35	71.44	71.18
	88.57	84.76	84.89	81.46	81.84	89.46
	90.72	96.69	79.81	81.33	76.01	104.94
	100.24	115.72	95.04	90.34	80.07	98.21
	112.93	111.03	97.58	92.63	97.45	93.52
	105.06	103.03	94.91	93.52	89.07	98.46
	99.10	98.34	97.70	94.53	97.07	96.18
	111.53	107.60	113.18	94.40	91.36	100.11
	97.83	103.03	103.41	96.69	85.78	102.53
	99.73	104.81	83.75	105.32	84.25	101.38
	92.09	103.82	94.33	92.00	87.94	99.59
	103.05	89.58	112.19	104.17	102.44	121.95
	101.41	88.89	87.51	91.83	95.28	108.14
	103.39	95.11	72.24	78.02	90.96	113.83
	86.82	99.68	87.51	88.20	95.37	98.73
	94.07	94.24	83.54	95.62	98.30	110.81
	104.60	96.49	90.96	104.08	111.42	107.53
	126.18	99.08	88.63	90.79	99.34	97.18
	94.93	102.18	100.03	93.38	95.80	115.04
	93.47	102.36	98.13	95.71	105.12	113.92
	103.14	98.92	103.14	104.35	93.64	101.94
	100.81	95.30	89.57	95.00	96.21	114.53
	95.00	89.42	85.95	101.41	97.26	117.09
	109.93	98.17	98.77	96.13	104.50	107.37
	93.79	109.48	101.86	97.41	99.00	99.90
	72.31	108.80	95.38	104.50	80.98	123.80
	110.76	98.24	94.93	101.86	100.58	113.40
	92.14	93.49	76.75	110.76	94.47	127.87
	117.32	112.19	95.98	98.39	101.86	102.01
	104.80	110.16	99.00	108.50	121.24	132.93
Mean	**100.00**	**99.94**	**93.07**	**95.49**	**94.20**	**106.12**
STDEV	**10.15**	**7.74**	**9.30**	**8.00**	**10.25**	**12.15**

Table 7.3.2: The MTT content of C3A cells after 24 hours incubation

Groups	Control	0.1µg/ml	1.0 µg/ml	10 µg/ml	100 µg/ml	1000 µg/ml
	92.17	102.82	102.82	85.15	76.77	51.41
	114.82	87.87	89.23	87.42	71.11	36.46
	86.97	77.45	76.32	33.29	80.17	36.91
	96.25	101.23	107.57	108.03	89.46	41.22
	98.74	91.49	101.91	97.61	91.95	44.61
	102.82	83.34	101.91	96.93	99.87	50.73
	101.01	108.71	99.19	112.33	104.40	43.71
	110.29	129.99	120.48	99.65	89.00	31.03
	96.93	101.46	103.95	98.74	96.02	39.63
	94.53	83.52	83.90	82.19	76.88	47.65
	100.04	101.37	89.03	96.43	88.65	39.48
	98.71	102.51	91.12	87.32	78.59	46.89
	95.67	94.15	97.76	92.26	84.09	42.14
	90.93	95.67	89.22	97.00	80.30	46.70
	95.86	105.35	97.38	98.14	82.57	44.61
	130.98	97.38	88.65	88.27	96.43	42.90
	104.78	98.71	94.91	94.53	95.29	55.43
	94.72	104.78	101.18	110.48	94.15	48.03
	93.77	101.18	101.94	92.26	93.58	48.22
	93.77	95.11	105.52	92.14	93.92	41.61
	90.36	100.76	109.08	102.25	92.73	44.73
	88.42	113.69	106.56	101.95	85.16	50.08
	99.12	103.88	109.97	107.00	105.37	46.52
	111.76	107.15	102.39	118.89	125.28	45.92
	115.03	108.19	111.76	109.97	118.30	53.95
	100.02	114.88	112.20	102.99	138.80	50.83
	104.62	101.21	132.27	119.19	109.68	62.86
	96.90	98.23	128.10	110.57	98.68	57.66
Mean	**100.00**	**100.43**	**102.01**	**97.25**	**94.19**	**46.14**
STDEV	**9.53**	**10.46**	**12.47**	**15.89**	**15.21**	**6.76**

Table 7.3.3: The MTT content of C3A cells after 48 hours incubation

Groups	Control	0.1µg/ml	1.0 µg/ml	10 µg/ml	100 µg/ml	1000 µg/ml
	102.56	87.03	81.77	92.05	87.71	60.53
	84.97	93.65	99.36	89.31	92.05	46.14
	104.84	95.25	95.25	95.25	83.83	52.08
	103.70	146.41	102.33	100.05	98.90	48.88
	105.53	106.44	101.19	97.53	103.24	56.88
	109.18	103.47	100.50	109.41	95.71	55.96
	99.59	111.69	106.67	114.89	94.56	53.91
	100.05	108.50	104.84	100.05	101.87	55.05
	96.62	104.39	105.53	118.09	87.03	52.76
	92.96	95.25	92.28	100.96	87.94	51.16
	80.23	93.81	84.97	94.13	74.23	84.33
	96.97	110.23	93.49	96.65	71.07	92.86
	92.86	114.34	116.23	103.60	65.07	93.49
	98.23	109.60	92.86	96.65	60.33	83.70
	97.92	107.08	100.76	99.18	43.59	113.08
	96.34	106.76	126.34	97.92	90.65	102.65
	98.23	108.34	108.02	108.34	97.28	117.81
	115.60	113.71	110.87	122.24	111.18	93.81
	114.34	116.87	136.77	108.65	121.29	98.86
	109.29	120.97	108.97	108.34	98.55	107.39
	143.98	86.79	93.60	91.45	89.65	56.84
	103.82	97.90	102.38	102.56	101.67	51.82
	85.89	89.12	104.90	102.74	105.25	51.28
	97.90	103.46	104.36	102.74	104.72	46.62
	100.05	92.34	106.51	100.95	109.02	42.14
	92.52	92.88	98.26	110.27	117.63	41.78
	91.81	87.32	103.10	98.44	99.34	56.84
	92.70	89.30	110.27	100.95	103.64	45.19
	98.80	97.90	98.26	114.76	105.79	44.83
	92.52	88.22	88.40	90.91	97.54	52.72
Mean	**100.00**	**102.63**	**102.63**	**102.30**	**93.34**	**67.05**
STDEV	**11.51**	**12.92**	**11.13**	**8.25**	**16.90**	**24.10**

Table 7.3.4: The ATP content of C3A cells after 1 hour incubation

Groups	Control	0.1 µg/ml	1 µg/ml	10 µg/ml	100 µg/ml	1000 µg/ml
	91.78	131.14	113.16	91.55	46.32	14.74
	112.18	117.22	117.80	75.18	55.36	24.36
	79.16	141.79	119.61	94.35	60.68	24.82
	95.33	121.34	108.74	95.53	41.60	20.20
	121.55	125.20	126.61	98.67	0.00	0.00
	89.27	132.57	111.19	118.57	91.07	79.02
	103.09	109.76	102.52	116.64	89.50	56.28
	108.96	101.23	106.93	104.79	73.41	48.01
	91.13	112.82	107.24	91.08	75.69	41.68
	107.56	121.94	113.32	99.59	91.97	54.36
	99.57	113.38	109.75	77.00	121.69	106.18
	100.01	123.64	113.07	115.28	135.68	115.02
	108.43	106.16	122.25	126.42	136.19	122.24
	103.25	122.89	121.56	118.44	122.43	123.99
	88.73	116.28	128.35	130.32	127.19	93.12
Mean	**100.00**	**119.82**	**114.81**	**103.56**	**84.58**	**61.60**
STDEV	**10.98**	**10.61**	**7.60**	**16.89**	**39.74**	**42.10**

Table 7.3.5: The ATP content of C3A cells after 24 hours incubation

Groups	Control	0.1 µg/ml	1 µg/ml	10 µg/ml	100 µg/ml	1000 µg/ml
	128.29	154.62	141.11	78.92	106.48	13.11
	43.25	159.29	63.74	80.96	101.52	27.26
	112.05	159.32	79.86	98.01	106.82	21.24
	119.59	118.08	81.46	58.74	111.97	19.98
	96.82	150.88	75.92	70.00	104.52	22.43
	109.80	111.98	146.87	113.14	66.32	5.85
	96.64	113.23	107.99	115.22	72.83	5.45
	106.56	111.34	126.44	112.28	75.49	5.16
	92.98	117.42	123.36	111.80	58.24	4.60
	94.01	107.02	126.15	114.86	57.77	3.76
	95.32	97.28	162.36	153.69	120.62	33.41
	91.89	100.94	172.19	160.71	149.03	37.66
	90.89	131.74	160.54	175.36	142.19	36.48
	109.37	167.37	163.16	159.43	123.16	33.03
	112.53	150.59	151.55	154.53	143.68	32.57
Mean	**100.00**	**130.07**	**125.51**	**117.18**	**102.71**	**20.13**
STDEV	**19.29**	**24.32**	**36.03**	**36.50**	**30.70**	**12.90**

Table 7.3.6: The ATP content of C3A cells after 48 hours incubation

Groups	Control	0.1 µg/ml	1 µg/ml	10 µg/ml	100 µg/ml	1000 µg/ml
	89.96	79.79	81.10	91.53	44.89	3.96
	93.37	78.90	82.96	43.97	43.31	5.87
	92.76	88.71	72.60	90.33	46.22	3.88
	98.87	95.70	85.20	41.57	24.29	4.43
	125.05	84.69	99.65	42.33	37.53	2.97
	78.81	115.59	104.51	109.03	41.40	1.54
	102.25	101.75	101.33	123.37	37.81	1.40
	103.70	84.04	113.19	96.90	53.34	1.78
	106.77	91.40	85.26	109.85	53.94	1.54
	108.46	102.46	104.70	101.20	61.30	1.87
	95.02	100.82	110.36	121.34	80.54	14.53
	84.13	110.35	122.85	121.38	94.24	16.43
	108.24	127.45	123.33	128.07	89.34	12.78
	100.35	123.70	124.25	129.45	84.37	14.13
	112.27	112.76	130.74	108.35	92.78	12.41
Mean	**100.00**	**99.88**	**102.80**	**97.24**	**59.02**	**6.64**
STDEV	**11.67**	**15.59**	**18.21**	**30.83**	**23.16**	**5.64**

Table 7.3.7: The content of adenylate kinase enzyme after 1 hour incubation using adenylate kinase assay

Groups	Control	0.1 µg/ml	1 µg/ml	10 µg/ml	100 µg/ml	1000 µg/ml
	119.60	118.45	106.96	77.31	31.97	10.85
	99.74	108.84	121.60	71.92	37.55	9.10
	99.40	83.94	96.39	67.14	37.94	8.49
	102.12	89.52	90.53	83.56	16.37	28.79
	79.13	83.63	80.44	85.67	11.01	37.00
	85.43	119.37	96.06	109.13	30.72	15.40
	80.86	129.37	123.00	114.41	47.39	13.10
	103.36	125.34	132.84	78.38	49.20	14.25
	111.48	112.78	131.67	133.57	38.30	16.98
	118.87	131.06	132.93	128.68	61.28	18.01
	124.83	137.32	127.23	114.31	69.90	30.23
	81.83	82.84	95.37	85.51	51.91	24.89
	100.44	92.89	76.23	81.42	50.72	24.77
	101.18	84.29	82.14	70.38	59.56	23.90
	91.71	71.33	81.75	79.12	58.05	30.39
Mean	**100.00**	**104.73**	**105.01**	**92.03**	**43.46**	**20.41**
STDEV	**6.46**	**9.66**	**9.50**	**9.77**	**7.40**	**3.94**

Table 7.3.8: The content of adenylate kinase enzyme after 24 hours incubation using adenylate kinase assay

Groups	Control	0.1 µg/ml	1 µg/ml	10 µg/ml	100 µg/ml	1000 µg/ml
	119.47	112.97	112.87	99.15	95.52	8.03
	101.66	90.51	91.27	79.94	85.61	6.49
	93.43	98.38	88.98	69.68	87.52	5.23
	93.24	88.97	88.52	64.92	69.16	5.29
	92.20	79.63	85.71	65.25	70.46	3.42
	101.80	97.18	84.38	90.47	65.16	9.07
	97.41	106.40	101.84	77.92	63.57	10.84
	92.26	101.36	97.80	110.73	58.17	15.60
	111.57	105.91	106.32	93.36	69.09	9.57
	96.97	107.61	97.55	82.93	77.75	13.72
	104.68	107.56	94.25	82.46	105.27	20.67
	102.32	102.09	93.94	74.19	97.54	20.94
	92.11	102.65	86.90	75.90	87.41	18.91
	99.98	93.09	98.23	75.83	96.88	18.67
	100.91	104.18	95.50	81.74	83.19	22.51
Mean	**100.00**	**99.90**	**94.94**	**81.63**	**80.82**	**12.60**
STDEV	**3.45**	**3.92**	**3.54**	**5.61**	**6.45**	**2.91**

Table 7.3.9: The content of adenylate kinase enzyme after 48 hours incubation using adenylate kinase assay

Groups	Control	0.1 µg/ml	1 µg/ml	10 µg/ml	100 µg/ml	1000 µg/ml
	105.51	112.08	111.16	97.77	69.85	1.83
	109.31	90.30	92.07	84.65	57.08	2.24
	89.97	90.70	87.32	69.55	52.48	1.97
	95.82	87.79	85.43	79.22	56.81	1.96
	99.38	93.71	94.94	81.36	51.51	1.94
	99.51	58.86	139.77	118.95	84.62	9.87
	85.43	93.63	131.37	109.59	74.28	9.22
	90.58	93.30	135.08	87.71	88.46	10.82
	121.78	127.58	127.41	119.96	86.94	14.92
	102.70	100.57	93.94	94.30	92.57	10.57
	52.95	110.21	110.91	102.67	70.06	6.03
	99.50	105.94	107.77	90.15	70.58	7.39
	113.95	103.54	105.05	93.71	69.20	8.24
	115.62	105.09	96.17	93.77	63.18	7.65
	117.98	109.14	104.64	89.98	68.08	11.22
Mean	**100.00**	**98.83**	**108.20**	**94.22**	**70.38**	**7.06**
STDEV	**7.30**	**6.63**	**7.66**	**6.07**	**5.67**	**1.82**

Table 7.3.10: Serum biochemical test results, n=10/group

	Urea (mmol/L)	Creatinine (µmol/L)	Albumin (g/L)	ALP (IU/L)	Uric Acid (mmol/L)	Total Bilirubin (µmol/L)	ALT (IU/L)	AST (IU/L)	Total Protein (g/L)
Control	6.4	63.00	12.00	109.00	0.58	2.0	69	131	69
	8	71	15	121	0.72	3.0	67	189	90
	8	66	16	128	0.54	2.0	102	187	75
	8	67	13	124	0.58	2.0	35	110	71
	7	**67**	**14**	**121**	**0.61**	**2.3**	**68**	**154**	**76**
CCl_4 induced group	7	64	15	203	0.36	2.0	100	291	75
	7	65	16	147	0.49	3.0			87
	8	61	16	98	0.24	8.0			91
	7	64	16	197	0.34		187	506	77
	7	59	13	161	0.42	2.0	185	149	66
	7	57	14	111	0.50	2.0	133	407	70
	7	60	13	143	0.51	2.0	73	134	65
	7	64	13	155	0.60	2.0	120	243	78
	7	**62**	**15**	**152**	**0.43**	**3.0**	**133**	**288**	**76**

10mg/kg GRT group	8	65	19	239	0.41	1.8	52	103	82
	8	51	14	102	0.40	2.1	77	259	82
	8	58	17	185	0.33	1.8	60	286	90
	8	52	17	125	0.23	2.7	66	313	86
	9	65	20	162	0.41	1.8	41	91	83
	5	49	16	83	0.31	1.8	48	133	78
	7	58	18	168	0.37	2.4	60	178	90
	5	49	12	121	0.31	1.8	40	173	71
	5	58	15	111	0.23	1.8	63	185	81
	7	**56**	**16**	**144**	**0.33**	**2.0**	**56**	**191**	**83**
100 mg/kg GRT group	7	52	21	89	0.35	1.8	79	241	87
	10	57	22	68	0.28	2.1	46	176	100
	10	54	24	83	0.47	3.2	54	159	102
	6	58	14	108	0.35	1.8	67	206	69
	7	57	16	117	0.50	1.8	48	101	80
	8	51	18	114	0.31	1.8	44	112	78
	7	69	16	127	0.60	1.8	87	142	79
	8	**57**	**19**	**101**	**0.41**	**2.0**	**61**	**162**	**85**

300 mg/kg GRT group	8	60	17	109	0.53	1.8	99	381	87
	9	58	20	108	0.40	2.3	122	614	89
	7	52	19	108	0.39	2.8	184	685	92
	8	53	21	70	0.37	1.8	66	219	93
	8	64	16	149	0.69	1.8	90	205	83
	7	68	15	141	0.71	1.8	89		86
	8	64	16	137	0.66	2.0	162	656	87
	6	49	14	132	0.28	1.8	45	157	82
	7	52	16	116	0.45	1.8	127	311	84
	8	**58**	**17**	**119**	**0.50**	**2.0**	**109**	**404**	**87**

Table 7.3.11: 90 days weekly body weights of the Sprague Dawley rats

		Baseline	Week 1 1/5/2017	Week 2 8/5/2017	Week 3 15/5/2017	Week 4 22/5/2017	Week 5 29/5/2017	Week 6 5/6/2017	Week 7 12/6/2017
Control	1								
	2	79	102	123	146	153	157	164	178
	3	120	149	165	184	198	202	218	216
	4	67	89	118	139	146	189	202	217
	5	77	100	122	131	153	154	162	171
	6	74	110	136	155	181	193	214	219
	7	67	93	121	140	170	184	205	207
	8	85	119	147	169	198	211	234	232
	9	74	109	143	158	188	201	215	227
	10	69	84	106	126	168	181	192	206
	Mean	**79**	**106**	**131**	**150**	**173**	**186**	**201**	**208**
	STDEV	**6**	**13**	**16**	**17**	**16**	**20**	**24**	**22**
	SEM	**6**	**6**	**6**	**6**	**7**	**7**	**8**	**7**

CCl_4 induced group	11	76	94	119	129	148	159	161	174
	78	83	104	121	150	160	178	175	180
	13	107	134	153	178	183	205	205	207
	14	65	92	107	128	145	157	166	168
	15	74	104	120	141	147	166	166	172
	16	94	139	156	192	200	230	241	258
	17	70	92	113	143	166	190	201	212
	18	77	108	133	164	181	201	210	223
	19	82	107	123	151	174	198	215	233
	20	61	78	105	140	163	198	210	222
	Mean	**79**	**105**	**125**	**152**	**167**	**188**	**195**	**205**
	STDEV	**11**	**20**	**17**	**20**	**18**	**20**	**24**	**28**
	SEM	**4**	**6**	**6**	**7**	**6**	**7**	**8**	**10**

10mg/kg treated group	21	58	79	115	140	161	195	203	217
	22	75	97	122	143	154	163	168	171
	23	90	116	134	151	156	169	175	177
	24	78	102	122	137	147	172	177	175
	25	77	102	119	135	152	169	172	174
	26	97	133	171	182	216	225	251	258
	27	93	132	165	180	194	212	228	240
	28	82	118	149	171	192	206	222	226
	29	83	120	151	182	209	214	226	244
	30	53	63	80	112	132	165	178	198
	Mean	**79**	**106**	**133**	**153**	**172**	**189**	**200**	**208**
	STDEV	**15**	**26**	**34**	**29**	**33**	**23**	**31**	**32**
	SEM	**4**	**7**	**9**	**8**	**9**	**8**	**9**	**10**

100mg/kg treated group	31	59	83	98	100	99	106	122	134
	32	62	83	103	123	149	168	172	182
	33	83	114	129	139	154	166	167	170
	34	78	107	124	137	154	163	167	172
	35	77	104	122	135	154	165	173	172
	36	99	132	162	182	211	226	244	251
	37	91	127	153	179	203	205	217	234
	38	91	127	153	174	190	212	230	224
	39	82	121	151	176	205	216	237	248
	40	59	75	95	129	153	174	189	203
	Mean	**78**	**107**	**129**	**147**	**167**	**180**	**192**	**199**
	STDEV	**14**	**22**	**26**	**24**	**26**	**24**	**28**	**30**
	SEM	**4**	**7**	**8**	**9**	**11**	**11**	**12**	**12**

300 mg/kg treated group	41	56	71	95	121	148	167	187	198
	42	66	86	104	128	147	166	172	179
	43	82	106	115	138	149	168	171	177
	44	80	105	130	144	160	173	184	186
	45	79	102	121	140	156	165	166	173
	46	118	158	194	219	244	262	285	298
	47	88	129	154	173	204	215	230	242
	48	74	101	129	159	188	213	223	234
	49	76	117	146	166	189	201	221	225
	50	60	83	112	137	163	182	205	220
	Mean	**78**	**106**	**130**	**152**	**175**	**191**	**204**	**213**
	STDEV	**20**	**26**	**30**	**30**	**32**	**33**	**39**	**40**
	SEM	**5**	**8**	**9**	**9**	**10**	**10**	**12**	**12**

	Week 8 19/6/2017	Week 9 26/6/2017	Week 10 3/7/2017	Week 11 10/7/2017	Week 12 17/7/2017	Week 13 24/07/17	Week 14 31/7/17
Control	183	184	185	188	197	203	200
	221	229	229	231	236	237	238
	225	233	240	250	259	262	262
	179	176	188	188	195	202	198
	230	237	249	253	273	274	280
	219	225	236	244	264	271	272
	251	254	270	275	295	295	292
	235	246	258	271	287	285	292
	211	219	235	235	255	255	261
	217	**223**	**232**	**237**	**251**	**254**	**255**
	25	**28**	**28**	**32**	**36**	**33**	**32**
	8	**9**	**10**	**11**	**12**	**11**	**12**

CCl_4 induced group	176	183	189	187	203	201	205
	191	189	195	199	199	198	201
	217	216	225	221	237	234	237
	174	174	186	178	188	186	196
	177	180	192	187	197	192	207
	260	282	280	294	302	305	314
	219	230	237	249	267	263	272
	236	248	261	265	287	287	300
	250	257	265	276	283	298	300
	240	246	252	266	278	282	284
	214	**220**	**228**	**232**	**244**	**250**	**257**
	29	**34**	**31**	**37**	**37**	**41**	**38**
	10	**12**	**11**	**14**	**14**	**15**	**15**

10mg/kg treated group	232	239	256	261	272	280	280
	177	184	179	189	194	197	200
	182	184	193	187	189	199	194
	191	185	196	194	197	206	202
	185	183	193	191	199	205	204
	271	282	293	311	313	321	318
	246	263	262	282	290	295	300
	240	244	255	261	275	275	284
	247	258	268	274	289	290	296
	207	223	233	241	254	263	269
	218	**225**	**233**	**239**	**247**	**250**	**252**
	31	**35**	**34**	**41**	**40**	**40**	**40**
	11	**12**	**12**	**14**	**15**	**15**	**15**

100mg/kg treated group	141	153	159	167	171	177	181
	183	190	193	197	202	203	205
	180	172	179	186	186	188	191
	181	178	189	187	200	200	198
	180	176	188	186	193	196	195
	269	277	292	296	309	316	313
	241	255	262	270	287	282	295
	247	249	265	269	280	286	283
	265	269	285	288	309	306	313
	223	227	285	248	264	277	278
	211	**215**	**230**	**229**	**240**	**250**	**245**
	33	**37**	**38**	**40**	**43**	**43**	**16**
	14	**14**	**17**	**16**	**17**	**17**	**18**

300 mg/kg treated group	212	222	235	240	256	261	262
	184	191	196	197	205	204	206
	186	185	197	196	205	201	205
	194	189	200	201	203	210	207
	178	179	183	186	193	192	191
	320	325	347	343	346	346	355
	257	261	275	279	296	301	302
	248	252	263	265	281	286	289
	239	244	256	263	271	277	279
	235	249	257	268	281	280	289
	225	**230**	**241**	**244**	**254**	**255**	**258**
	46	**47**	**52**	**50**	**50**	**50**	**53**
	14	**15**	**16**	**16**	**16**	**16**	**17**

	Week15 7/8/2017	Week 16 14/8/2017	Week 17 21/8/2017	Week 18 25/8/2017
Control	200	198	210	205
	247	239	245	246
	272	274	279	276
	195	200	199	199
	292	297	294	295
	282	280	287	288
	308	307	311	314
	292	302	306	307
	268	271	277	279
	262	**263**	**268**	**267**
	41	**40**	**41**	**42**
	13	**14**	**14**	**14**

CCl_4 induced group	197	200	204	247
	202	209	204	202
	240	243	242	208
	194	197	192	200
	201	203	200	195
	319	327	327	329
	275	283	288	288
	302	313	306	312
	314	312	320	320
	300	294	307	310
	261	**265**	**265**	**263**
	44	**45**	**47**	**49**
	17	**17**	**18**	**18**

10mg/kg treated group	294	295	297	295
	196	201	208	203
	194	197	201	198
	206	202	207	209
	198	206	207	209
	339	336	340	340
	316	318	317	320
	297	294	299	300
	299	301	306	298
	278	282	290	283
	258	**260**	**264**	**262**
	48	**45**	**46**	**45**
	18	**17**	**17**	**17**

100mg/kg treated group	180	189	188	191
	200	207	209	201
	190	193	193	197
	198	204	199	203
	192	201	196	199
	330	324..09	335	325
	294	301	306	302
	295	296	298	300
	319	321	318	322
	291	296	298	303
	257	**252**	**261**	**262**
	49	**47**	**49**	**47**
	19	**18**	**19**	**19**

300 mg/kg treated group	278	276	291	284
	213	207	211	212
	205	207	211	207
	207	214	214	209
	196	201	196	191
	381	375	387	381
	311	313	318	318
	301	304	307	308
	287	286	300	296
	301	301	308	311
	267	**268**	**272**	**270**
	59	**56**	**61**	**62**
	19	**19**	**20**	**20**

Table 7.3.12: 90 days food intake of the Sprague-Dawley rats

		Cage number	Week 1 Intake	Week 2 Intake	Week 3 Intake	Week 4 Intake	Week 5 Intake	Week 6 Intake	Week 7 Intake	Week 8 Intake
Control	1									
	2	C1(2)								
	3		778	296	296	272	283	294	282	287
	4	C2(1)								
	5		182	172	147	159	130	142	131	137
	6	C11(3)								
	7									
	8		836	583	504	406	447	447	456	490
	9	C12(3)								
	10		752	485	464	431	432	464	453	499
	Average		**637**	**384**	**353**	**317**	**323**	**337**	**330**	**353**
	STDEV		**305**	**185**	**164**	**126**	**148**	**150**	**156**	**174**
	SEM		**102**	**62**	**55**	**42**	**49**	**50**	**52**	**58**

CCl_4 induced group	11	C3(3)								
	12									
	13		657	538	438	389	480	408	394	437
	14	C4(2)								
	15		493	262	259	228	231	239	246	228
	16	C13(3)								
	17									
	18		799	523	492	441	432	458	447	491
	19	C14(2)								
	20		738	457	409	411	357	342	395	314
	Average		**677**	**414**	**387**	**360**	**340**	**346**	**363**	**344**
	STDEV		**162**	**136**	**118**	**115**	**102**	**109**	**104**	**134**
	SEM		**51**	**43**	**37**	**37**	**32**	**35**	**33**	**42**
10mg/kg treated group	21	C5(2)								
	22									
	23		829	319	327	286	302	424	268	314
	24	C6(2)								
	25		887	436	429	462	580	506	401	375
	26	C15(3)								
	27									
	28		852	500	499	458	483	500	492	539
	29	C16(3)								
	30		785	486	474	464	444	462	457	479
	Average		**841**	**474**	**467**	**461**	**502**	**489**	**450**	**464**
	STDEV		**52**	**34**	**35**	**3**	**70**	**24**	**46**	**83**
	SEM		**16**	**11**	**11**	**1**	**22**	**8**	**15**	**26**

100mg/kg treated group	31	C7(3)								
	32									
	33		650	320	342	318	336	340	344	391
	34	C8(2)								
	35		872	302	275	269	385	408	283	276
	36	C17(3)								
	37									
	38		774	513	519	516	498	501	506	547
	39	C18(2)								
	40		663	361	399	303	347	339	351	353
	Average		**770**	**392**	**398**	**363**	**410**	**416**	**380**	**392**
	STDEV		**104**	**109**	**122**	**134**	**79**	**81**	**114**	**140**
	SEM		**33**	**34**	**39**	**42**	**25**	**26**	**36**	**44**
300mg/kg treated group	41	C9(2)								
	42									
	43		637	463	350	383	331	538	309	418
	44	C10(2)								
	45		874	876	529	563	565	574	476	472
	46	C19(3)								
	47									
	48		939	620	579	503	525	523	450	530
	49	C20(3)								
	50		821	455	462	420	458	457	527	416
	Average		**878**	**650**	**524**	**495**	**516**	**518**	**485**	**473**
	STDEV		**59**	**212**	**59**	**72**	**54**	**59**	**39**	**57**
	SEM		**19**	**67**	**19**	**23**	**17**	**19**	**12**	**18**

	Week 9 Intake	Week 10 Intake	Week 11 Intake	Week 12 Intake	Week 13 Intake	Week 14 Intake	Week 15 Intake	Week 16 Intake
Control	290	235	303	311	281	243	349	318
	163	149	152	144	131	137	138	145
	443	510	515	511	445	476	457	518
	460	512	534	519	481	477	456	542
	339	**352**	**376**	**371**	**335**	**333**	**350**	**381**
	140	**187**	**182**	**179**	**335**	**333**	**350**	**381**
	47	**62**	**61**	**60**	**112**	**111**	**117**	**127**
CCl_4 induced group	404	421	456	414	387	393	382	461
	235	240	257	255	247	237	229	254
	454	498	516	507	465	469	486	532
	338	302	320	334	296	347	288	407
	342	**346**	**364**	**365**	**349**	**361**	**346**	**414**
	109	**135**	**135**	**129**	**97**	**361**	**346**	**414**
	35	**43**	**43**	**41**	**31**	**114**	**109**	**131**

10mg/kg treated group	420	326	348	354	254	353	274	332
	502	359	301	507	439	581	384	584
	496	526	549	546	485	523	491	580
	458	479	516	514	455	457	448	514
	485	**455**	**455**	**522**	**408**	**478**	**399**	**502**
	24	**86**	**134**	**21**	**104**	**98**	**399**	**502**
	8	**27**	**43**	**7**	**33**	**31**	**126**	**159**
100mg/kg treated group	336	391	366	373	339	342	349	392
	404	298	273	261	274	341	261	291
	497	528	545	556	520	523	517	616
	335	363	382	370	339	378	352	374
	412	**397**	**400**	**396**	**368**	**396**	**370**	**418**
	81	**119**	**137**	**150**	**106**	**86**	**370**	**418**
	26	**37**	**43**	**47**	**34**	**27**	**117**	**132**

300mg/kg treated group	534	435	412	253	229	238	224	269
	570	492	592	474	380	475	418	518
	519	502	484	563	528	561	535	559
	446	469	521	508	454	482	462	461
	512	**488**	**533**	**515**	**398**	**439**	**410**	**452**
	63	**17**	**55**	**45**	**128**	**139**	**410**	**452**
	20	**5**	**17**	**14**	**40**	**44**	**130**	**143**

Table 7.3.13: 90 days water intake of the Sprague-Dawley rats

			Week 1 Intake	Week 2 Intake	Week 3 Intake	Week 4 Intake	Week 5 Intake	Week 6 Intake	Week 7 Intake	Week 8 Intake
Control	1									
	2	C1(2)								
	3		335	300	300	300	310	300	310	310
	4	C2(1)								
	5		190	160	150	170	170	165	160	160
	6	C11(3)								
	7									
	8		450	550	520	490	540	500	580	580
	9	C12(3)								
	10		470	530	470	533	480	500	510	520
	Average		**361**	**385**	**360**	**373**	**375**	**366**	**390**	**393**
	STDEV		**129**	**188**	**169**	**169**	**168**	**164**	**191**	**193**
	SEM		**43**	**63**	**56**	**56**	**56**	**55**	**64**	**64**

CCl_4 induced group	11	C3(3)								
	12									
	13		567	490	430	440	375	400	410	400
	14	C4(2)								
	15		431	310	290	290	300	290	330	310
	16	C13(3)								
	17									
	18		580	540	490	515	480	500	570	570
	19	C14(2)								
	20		370	350	330	408	430	400	320	360
	Average		**460**	**400**	**370**	**404**	**403**	**397**	**407**	**413**
	STDEV		**108**	**123**	**106**	**113**	**93**	**105**	**142**	**138**
	SEM		**34**	**39**	**33**	**36**	**29**	**33**	**45**	**44**
10mg/kg treated group	21	C5(2)								
	22									
	23		377	260	260	290	270	260	290	280
	24	C6(2)								
	25		470	320	330	304	350	345	400	300
	26	C15(3)								
	27									
	28		700	580	525	540	460	445	530	550
	29	C16(3)								
	30		430	580	480	550	500	495	500	520
	Average		**533**	**493**	**445**	**465**	**437**	**428**	**477**	**457**
	STDEV		**146**	**150**	**102**	**139**	**78**	**76**	**68**	**137**
	SEM		**46**	**47**	**32**	**44**	**25**	**24**	**22**	**43**

100mg/kg treated group	31	C7(3)								
	32									
	33		510	490	510	490	445	460	480	530
	34	C8(2)								
	35		500	330	290	290	300	290	320	300
	36	C17(3)								
	37									
	38		585	610	580	510	580	550	610	560
	39	C18(2)								
	40		440	360	340	360	375	350	330	330
	Average		**508**	**433**	**403**	**387**	**418**	**397**	**420**	**397**
	STDEV		**73**	**154**	**155**	**112**	**145**	**136**	**165**	**142**
	SEM		**23**	**49**	**49**	**36**	**46**	**43**	**52**	**45**
300mg/kg treated group	41	C9(2)								
	42									
	43		340	340	300	320	285	290	290	290
	44	C10(2)								
	45		325	290	290	330	355	330	300	320
	46	C19(3)								
	47									
	48		540	630	630	600	585	560	620	580
	49	C20(3)								
	50		652	580	580	523	590	550	570	630
	average		**506**	**500**	**500**	**484**	**510**	**480**	**497**	**510**
	STDEV		**166**	**184**	**184**	**139**	**134**	**130**	**172**	**166**
	SEM		**53**	**58**	**58**	**44**	**42**	**41**	**54**	**53**

	Week 9 Intake	Week 10 Intake	Week 11 Intake	Week 12 Intake	Week 13 Intake	Week 14 Intake	Week 15 Intake	Week 16 Intake
Control	310	310	320	330	300	270	260	340
	170	170	200	190	160	140	160	150
	600	600	520	540	570	610	560	620
	500	500	490	510	540	480	500	530
	395	**395**	**383**	**393**	**393**	**375**	**370**	**410**
	192	**192**	**150**	**164**	**197**	**210**	**191**	**209**
	64	**64**	**50**	**55**	**66**	**70**	**64**	**70**
CCl_4 induced group	450	400	420	400	450	400	430	480
	290	290	310	320	330	320	340	270
	580	560	520	510	530	540	550	560
	310	330	390	370	380	360	340	360
	393	**393**	**407**	**400**	**423**	**405**	**415**	**418**
	162	**146**	**106**	**98**	**87**	**96**	**99**	**128**
	51	**46**	**34**	**31**	**27**	**30**	**31**	**41**

10mg/kg treated group	250	280	270	250	260	230	260	270
	290	320	350	320	320	290	320	340
	550	590	500	520	580	580	580	630
	520	510	500	455	500	470	510	530
	453	**473**	**450**	**432**	**415**	**393**	**418**	**443**
	142	**139**	**87**	**102**	**150**	**161**	**152**	**166**
	45	**44**	**27**	**32**	**47**	**51**	**48**	**53**
100mg/kg treated group	480	480	470	450	490	450	500	480
	310	290	320	310	310	340	280	290
	610	590	570	560	620	630	600	670
	350	360	370	370	400	340	340	350
	423	**413**	**420**	**413**	**455**	**440**	**430**	**448**
	163	**157**	**132**	**131**	**132**	**137**	**147**	**168**
	52	**50**	**42**	**41**	**42**	**43**	**46**	**53**

300mg/kg treated group	290	310	310	300	330	290	290	340
	300	290	330	300	320	290	340	300
	520	510	580	550	540	560	580	610
	540	570	570	540	550	540	580	640
	453	**457**	**493**	**463**	**435**	**420**	**448**	**473**
	133	**147**	**142**	**142**	**127**	**150**	**154**	**177**
	42	**47**	**45**	**45**	**40**	**48**	**49**	**56**

Table 7.3.14: Liver weights of Sprague-Dawley rats at termination

	Liver weight (g)				
	Control	**CCl_4**	**10 mg/kg**	**100 mg/kg**	**300 mg/kg**
		9.09	7.63	7.86	7.24
	9.61	10.88	9.1	7.17	7.29
	9.21	9.62	8.97	8.9	10.15
	10.44	12.64	8.69	8.12	7.7
	7.04	9.57	14.52	8.42	11.93
	12.01	13.54	11.49	12.41	11.88
	10.51	13.73	13.13	10.72	14.44
	12.89	14.11	13.75	11.13	13.7
	11.13	14.77	10.1	12.92	13.09
	10.62	13.84	13.54	14.85	10.26
Mean	10.38	12.18	11.09	10.25	10.77
STDEV	1.69	2.17	2.50	2.56	2.68

Table 7.3.15: Histopathology report of the liver, Vetdiagnostix Pathology Laboratories

	Males	Females
Control	Livers: in two of the 4 series there is moderate hepatocellular hydropic and microvesicular vacuolar degeneration with proliferation of sinusoidal Kupfer cells.	Livers: moderate micro and macrovesicular vacuolar degeneration evident in 3 of 4 series. In one series dilation and duplication of central veins with capillary canal formation evident.
CCl_4	Livers: in all 4 series there is moderate to severe periacinar hepatocellular micro and macrovesicular degeneration with single cell and clustered cell necrosis. Periportal hepatocytes exhibit moderate hydropic degeneration. Proliferation of sinusoidal Kupfer cells. Into series there is mild sinusoidal leukocytosis.	Livers: moderate to severe micro and macrovesicular hepatocellular vacuolar degeneration with single cell and clustered cell necrosis. Small narrow rims of more viable periportal hepatocytes noted. In one series there is periportal accumulation of granular brown pigment within portal hepatocytes. Similar pigment encountered in sinusoidal macrophages and individual portal macrophages.
10mg/kg	Livers: mild hepatocellular hydropic degeneration into of the 4 series. In the other 2 series there is moderate hydropic and vacuolar degeneration with proliferation of sinusoidal Kupfer cells. In one series there is dilation of central veins with capillary canal formation.	Livers: mild hepatocellular hydropic and microvesicular vacuolar degeneration with proliferation of sinusoidal Kupfer cells evident in 3 of 5, while moderate vacuolar degeneration evident in 2 of 5. Scant portal inflammatory infiltrates of individual mononuclear cells in all 5 series. In one series there is dilation and duplication of central veins and in the same series midzonal and periportal hepatocytescontaining intracytoplasmic granular brown pigment resembling iron/lipofuscin. Similar pigment evident within periportal sinusoidal macrophages and portal macrophages.

100mg/kg	Livers: mild hepatocellular micro and macrovesicular vacuolar degeneration noted in 2/5 series. In 3/5 series there is moderate hepatocellular vacuolar degeneration which is most outspoken in the centrizonal aspects of the hepatic lobules.	Livers: in one series there is periportal accumulation of granular brown pigment within hepatocytes with clusters of pigment engorged macrophages in the periportal hepatic sinusoids and portal tracts. Based on the appearance of the pigment in H&E stained sections one must consider iron and lipofuscin as the most likely differentials. These pigment accumulations are associated with hepatocellular degeneration. The other 2 series exhibit mild hepatocellular hydropic degeneration.
300mg/kg	Livers: in 3 of the 4 series there is moderate hepatocellular hydropic plus macro and microvesicular vacuolar degeneration. Proliferation of sinusoidal Kupfer cells.	Livers: in 1 of the 5 series there is periportal intracytoplasmic accumulation of golden brown pigment (iron?) within hepatocytes. There is associated proliferation of sinusoidal Kupfer cells and pigment engorged macrophages evident in sinusoids and portal tracts.

Table 7.3.16: Liver NF-KB expression from two independent experiments

	NF-kB band Intensity	B-Actin	Normalized NF-kB	% NF-kB	%Av.
Control	350472.0	886509.0	0.4	100.0	84.8
	255952.0	929033.0	0.3	69.7	
CCl_4	479009.0	1573640.0	0.3	77.0	82.4
	471968.0	1361036.0	0.3	87.7	
10 mg/kg	337936.0	1080432.0	0.3	79.1	93.1
	355806.0	839760.0	0.4	107.2	
100 mg/kg	238576.0	907218.0	0.3	66.5	43.9
	222938.0	862372.0	0.3	65.4	
300 mg/kg	266382.0	1049209.0	0.3	64.2	28.8
	205680.0	1486400.0	0.1	35.0	
Control	264792.0	886509.0	0.3	100.0	93.8
	243342.0	929033.0	0.3	87.7	
CCl_4	437840.0	1573640.0	0.3	93.2	100.6
	439056.0	1361036.0	0.3	108.0	
10 mg/kg	314560.0	1080432.0	0.3	97.5	113.9
	327056.0	839760.0	0.4	130.4	
100 mg/kg	203694.0	907218.0	0.2	75.2	74.5
	190096.0	862372.0	0.2	73.8	
300 mg/kg	217170.0	1049209.0	0.2	69.3	50.6
	141949.0	1486400.0	0.1	32.0	

Table 7.3.17: Expression of Caspase 3 from three independent experiments

	Caspase 3 intensity	B-Actin	Normalized Caspase 3	(%) Caspase 3	AV.
Control	1219710.0	434709.0	2.8	100.0	70.2
	1280480.0	1129474.0	1.1	40.4	
CCl_4	1728969.0	1538472.0	1.1	40.1	39.4
	1434690.0	1582659.0	0.9	32.3	
	1751145.0	1364048.0	1.3	45.8	
10 mg/kg	1865580.0	1285167.0	1.5	51.7	55.7
	1812360.0	1003314.0	1.8	64.4	
	1455498.0	1016691.0	1.4	51.0	
100 mg/kg	1108980.0	1026798.0	1.1	38.5	55.2
	1705800.0	924017.0	1.8	65.8	
	1543235.0	895242.0	1.7	61.4	
300 mg/kg	1844096.0	694200.0	2.7	94.7	106.3
	1623600.0	752724.0	2.2	76.9	
	2809726.0	679932.0	4.1	147.3	
Control	2088725.0	452466.0	4.6	164.5	118.4
	1450319.0	715470.0	2.0	72.2	
CCl_4	1935417.0	732816.0	2.6	94.1	84.7
	1719642.0	886212.0	1.9	69.2	
	2012339.0	788508.0	2.6	91.0	
10 mg/kg	1887378.0	1124914.0	1.7	59.8	51.1
	1744649.0	1292443.0	1.3	48.1	
	1306433.0	1022618.0	1.3	45.5	
100 mg/kg	1483012.0	1126761.0	1.3	46.9	49.9
	1617040.0	1120245.0	1.4	51.4	
	1487294.0	1032462.0	1.4	51.3	
300 mg/kg	1642676.0	902652.0	1.8	64.9	74.8
	1416679.0	756261.0	1.9	66.8	
	1462412.0	561330.0	2.6	92.9	

Control	716400.0	1129474.0	0.6	22.6	22.6
CCl_4	781014.0	1538472.0	0.5	18.1	17.4
	748116.0	1582659.0	0.5	16.8	
	658098.0	1364048.0	0.5	17.2	
10 mg/kg	590868.0	1285167.0	0.5	16.4	17.8
	595008.0	1003314.0	0.6	21.1	
	452200.0	1016691.0	0.4	15.9	
100 mg/kg	453492.0	1026798.0	0.4	15.7	19.5
	556852.0	924017.0	0.6	21.5	
	535591.0	895242.0	0.6	21.3	
300 mg/kg	759960.0	694200.0	1.1	39.0	30.8
	544867.0	752724.0	0.7	25.8	
	528530.0	679932.0	0.8	27.7	

Table 7.3.18: Expression of Bax in the liver

	Bax intensity	**B-Actin**	**Normalized Bax**	**(%) Bax**	**AV.**
Control	1023309.0	886509.0	1.2	100.0	95.6
	977262.0	929033.0	1.1	91.1	
CCl_4	922740.0	1573640.0	0.6	50.8	66.3
	1283974.0	1361036.0	0.9	81.7	
10 mg/kg	1774638.0	1080432.0	1.6	142.3	116.5
	878713.0	839760.0	1.0	90.7	
100 mg/kg	1059485.0	907218.0	1.2	101.2	71.2
	409572.0	862372.0	0.5	41.1	
300 mg/kg	938769.0	1049209.0	0.9	77.5	82.4
	1323157.0	1486400.0	0.9	77.1	
	869550.0	812760.0	1.1	92.7	

Table 7.3.19: Expression of CYP3A4 on the liver

	CYP3A4 intensity	B-Actin	Normalized CYP3A4	(%) CYP3A4	AV.
Control	2743966.00	7352858.00	0.37	100.00	97.79
	1491680.00	4181596.00	0.36	95.59	
CCl_4	2459170.00	4409640.00	0.56	149.44	104.96
	1713040.00	3647800.00	0.47	125.84	
	674695.00	4566800.00	0.15	39.59	
10 mg/kg	1982160.00	3675778.00	0.54	144.50	110.04
	1667250.00	5142240.00	0.32	86.88	
	2761440.00	7495005.00	0.37	98.73	
100 mg/kg	1452024.00	6193319.00	0.23	62.82	76.88
	2157215.00	7529680.00	0.29	76.77	
	2272920.00	6688668.00	0.34	91.06	
300 mg/kg	1508800.00	5506238.00	0.27	73.43	79.82
	1749710.00	5586555.00	0.31	83.93	
	1279194.00	4175184.00	0.31	82.10	
Control	1790712.00	4486835.00	0.40	100.00	72.95
	965406.00	5270440.00	0.18	45.90	
CCl_4	1248935.00	6964600.00	0.18	44.93	43.59
	1180411.00	3834402.00	0.31	77.13	
	183198.00	5266548.00	0.03	8.72	
10 mg/kg	1444794.00	7163520.00	0.20	50.54	37.24
	837160.00	6291870.00	0.13	33.34	
	881088.00	7930962.00	0.11	27.84	
100 mg/kg	376142.00	9466736.00	0.04	9.96	36.68
	1147320.00	6819120.00	0.17	42.16	
	1330056.00	5754060.00	0.23	57.92	
300 mg/kg	1252560.00	5526920.00	0.23	56.78	39.74
	664202.00	3539840.00	0.19	47.01	
	360168.00	5850760.00	0.06	15.42	

Control	4396858.00	8950662.00	0.49	100.00	197.89
	8339277.00	5739403.00	1.45	295.78	
CCl_4	5584200.00	7376727.00	0.76	154.10	85.34
	2611400.00	7990360.00	0.33	66.53	
	896496.00	5156611.00	0.17	35.39	
10 mg/kg	3462066.00	5942844.00	0.58	118.59	213.37
	5346167.00	4426464.00	1.21	245.87	
	5049564.00	3729082.00	1.35	275.65	
100 mg/kg	3866204.00	3842524.00	1.01	204.82	420.85
	8921488.00	2414760.00	3.69	752.10	
	10717080.00	7138387.00	1.50	305.63	
300 mg/kg	11516904.00	4895350.00	2.35	478.92	336.49
	5820605.00	3729168.00	1.56	317.74	
	4616392.00	4416069.00	1.05	212.80	

CHAPTER 8

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