


THE EFFECT OF ROOIBOS ON TRACE  
ELEMENTS ABSORPTION AND BIOCHEMICAL  
PARAMETERS – A MURINE MODEL

CARINE KUNSEVI-KILOLA

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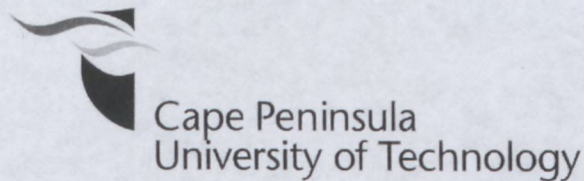
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**The effect of Rooibos on trace elements absorption and biochemical parameters – A murine model**

by

**CARINE KUNSEVI-KILOLA**

**Thesis submitted in fulfillment of the requirements for the degree  
Master Technologiae: Biomedical Technology**

In the

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**Bellville, South Africa**

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## DECLARATION

I, Carine Kunsevi Kilola, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Carine KK

Signed

03/03/2014

Date

## ABSTRACT

Over the past few decades, it has been shown that various critical diseases including heart disease, cancer, and diabetes associated with free radical generation and low endogenous antioxidant capacity, lead to oxidative stress and cell injury. In recent years, numerous studies have also reported that antioxidants, present in various beverages, vegetables and some foods have attracted a significant research interest due to their potential benefits to human health. However, epidemiological evidence shows a correlation between the intake of food rich in antioxidants and the reduced incidence of some mortality of chronic diseases, certain cancers and coronary heart disease. The aims of this study were to determine the effects of rooibos teas (fermented and unfermented) and green tea as a comparison on the biochemical parameters and the trace element absorption in a rat model.

In this study 4 groups of experimental animals were used. All groups had *ad libitum* access to standard rat chow. Group A, the controls (11 animals), were fed with tap water; group B (11 animals) were fed with the liquid extract of fermented rooibos tea; group C (9 animals) were fed with the liquid extracts of unfermented rooibos and group D (9 animals) were fed with the liquid extract of green tea. All groups were fed for a period of 10 weeks. After the feeding period, the animals were sacrificed by euthanization with intraperitoneal injections of pentobarbital. Blood was sampled by cardiac puncture and centrifuged to obtain the serum. Some elemental analyses were performed with X-ray emission and backscattering. ICP-OES was used to determine the magnesium content. For X-ray emission, backscattering and ICP-OES analyses, 100  $\mu$ L of each serum sample in a group were added to 2 mL freeze-drying tube. Of the combined specimen, 100  $\mu$ L was used for the magnesium determination by ICP-OES. The remainder of the combined serum specimens for each group were freeze-dried at -80  $^{\circ}$ C and then pressed into a pellet. The pellet was coated with carbon and analyzed using X-ray emission and backscattering. The elemental X-rays of P, S, Ca, Mn, Fe, Cu, Co, Zn, Mo, Ca and Se emitted were quantified to obtain the respective concentrations. Biochemical chemistry analyses were performed on each serum sample of each animal. The biochemical parameters tested for were total protein, albumin, globulin, total bilirubin, lactate dehydrogenase, blood urea nitrogen, uric acid, total cholesterol, aspartate aminotransferase, alanine aminotransferase, creatine phosphokinase and creatinine.

The P concentration increased ( $p=0.028$ ) when fed with the fermented rooibos tea liquid extract and S content increased when fed with the - the unfermented tea liquid extract ( $p=0.041$ ). The concentrations of Cl and Cr were not affected ( $p>0.05$ ) by any of tea liquid extracts. The

unfermented rooibos tea liquid extract and the green tea indicated a decrease in the concentrations of Fe ( $p=0.031$  and  $p=0.032$ , respectively) and Mn ( $p=0.041$  and  $p=0.034$ , respectively). The concentrations of Fe, Zn and Cu in the serum increased when feeding with fermented rooibos tea liquid extract ( $p=0.024$ ;  $p=0.030$  and  $p=0.015$ , respectively) while Se, Mo and Mg concentrations were decreased by the liquid extracts of the fermented, unfermented and green teas ( $p=0.014$ ,  $p=0.017$  and  $p=0.011$ ;  $p=0.024$ ,  $p=0.026$  and  $p=0.019$ ;  $p=0.031$ ,  $p=0.034$  and  $p=0.025$ , respectively).

Concerning the biochemical parameters, the total protein, globulin and the uric acid contents in the serum sample were slightly affected with the green tea extract ( $p=0.041$ ,  $p=0.039$  and  $p=0.047$  respectively). The albumin, lactate dehydrogenase, blood urea nitrogen, the total cholesterol, the alanine aminotransferase and the aspartate aminotransferase concentrations were not affected ( $p>0$ ) by any of the tea liquid extracts. However, the total bilirubin content was decreased ( $p=0.012$ ) when feeding with the fermented rooibos group while the creatine phosphokinase and the creatinine contents were decreased ( $p=0.042$  and  $p=0.033$ , respectively) when feeding with the unfermented rooibos tea liquid extract.

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## DEDICATION

I dedicate this dissertation to my family, especially to my...  
Dad and my lovely Moms, Honoré KUNSEVI, Françoise BULEMBI and Henriette MADITUKA,  
for instilling in me the importance of hard work and higher education;  
to my sisters: Solange, Nene, Nina and Ritha and my brothers: Tresor, Felix, Yannick and Petit  
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## LIST OF ABBREVIATIONS, TERMS AND CONCEPTS

<b>ALB</b>	Albumin, the most abundant protein in the blood plasma, produced in the liver and comprising about half of the blood serum protein.
<b>ALT</b>	Alanine aminotransferase formerly called serum glutamic pyruvic transaminase (SGPT) measures the amount of this enzyme in the blood. ALT is found mainly in the liver, but also in smaller amounts in the kidneys, heart, muscles and pancreas.
<b>AST</b>	Aspartate aminotransferase formerly, called serum glutamic oxaloacetic transaminase (SGOT), measures the amount of this enzyme in the blood. AST is normally found in red blood cells, liver, heart, muscle tissue, pancreas and kidneys.
<b>Antioxidants</b>	Compounds that act as scavengers of free radicals.
<b>BS analysis</b>	Backscattering is the reflection of electromagnetic waves, particles, or signals back to the direction from which they came. It is a diffuse reflection due to scattering, as opposed to specular reflection like a mirror.
<b>Bilirubin</b>	Metabolite (breakdown product) of haemoglobin present in the bloodstream and liver of all mammals.
<b>BCG</b>	Bromocresol green
<b>BUN</b>	blood urea nitrogen. Urea nitrogen is what forms when protein breaks down.
<b>CK</b>	Creatine kinase, an enzyme in muscle, brain, and other tissues that catalyzes the transfer of a phosphate group from adenosine triphosphate to creatine, producing adenosine diphosphate and phosphocreatine ( $ATP + creatine = ADP + phosphocreatine$ ).
<b>CPK</b>	Creatine phosphokinase (CPK) is an enzyme found mainly in the heart, brain, and skeletal muscle.
<b>Crea</b>	Creatinine, a nitrogenous compound formed as the irreversible end product of creatine metabolism. It is formed in the muscle in relatively small amounts, passes into the blood and is excreted in the urine.
<b>Enzyme</b>	A protein that helps to speed up chemical reactions
<b>4-AAP</b>	4-Aminoantipyrine
<b>Flavonoids</b>	The flavonoids are polyphenolic compounds possessing 15 carbon atoms consisting of two benzene rings joined by a linear

three carbon chain. They are compounds found in fruits, vegetables, and certain beverages that have diverse beneficial biochemical and antioxidant effects. Their dietary intake is quite high compared to other dietary antioxidants like vitamins C and E. The antioxidant activity of flavonoids depends on their molecular structure.

<b>FR</b>	Fermented rooibos
<b>Free radical</b>	Any species capable of independent existence that contains one or more unpaired electrons.
<b>GRT or GR</b>	Green Tea.
<b>ICP-OES</b>	Inductively coupled plasma optical emission spectrometry (ICP-OES) is an analytical technique used for the detection of trace metals.
<b>LDH</b>	Lactate dehydrogenase, an enzyme predominantly found in the cytoplasm of almost all body tissues (the liver, kidneys, striated muscle, and heart muscle), where its main function is to catalyze the oxidation of L-lactate to pyruvate.
<b>Macro elements</b>	Elements that are present in quantities of grams per 100 (as percentage) such as Na, Mg, K, Ca, P and S
<b>MDLs</b>	Stands for minimum detection limits which are the minimum amount of a species that can be determined in a given mass of a matrix.
<b>NADPH</b>	Nicotinamide adenine dinucleotide phosphate
<b>Oxidative Stress</b>	Oxidative stress is caused by an imbalance between the production of reaction oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage.
<b>PIXE</b>	Particle-Induced X-ray Emission, a technique used in the determining of the elemental make-up of a material or sample.
<b>Radical</b>	Chemical species with an unpaired electron
<b>Redox (reaction)</b>	A chemical reaction (reduction and oxidation) between two substances in which one substance donates an electron and the other substance accepts the electron. Oxidation is therefore the loss of electrons or an increase in oxidation state by a molecule, atom or ion and the reduction is the gain of electrons or a decrease in oxidation state by a molecule, atom or ion.

<b>Reactive oxygen species</b>	compounds with an oxygen atom which contains one or more unpaired electrons.
<b>Rooibos</b>	South African herbal plant
<b>SRC</b>	Standard rat chow
<b>T-Chol</b>	Total Cholesterol
<b>T-Pro</b>	Total protein
<b>Trace elements</b>	Elements such as Cu, Fe, Mn Zn, Ni, Cr and Se, that occur in very small quantities (parts per million or at time parts per billion) in the body but is nonetheless important for many biological processes.
<b>UFR</b>	Unfermented rooibos.
<b>Urea</b>	The final nitrogenous product in the breakdown of proteins by the body, during which amino groups (NH <sub>2</sub> ) are removed from amino acids and converted into ammonium ions (NH <sub>4</sub> ), which are toxic at high concentrations.
<b>Uric acid</b>	Nitrogenous end product of protein and purine metabolism, present in the blood and urine and produced by the breakdown of amino acids in the liver.

## CHAPTER ONE

### INTRODUCTION AND LITERATURE REVIEW

#### 1.1 Introduction

Research on antioxidants and oxidative stress has attempted to clarify the aetiology of certain disease conditions such as heart disease, cancer, muscular degeneration, diabetes, nutrient deficiency of people and animals which were previously not well understood (Cadenas & Packer, 2002). Present-day nutritional life style causes a multitude of disorders and metabolic dysfunctions in humans as well as animals. The decomposition and absorption of food are effected by enzymes that take part in the chemical reactions reduction and oxidation, generally referred to as redox reactions, for generating energy (Maret, 2003).

Chemical species termed free radicals are molecular fragments that react spontaneously within one of a thousandth of a second with adjacent molecules (Trachootham *et al.*, 2008). These are formed during the oxidation/reduction (redox) chemical reaction process. A free radical is defined as any atom (for example oxygen, nitrogen) or molecule capable of independent existence with at least one unpaired electron in the outermost shell (Lippard & Berg, 1994). A free radical is formed once a covalent bond connecting entities is broken and one electron remains with each newly formed atom (McMurray, 1995). Free radicals are highly reactive due to the presence of the unpaired electron(s) and are generally grouped as reactive oxygen species (ROS). This is however necessary for breaking up proteins, sugars and fats structures foreign to the body. This process ensures timeous release of energy through oxidation in the cell. Enzymes are proteins that enable or accelerate these chemical processes. Without these enzymes, a well-regulated metabolism would not be possible (Trachootham *et al.*, 2008). An example is the reaction of the enzyme superoxide dismutase, an inherent free radical scavenger, which catalyzes the dismutation of the superoxide radical. This reaction is illustrated below. M is the metal ion and SOD is superoxide dismutase.

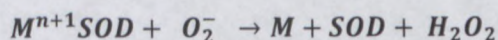
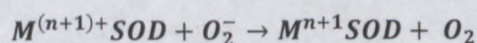


Figure 1.1 Reactions of the enzyme superoxide dismutase (SOD), a free radical scavenger, with metal ions such as Fe, Cu etc represented as M.

If free radicals such as superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radical ( $OH^*$ ) are not completely neutralized at the "moment of creation", they may react with intracellular nucleic acids and cell damage will occur (Valko *et al.*, 2007).

Personal health can decline due to an excess of free radical activities arriving from metabolic dysfunction, for example toxins in food (like insecticides), air borne pollutants (such as smoking) or by impairing the scavenger system. Pathological conditions can be initiated with minor inflammation, which can subsequently become severe (Panickar & Anderson, 2011). Inherent "free radical scavengers," such as SOD therefore neutralize (or detoxify) the oxidant and hence is called an antioxidant (Panickar & Anderson, 2011). An antioxidant is thus a molecule which has the ability to slow or prevent the oxidation of other molecules (Dugas & Penney, 1989; Lippard & Berg, 1994; Podda & Grundmann-Kollmann, 2008). It therefore acts as a protective mechanism for the metabolism of reactive oxygen species.

## 1.2 Antioxidants

Antioxidants can broadly be classified into two categories; those soluble in aqueous medium, the hydrophilic antioxidants, and those soluble in organic medium, the hydrophobic antioxidants (McMurray, 1995; Podda & Grundmann-Kollmann, 2008). As a general rule, water-soluble antioxidants react with oxidants in the cell cytoplasm and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation (Housecroft & Constable, 1995; McMurray, 1995; Martini, 2007). These compounds may be produced in the body or obtained from the diet. Boelsma *et al.* (2001) reported that the supplementation with nutrients such as vitamins, carotenoids and polyunsaturated fatty acids was shown to provide protection against ultraviolet light, although the sun-protection factor was relatively small compared with that of topical sunscreens. The different antioxidants are present at a wide range of concentrations in body fluids and tissues, with some, such as glutathione or ubiquinone are mostly present within cells, while others, such as uric acid are more evenly distributed throughout the body (Dugas & Penney, 1989; Lippard & Berg, 1994).

Halim *et al.* (1997) demonstrated that liver function tests, disturbed by carbon tetrachloride (CCl<sub>4</sub>), were significantly modulated by antioxidants such as vitamins E and C and subsequent histopathological examination showed that antioxidants ameliorated the necrotic and fibrotic changes caused by CCl<sub>4</sub>. Treatment with antioxidants was also shown to modulate the toxic effect of CCl<sub>4</sub> on the lipid profile (a group of blood tests that determines risk of coronary heart disease, includes total cholesterol, HDL-cholesterol, etc.) and malondialdehyde content. Administration of antioxidants like vitamins E and C could play an important role in prophylaxis against lipid peroxidation and consequently liver fibrosis caused by free radicals (Halim *et al.*, 1997).

Enzymes and other antioxidants are the basic components of this scavenger system where various elements such as metal ions, are incorporated in chemical structures of these enzymes (Heim *et al.*, 2002). The concentration of these ions in the parts per million ranges, are referred to as trace elements. Therefore, the absorption of trace elements is necessary to initiate chemical reactions between enzymes and antioxidants. Without this harmonious interaction, the entire metabolism and the removal waste cannot be accomplished.

Concentrations of elements can have significant effect on a biological system, for example, an excessively high concentration of metals would result in the inhibition of the enzyme activity (Prohaska *et al.*, 2000) while an excessively low concentration would result in the disturbances in vitamin synthesis and a reduction in metabolism. In this context, excessive supplementation with zinc, for example, is known to cause hypochromic anemia by inducing a functional copper deficiency which can lead to ataxia (Prohaska, 2000). It is therefore important that all trace elements are biologically available in the correct concentrations and the appropriate ratio to each other. For that reason, trace elements cannot be considered individually and isolated from each other. For example, Cu-deficiency (CuD) leads to heart hypertrophy (Zhou, 2008).

The growing interest in the replacement of synthetic food antioxidants by natural ones has promoted research on vegetable sources and the screening of raw materials for identifying new antioxidants. Antioxidants are also widely needed to prevent deterioration of oxidizable products such as cosmetics, pharmaceuticals and plastics. Therefore, oxidation reactions are not the only exclusive concerns for the food industry but also irradiated food, obesity and genetically modified food. Polyphenols are the majority of compounds in plants with antioxidant activity (Moure *et al.*, 2001). In addition, antioxidants with biomedical properties such as anticarcinogenicity, antimutagenicity, antiallergenicity and anti-aging activity have been reported for natural and synthetic compounds (Moure *et al.*, 2001).

Although oxidation reactions are crucial for life (Mader, 2004), it can also be damaging (Vertuani *et al.*, 2004). Therefore, plants and animals preserve complex systems of multiple types of antioxidants (for example, glutathione, vitamin C, and vitamins E) in addition to enzymes such as catalase, superoxide dismutase and various peroxidases (Jacob, 1996; Wolf, 2005; Brigelius-Flohé & Davies, 2007).

Oxidative stress results from a disproportion in quantity between oxidants and antioxidants in favour of the oxidants, potentially leading to biological damage. During the aerobic metabolism process, oxidants are formed as normal products, but under pathophysiological

conditions they can be produced at elevated rates. Antioxidant defense entails numerous strategies, both enzymatic and non-enzymatic. In the lipid stage, tocopherols and carotenes, including oxy-carotenoids are of interest, as are vitamin A and ubiquinol. In the aqueous stage, there are ascorbate, glutathione and other compounds. Over and above the cytosol, the nuclear and mitochondrial matrices and extracellular fluids are protected. Overall, these low molecular mass antioxidant molecules add considerably to the defense provided by the enzymes superoxide dismutase, catalase and glutathione peroxidases (Sies, 1997).

The deleterious effect in the use of oxygen, as part of the process for generating metabolic energy, is the production of reactive oxygen species (ROS), which result in damage to biomolecules (for example, lipid, protein, amino acids and DNA). In this process, the superoxide anion is produced as side-effect of several steps in the electron transport chain (Fang *et al.*, 2002). Therefore, organisms contain a complex network of antioxidant metabolites and enzymes that work together in order to prevent oxidative damage to cellular component such as DNA, proteins and lipids (Lippard & Berg, 1994).

Generally, antioxidant systems have the responsibility of either preventing these reactive species from being formed or removing them before they can damage vital components of the cell (Vertuani *et al.*, 2004). Considerate free radical biology is indispensable for designing an optimal nutritional counter measure against space radiation-induced cytotoxicity. Free radicals (for example, superoxide, nitric oxide, and hydroxyl radicals) and other reactive species (for example, hydrogen peroxide, peroxynitrite and hypochlorous acid) are produced in the body, principally as a result of aerobic metabolism. Antioxidants (for example, glutathione, arginine, citrulline, taurine, creatine, selenium, zinc, vitamin E, vitamin C, vitamin A, and tea polyphenols) and antioxidant enzymes (for example, superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidases) exert synergistic actions in scavenging free radicals.

Over the past three decades, a large number of epidemiological studies showed that malnutrition (for example, dietary deficiencies of protein, selenium, and zinc) or excess of certain nutrients (for example, iron and vitamin C) gave rise to the oxidation of biomolecules and cell injury (McMurray, 1995; Fang *et al.*, 2002). Nutritional antioxidants such as vitamins have a potential role in preventing cardiovascular disease and cancers (Hercberg *et al.*, 1998; Devasagayam *et al.*, 2004). These are useful radioprotectors and play an important role in preventing many human diseases (for example, cancer, atherosclerosis, stroke, rheumatoid arthritis, neurodegeneration and diabetes). Accordingly, De Rijk *et al.* (1997)

suggested that a high intake of dietary vitamin E may protect against the occurrence of Parkinson disease. An antioxidant is “any substance that, when present at low concentration compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate” (Gutteridge & Halliwell, 1989; Sies, 1997). Hence, the knowledge of enzymatic and non-enzymatic oxidative defense mechanisms serve as a guide principle for establishing the most effective nutrition support to ensure the biological safety of manned space missions (Fang *et al.*, 2002).

Oxidants can damage cells by starting a chemical chain reaction such as lipid peroxidation, or by oxidizing DNA or proteins (Sies, 1993; Lippard & Berg, 1994; Chaudiere & Ferrari-Iliou, 1999). Damage to DNA can cause mutations and possibly cancer, if not reversed by DNA repair degradation (Sies, 1993; Lippard & Berg, 1994; Cadenas & Davies, 2000).

The relative importance and interactions between these different antioxidants is a complex area, with the various metabolites and enzyme systems having synergistic and interdependent effects on one another. The action of one antioxidant may depend on the proper function of other members of the antioxidant system (Lippard & Berg, 1994; Ďuračková, 2010). The amount of protection provided by any one antioxidant therefore depends on its concentration, its reactivity towards the particular reactive oxygen species being considered; and, the status of the antioxidants with which it interacts (Halliwell, 2011).

### **1.3 Trace elements and enzymes**

Enzymes are large biological molecules responsible for thousands of chemical interconversions that sustain life. From the digestion of food to the synthesis of DNA, they are highly selective catalysts, greatly accelerating both the rate and specificity of metabolic reactions (Andreini *et al.*, 2008). Most enzymes are proteins, although some catalytic RNA molecules have been identified. Enzymes adopt a specific three-dimensional structure and may employ organic (for example, biotin) and inorganic (for example, magnesium ion,  $Mg^{2+}$ ) cofactors to assist in catalysis (Andreini *et al.*, 2008; Andreini *et al.*, 2009). Substrates, in enzymatic reaction, are the molecules at the beginning of the process converted into different molecules called products. In a biological cell, almost all chemical reactions need enzymes in order to occur at rates sufficient for life. Since enzymes are selective for their substrates and speed up only a few reactions from among many possibilities, the set of enzymes made in a cell determines which metabolic pathways occur in that cell. There exist presently many enzymes of which the structures have not yet been elucidated.

Cofactors are mostly metal ions or coenzymes and are either inorganic or organic in nature. Cofactors assist enzymes during the catalysis of reactions (Adugna *et al.*, 2004). Coenzymes are non-protein organic molecules that are mostly derivatives of vitamins soluble in water by phosphorylation. They bind apoenzyme to proteins to produce an active holoenzyme. Apoenzymes are enzymes that lack their necessary cofactor(s) for proper functioning. The binding of the enzyme to a coenzyme forms a holoenzyme. Holoenzymes are the active form of an apoenzyme (Adugna *et al.*, 2004). Hence, cofactors can be metals or coenzymes with the primary function of assisting in enzyme activity. They are able to assist in performing necessary reactions without which the enzyme function cannot be realized. They are divided into coenzymes and prosthetic groups. A holoenzyme refers to a catalytically active enzyme that consists of both apoenzyme (enzyme without its cofactor(s)) and cofactor (Andreini *et al.*, 2008). There are two groups of cofactors: metals and small organic molecules referred to as coenzymes. Coenzymes usually obtained from vitamins. Prosthetic groups refer to tightly bound coenzymes, while co-substrates refer to loosely bound coenzymes that are released in the same way as substrates and products. Loosely bound coenzymes differ from substrates in that the same coenzymes may be used by different enzymes in order to bring about proper enzyme activity (Wang *et al.*, 2005). Enzymes without their necessary cofactors are called apoenzymes, which are the inactive form of an enzyme.

Metal ions are known as the common cofactors. In some enzymes, their function as catalyst cannot be carried out if a metal ion is not available to be bound to the active site. In daily nutrition, this kind of cofactor plays a role as the essential trace elements such as: iron ( $\text{Fe}^{3+}$ ), manganese ( $\text{Mn}^{2+}$ ), cobalt ( $\text{Co}^{2+}$ ), copper ( $\text{Cu}^{2+}$ ), zinc ( $\text{Zn}^{2+}$ ), selenium ( $\text{Se}^{2+}$ ), and molybdenum ( $\text{Mo}^{5+}$ ). Hence, the application of metal ions in biological catalysis is of cardinal importance. For example, organisms need Co for the vitamin B<sub>12</sub> synthesis; trace elements such as Cu, Zn and Mn are also needed for the "superoxide-dismutase (SOD)" enzymes which catalyze the dismutation of superoxide ion into oxygen and hydrogen peroxide. As such, SOD enzymes are important antioxidants in the defense of nearly all cells exposed to oxygen. Therefore, an abnormality in trace elements will inevitably result in the failure of a specific chain of action.

All of these trace elements are biologically important and have a dynamic, bio-activating or regulating effect (Barandier *et al.*, 1999). Some compounds contribute to antioxidant defense by chelating transition metals, such as Fe, Cu, Zn and Se (Lippard & Berg, 1994; Chaudière & Ferrari-Iliou, 1999), which are classed as trace elements and preventing them from catalyzing the production of free radicals in the cell. Particularly important is the ability to

sequester iron, which is the function of iron-binding proteins such as transferrin and ferritin. Se and Zn are commonly referred to as antioxidant elements but these chemical elements have no antioxidant action themselves and are instead required for the activity of some antioxidant enzymes (Bray & Bettger, 1990; Jacob *et al.*, 1999).

The elements predominantly present in enzyme systems are Mg, Zn, Fe, Mn, Ca, Co, Mo, W and Cu (Andreini *et al.*, 2004; Andreini *et al.*, 2008; Andreini *et al.*, 2009). In a survey performed by these authors, the distributions of ions of these elements across the 6 classes of enzymes, that is, oxidoreductases (EC1), transferases (EC2), hydrolases (EC3), lyases (EC4), isomerases (EC5) and ligases (EC6) have been established. This distribution is summarized in Table 1.1.

**Table 1.1** The distribution, as a percentage of the metal-containing enzymes, of the ions of Mg, Zn, Fe, Mn, Ca, Co, Mo, W and Cu in the six classes of enzymes (based on the data of Andreini *et al.*, 2004; Holliday *et al.*, 2005; Holliday *et al.*, 2007 and Adreini *et al.*, 2008). EC1 to EC6 are the oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. Zero indicates that the functioning of the enzyme class does not depend on metal ions.

Metal ions	Enzymes					
	EC1 Oxidoreductases	EC2 Transferases	EC3 Hydrolases	EC4 Lyases	EC5 Isomerases	EC6 Ligases
Mg <sup>2+</sup>	5	45	23	9	4	14
Zn <sup>2+</sup>	10	12	66	8	2	2
Fe (Fe <sup>2+</sup> , Fe <sup>3+</sup> )	81	5	6	5	3	0
Mn <sup>2+</sup>	11	41	23	10	10	5
Ca <sup>2+</sup>	7	10	69	14	0	0
Co <sup>4+</sup>	1	33	38	8	12	0
Mo/W (Mo <sup>4+</sup> , Mo <sup>+6</sup> W <sup>6+</sup> )	88	6	0	6	0	0
Cu (Cu <sup>+</sup> , Cu <sup>2+</sup> )	93	7	0	0	0	0
Metallic	44	40	39	36	36	59
Non-metallic	56	60	61	64	64	41

It is important to note is that the metal ions are only present in about 40% of the enzymes in each of the classes. This means that 60% of the enzyme class activity is not dependent on metal ions and are therefore only organic in nature, except where P is incorporated into the enzyme. Furthermore, the sum of the percentages of a particular ion present in the enzyme classes amounts to 100%. Where the combined percentages of the ions amount to more than 100%; it indicates that these ions are all present in the same enzyme.

Various preliminary inferences can now be made on the distribution of these common ions. For the purpose of this study, these inferences will be classified as inclusive in which instance the activity will be considered with respect to the metal ion and as exclusive where the activity will be considered with respect to the enzyme class. The minimum concentration of 10% is arbitrarily considered as significant to affect the activity of the enzyme and would yield a notable malfunction. Furthermore, this does not preclude the fact that significant abnormalities<sup>1</sup> will not affect the activity of the non-metallic enzymes in the specific classes.

#### Magnesium (Mg)

Magnesium is important as a cofactor in several enzymatic reactions contributing to stable cardiovascular hemodynamics and electrophysiologic functioning. Its deficiency is common and can be associated with risk factors and complications of heart failure (Douban *et al.*, 1996). Typical therapy for heart failure (digoxin, diuretic agents, and angiotensin-converting-enzyme inhibitors) are influenced by or associated with significant alteration in magnesium balance. Magnesium toxicity rarely occurs except in patients with renal dysfunction. Mg plays a pivotal role in the functioning of both the enzyme classes transferases and hydrolases (EC2 and EC3, respectively) and to a lesser extent in ligases (EC6) (Bains, 2010). Hence, an abnormality in the concentration of Mg will significantly affect the activity of enzymes in these classes. In the Mg ion, the bonding between the nucleus and the valence is relatively stronger than in the instance of elements such as Na and K and hence it is expected that the role of the ion in transferases would be more prominent than in hydrolases. Also, the ion would not play a prominent role in oxidoreductases due to its low redox potential and if present, would only have a structural function.

#### Zinc (Zn)

In the case of Zn however, the d-orbital is completely filled (Cotton *et al.*, 1988). The formation of the  $Zn^{2+}$  is therefore stable (Anderson & Dowd, 1987). As a result, this ion is the only zinc ion formed. Furthermore, groups with lone pairs of electrons such as the water molecule and the amino functional group would easily bond with the ion. Thus,  $Zn^{2+}$  would play a pivotal role in hydrolases and to an extent dehydrogenases. Also, transferases such as those containing the amino group ( $-NH_2$ ), would also easily bond with the ion. This bonding would be less prominent than in the case of the water molecule since O is more electronegative than N (Cotton *et al.*, 1988). Examples of enzymes with Zn as cofactor are

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<sup>1</sup> Abnormality in this study is defined as either the lack or excess of a substance responsible for a particular function. The definition therefore indicates a hypo- or hyper- concentration state. Thus the substances are not absent, but present in either a decreased or increased concentration.

glutamate dehydrogenase, alcohol dehydrogenase, lactate dehydrogenase, carbonic anhydrase, alkaline phosphatase, DNA polymerase, RNA polymerase, delta-alanine dehydratase, superoxide dismutase and pancreatic carboxypeptidase. Lactate dehydrogenase and alkaline phosphatase are two of the biochemical parameters that are analysed in this study.

#### Iron (Fe)

The d-orbital of Fe is not completely filled or half-filled as in the case of Zn (Cotton *et al.*, 1988) and hence more than one oxidation state of Fe is available. These are Fe<sup>2+</sup> and Fe<sup>+3</sup>. These oxidation states involve an exchange of electrons and for this reason Fe would form a part of mostly enzyme systems that involves oxidation and reduction, that is, the oxidoreductases (EC1).

#### Manganese (Mn)

Mn has the stable oxidation of Mn<sup>2+</sup> and therefore should have similar characteristics to that of Mg. However, in the case of Mn the d-orbital is being filled and Mn would also form part of the structures of enzymes. Excess concentrations of manganese interfere with the absorption of dietary iron and long-term exposure to excess levels may result in iron-deficiency anemia. Increased manganese intake impairs the activity of copper metallo-enzymes such as caeruloplasmin (Blaurock-Busch & Griffin, 1996).

#### Calcium (Ca)

Ca is in the same group as Mg and therefore is expected to exhibit similar characteristics. However, in the case of Ca, the valence electrons are further away from the nucleus of Ca than the valence electrons of Mg from the nucleus of Mg. Thus bonding between Ca<sup>2+</sup> and enzymes would be weaker than the bonding between Mg<sup>2+</sup> and enzymes. Hence Ca would be less suitable for bonding in transferases than Mg but more suitable for bonding in hydrolases.

#### Cobalt (Co)

The cobalt ion (Co<sup>4+</sup>) is stable. Therefore, Co should form part of transferases and hydrolases.

#### Molybdenum (Mo)

Mo ionization is governed, as in the case of Fe, by the change in the oxidation states of 4+ and 6+. Mo therefore, forms part in reactions where oxidation and reduction are prominent.

However, the ion should be present in the oxidoreductases (EC1). To an extent, another element, tungsten, W, is also highly correlated with the activity of the oxidoreductases.

Copper (Cu)

The Cu ion exhibits the same characteristics of Fe, but has a higher redox potential. As a result, Cu should be more prominent in EC1 than Fe or Mo. Cu is therefore exclusively incorporated into the structure of EC1. Some of these reactions are given in figure 2. Furthermore, variation in Cu concentration would not affect the activities of enzyme classes EC3 to EC6 and minimally that of EC2. To a lesser extent, this is also the case with Mo. In addition, the correlation of the Fe concentration to all the classes of enzyme corresponds to the correlation of Mo with all the classes of enzymes. In a similar manner, Mg and Mn correlations correspond with respect to the classes of enzymes (Andreini, 2008).

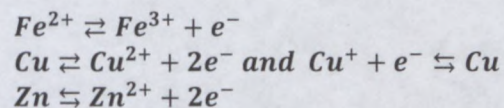


Figure 1.2 Reactions of metal ions of Fe, Cu and Zn.

#### 1.4 Biochemical parameters: measured and calculated

The clinical parameters of interest are function tests which are normally used to assess the health of an organ. These functions tests have been grouped to specifically indicate whether enzymes in an organ such as the heart (always called cardiac enzymes), the liver and the kidney are functioning properly. The physiological role of the heart, the liver and the kidney has been reported in detail by various authors (Coven *et al.*, 2003; Mader, 2004; Silverthorn *et al.*, 2007). The functional condition of the organ is assessed based on the abnormality in the results of these tests.

##### 1.4.1 Measured biochemical parameters

Total protein:

Measurement of total protein is used in the diagnosis and treatment of a variety of abnormalities in the liver, kidney or bone marrow, as well as other metabolic and nutritional disorders such as malnutrition. The measurement is an indication of the concentration of the two principle groups of proteins in the blood: albumin and globulin (Lo *et al.*, 2013).

Albumin:

Measurements of albumin are used in the diagnosis and treatment of numerous diseases involving the liver or kidney. Albumin constitutes more than half the total protein present in serum. Approximately 35% of the total albumin is found in the intravascular compartment.

The remainder is found in the extravascular compartments; the interstitial spaces, mainly of the muscles and skin. Albumin does not diffuse freely through intact vascular endothelium and is therefore the major protein providing the critical colloid osmotic (oncotic) pressure (COP). The passage of water and diffusible solutes through the capillaries is dependent on the COP. Albumin accounts for 70% of the colloid osmotic pressure and therefore exerts a greater osmosis than most of the molecules dissolved in the plasma (for this reason it cannot be completely replaced by inert substances such as dextran). Albumin has a negative charge ( $9^-$ ) at normal blood pH and attracts and retains cations, especially  $\text{Na}^+$  and to an extent  $\text{K}^+$  in the vascular compartment (Sadava *et al.*, 2011). This condition is referred to as the Gibbs–Donnan effect. Chloride ( $\text{Cl}^-$ ) ions are also bind to albumin and its negative charge increases and so ability to retain  $\text{Na}^+$  ions inside the capillaries. This enhanced osmosis causes the COP to be 50% greater than it would be by protein concentration alone.

Albumin functions in the transport of bilirubin, hormones, metals, vitamins and drugs. It plays an important role in lipid metabolism by binding fatty acids and so rendering these acids soluble in the plasma. This is one reason why hyperlipidemia occurs in clinical situations of hypoalbuminaemia. The binding of hormones by albumin regulates the amount of free hormone available at any time. Because of its negative charge, albumin is also able to furnish some of the anions needed to balance the cations of the plasma. This is the main protein made by the liver and it circulates in the bloodstream. The ability to make albumin (and other proteins) is affected in some types of liver disorders. A low level of blood albumin occurs in some liver disorders. It helps keep the blood from leaking out of blood vessels (Lo *et al.*, 2013).

#### Total bilirubin:

Measurements of the levels of bilirubin are used in the diagnosis and treatment of liver, hemolytic hematological and metabolic disorders, including hepatitis and gall bladder block. It gives bile its yellow/green colour. A high level of bilirubin in the blood give rise to a condition called jaundice ('yellow'). Bilirubin is made from haemoglobin after 120 days when red blood cell is broken down. Haemoglobin is found in red blood cells and is released when the red blood cells break down. Liver cells take in bilirubin and attach sugar molecules to it. This is then called 'conjugated' bilirubin which is passed into the bile ducts (Lo *et al.*, 2004).

#### Lactate dehydrogenase (LDH):

Elevated levels of LDH and changes in the ratio of the LDH isoenzymes usually indicate some type of tissue damage. LDH levels typically will rise as the cellular destruction begins,

peak after some time period and then begin to fall. For example, when a person has a heart attack, blood levels of total LDH will raise within 24 to 48 hours, peak in 2 to 3 days and return to normal in 10 to 14 days. LDH levels are elevated in a wide variety of conditions reflecting its widespread tissue distribution. Low and normal levels of LDH do not usually indicate a problem. Low levels are sometimes seen when a person ingests large amounts of ascorbic acid (vitamin C) (Hanukoglu, 1992).

Blood urea (nitrogen):

Urea is formed in the liver by the cyclic process called the urea cycle or the ornithine cycle (Sadava *et al.*, 2011). The amino acids that are not needed in the body are deaminated by enzyme oxidase, producing ammonia ( $\text{NH}_3$ ). The ammonia formed is converted to urea. The urea is distributed throughout the entire body water. Some of it is recycled through the enterohepatic circulation. Urea is also lost through the gastrointestinal tract, lungs, and skin. However, the bulk of the urea concentration is excreted by the kidney and starts with glomerular filtration. At high flow rates, about 40% of the filtered urea load is reabsorbed and at low flow rates, about 60% is absorbed. Hence, the blood urea reveals important information on how well kidneys and liver are working. If a blood urea nitrogen test reveals that urea nitrogen levels are higher than normal, it probably indicates that kidneys are not working properly. Or it could point to high protein intake, inadequate fluid intake or poor circulation. If a blood urea nitrogen test shows lower than normal levels, it could indicate liver disease or damage, or malnutrition. But low blood urea nitrogen (BUN) level would not likely be the first indication of liver disease because the blood urea nitrogen test is not used as a screening test for that disorder (Sadava *et al.*, 2011).

Uric acid:

Uric acid is the byproduct of protein digestion that occurs in the liver and is normally removed from the blood stream and excreted by the kidneys (Mader, 2004). In the case of excess of uric acid deposited in the joints in crystal form, a painful arthritic condition known as gout will be created. The production of uric acid can however be controlled by the amount of protein that has been metabolized by the body. The body's alkalinity and urine pH is a component in the quantity of uric acid produced (Sadava *et al.*, 2011).

Total cholesterol:

Cholesterol is an essential structural component of animal cell membranes that is required to establish proper membrane permeability and fluidity. Cholesterol is required to build and maintain membranes where it modulates membrane fluidity over the range of physiological temperatures (Sadava *et al.*, 2011). It is the hydroxyl group of the cholesterol molecule that interacts with the polar head groups ( $\text{PO}_4^{3-}$ ) of the membrane phospholipids and sphingolipids. Through the interaction with the phospholipid fatty-acid chains, cholesterol increases membrane packing, which reduces membrane fluidity (Ohvo-Rekilä *et al.*, 2002). A high concentration of cholesterol can cause the internal diameters of blood vessels causing a constriction. The result is that blood flow is hampered and can lead to cardiac complications.

Alanine aminotransferase:

Alanine aminotransferase, sometimes termed alanine transaminase, is classed under EC2. Abnormality in the concentration of ALT has been used in the diagnosis of certain liver diseases (for example, viral hepatitis and cirrhosis) and heart diseases. This is an enzyme that helps to process proteins. Large amounts of ALT occur in liver cells. When the liver is injured or inflamed (as in hepatitis), the blood level of ALT usually rises (Ghouri *et al.*, 2010).

Aspartate aminotransferase:

Aspartate aminotransferase, also termed aspartate transaminase, abnormalities are used in the diagnosis of certain types of liver and heart disease. This is an enzyme usually found inside liver cells. When a blood test detects high levels of this enzyme in the blood, it usually means the liver is injured in some way. However, AST can also be released if heart or skeletal muscle is damaged. For this reason ALT is usually considered to be more specifically related to liver problems (Ghouri *et al.*, 2010).

Creatine phosphokinase & Creatinine:

Creatine phosphokinase, also known as creatine kinase (CK) or phospho-creatine kinase (PCrK), and sometimes incorrectly as creatinine kinase, is an enzyme of the transferase class (EC2). The enzyme is expressed by various tissues and cell types. Creatine phosphokinase catalyses the conversion of creatine and consumes adenosine triphosphate (ATP) to create phosphocreatine (PCr) and adenosine diphosphate (ADP). This CPK enzyme reaction is reversible, so that ATP can also be generated from PCr and ADP. Elevation of CPK is an indication of damage to muscle. It is therefore indicative of injury to or onset of rhabdomyolysis, myocardial infarction, myositis and myocarditis. (Ghouri *et al.*, 2010)

Creatinine is a non-protein end product of the metabolism of creatine, a compound involved in supplying energy for muscle contraction. Creatinine appears in serum in amounts proportional to the body's muscle mass. Creatinine is easily excreted by the kidneys, with its serum level directly related to the glomerular filtration rate. Since serum creatinine levels normally remain constant, elevated levels usually indicate diminished renal function (Sadava *et al.*, 2011). Creatinine determinations are useful in the diagnosis and treatment of acute and chronic renal disease as well as monitoring renal dialysis. Creatinine is a break-down product of creatine phosphate in muscle and is usually produced at a fairly constant rate by the body (depending on muscle mass).

#### 1.4.2 Calculated biochemical parameters

Globulin:

The globulin fraction includes hundreds of serum proteins including carrier proteins, enzymes, complement and immunoglobulins. Most of these are synthesized in the liver, although the immunoglobulins are synthesized by plasma cells (Sadava *et al.*, 2011). Globulins are divided into four groups by electrophoresis. The four fractions are  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$  and  $\gamma$ , depending on their migratory pattern between the anode and the cathode. Increases in the globulin fraction usually result from an increase in immunoglobulins, since albumin and globulin are the two principle proteins; the concentration of GLB is calculated by the difference in total protein and albumin, that is:

$$\text{Globulin} = \text{total protein} - \text{albumin}$$

Albumin to globulin ratio:

The albumin/globulin ratio is the calculation of serum albumin compare to serum globulin level to determine whether there is an overproduction or underproduction of gamma-globulin. A low A/G ratio may be due to overproduction of gamma-globulin (monoclonal/polyclonal gammopathy, multiple myeloma or autoimmune diseases) (Sadava *et al.*, 2011) or due to low albumin (low production as in cirrhosis or excessive loss as in nephrotic syndrome or protein losing enteropathy etc.). In contrast, if the A/G ratio is high then one should look for diseases with low gamma-globulin production such as agammaglobulinaemia. To obtain this information, a serum electrophoresis or immunoelectrophoresis should be applied and a more specific abnormality can be determined.

BUN to creatinine ratio:

Both urea and creatinine are nitrogenous end products of metabolism. Urea is however the primary metabolite derived from dietary protein and tissue protein turnover. Creatinine, on

the other hand, is the product of muscle creatine catabolism. Both these molecules are relatively small and are distributed throughout total body water (Wittig *et al.*, 2006). The principle behind this ratio is the fact that both urea (BUN) and creatinine are freely filtered by the glomerulus in the kidney; however urea reabsorbed by the tubules can be regulated (increased or decreased) whereas creatinine reabsorption remains the same (minimal reabsorption). Wittig *et al.* (2006) suggested that a ratio of (1) greater than 20:1 occurs prerenally and is indicative of an increase in BUN absorption and BUN is thus disproportionately elevated relative to creatinine in the serum; (2) between 10:1 and 20:1 occurs postrenally and is the normal range but does not exclude postrenal diseases, though the BUN reabsorption may be within normal limits and (3) less than 10:1 n (Wittig *et al.*, 2006).

#### **1.4.3 Reference ranges for biochemical parameters**

In the majority of laboratory measurements, the combination of short term physiologic variation and analytic error is sufficient to render the interpretation of single determination difficult when the concentrations are in the borderline range (Wallach, 1996).

The cause of elevated enzymes in animals after laboratory habituation is unclear. For this reason Weber *et al.* (2002) specified that biochemical values, as cited in Table 1.2, provide valuable baseline information for use in further medical studies. When results are analyzed in the context of normal values, laboratory studies to evaluate infected individual rats are more informative (Weber *et al.*, 2002). It is important that healthy animals are used when establishing "normal values"; since it would be difficult to determine the health of experimental animals.

While comparisons between the experimental groups are preliminary, the results suggest a testable hypothesis that capture stress can induce. Such changes might be resolved after careful laboratory habituation (Weber *et al.*, 2002) (see table 1.2). These data allow for future comparison with other species and for better assessment of the impact of laboratory manipulations, including experimentally induced infectious diseases, on individual dusky-footed wood rats. All of the animals tested in this study appeared healthy; were normally hydrated; and, were in good body condition.

It is recognized that different analytical methods as well as environmental and technique related variables influence the values obtained for a particular parameter (Lang, 1993). Giknis & Clifford (2008) have compiled clinical laboratory data from approximately 30 studies

conducted in the control group. The data included are for information purposes only. According to the study done by Hall *et al.* (1997) on control values for clinical pathology data, there is a general agreement among clinical pathologists that concurrent control data for a parameter in question are best for comparison and determining a potential test article potential finding.

**Table 1.2: Summary of serum biochemical parameters for wild-caught *Neotoma fuscipes* and all animals combined (Weber *et al.*, 2002).**

Serum chemistry Parameter wild-caught <i>Neotoma fuscipes</i> and all animals combined	Range of Values as measured	Range of Values after conversion
Creatinine	0-4(mg/L)	0-4 (mg/L)
Blood Urea Nitrogen	100-320(mg/L)	100-320 (mg/L)
Total Protein	57-80(g/L)	57-80(g/L)
Albumin	12-42(g/L)	12-42 (g/L)
Alanine Transaminase	1-22(IU/l)	1-22 (IU/l)
Aspartate aminotransferase	55-329(IU/l)	55-329 (IU/l)
Alkaline Phosphatase	46-245(IU/l)	46-245 (IU/l)
Bilirubin Total	0-4(mg/dl)	5.1-17( $\mu$ mol/L)
Cholesterol	900-2270(mg/dl)	3.9-5.2 (mmol/L)

In fact, the joint scientific committee for International Harmonization of Clinical Pathology testing has emphasized that “The concurrent control data are more appropriate than historical references ranges for comparison with test material groups” (Kiehlbauch *et al.*, 2000). (see table 1.3 (Giknis & Clifford, 2008)).

**Table 1.3: Serum chemistry reference data for 8-16 weeks old male Wistar rats (Giknis & Clifford, 2008)**

Test	Range measured (8-16weeks old male wistar rats)	Range converted (8-16weeks old male wistar rats)
Total Protein(g/L)	52-71	52-71 (g/L)
Creatinine (mg/L)	2-5	177- 442( $\mu$ mol/L)
Bilirubin Total ( $\mu$ mol/L)	5.1-17	5.1-17( $\mu$ mol/L)
Alkaline Phosphatase (IU/l)	62-230	62-230 (IU/l)
Aspartate aminotransferase (IU/l)	74-143	74-143 (IU/l)
Alanine Transaminase(IU/l)	18-45	18-45 (IU/l)
Albumin(g/L)	34-48	34-48 (g/L)
Lactate dehydrogenase ( $\mu$ mol/L)	122-222	122-222 IU/l
Creatine phosphokinase (IU/l)	60-400	60-400 IU/L
Uric acid (mg/dL)	3.0-7.0	178-416 ( $\mu$ mol/L)

Historical control data can help to explain the severity of a particular finding and put that finding into perspective (Clemon, 1997). After clinical measurement, it was found that controls values were in an acceptable range when compared to the value obtained by other authors. Normal results vary, based on the laboratory and method used. These values are subjectively averaged from a variety of sources. There is a great range of values reported. This may be accounted for by variation in age, sex, breed or sampling technique and testing methodology. As such, the range limits are not firm boundaries and should be used as guidelines (see table 1.4 (Kiehlbauch *et al.*, 2000), .

**Table 1.4: Serum chemistry reference data for 8-16 week-old males Wistar rats (Kiehlbauch *et al.*, 2000).**

Parameters	Value ranges of 8-16 week-old males wistar rats measured and converted	
Albumin	3.8-4.8 g/dl (x10)	38-48 g/L
ALT	<49 IU/L	<49IU/L
AST	< 46 IU/L	< 46 IU/L
BUN	10-21 mg/dl (x 0.357)	3.57-7.5 mmol/L
Total Protein	5.6-7.6 g/dl (x 10)	56-76 g/L
Total Bilirubin	0.2-0.5 mg/dl (x17.1)	3.42-8.55umol/L
Total Cholesterol	40-130 mg/dl (x0.0259)	1.036-3.367mmol/L

In the previous sections, trace elements and clinical parameters were discussed. The primary aim of this work is to evaluate the effect of antioxidant teas on the absorption of these elements and clinical parameters.

## 1.5 Teas

### 1.5.1 Rooibos

#### 1.5.1.1 Origin and types

More than 300 years ago, the Khoi-San people, indigenous inhabitants, in the Cederberg mountainous regions of the Western Cape Province in South Africa were the first to collect wild rooibos extracts (*English: red bush*) and used it to make a tea.

They discovered that they could brew a sweet, tasty liquid from rooibos tea leaves and stems that were first cut, bruised with wooden hammers, wetted fermented in heaps, and finally sun-dried (Erickson, 2003). Botanists first recorded rooibos plants in 1772 when they were introduced to rooibos tea by the Khoi people for scientific classification (Erickson, 2003). The fermented type is called red rooibos tea because fermentation of the leaves and stems

Kingdom: *Plantae*

Division: Magnoliophyta  
Class: Magnoliopsida  
Order: *Fabaces*  
Family: *Fabaceae*  
Genus: *Aspalathus*  
Species: *Aspalathus linearis*  
Binomial name: *Aspalathus linearis*

containing polyphenolic compounds are oxidized rendering the product red. This distinctive colour led to the Afrikaans name “rooibos” (Joubert *et al.*, 2008). The unfermented type, often called “green” rooibos because of the coloration as it doesn’t undergo oxidation or fermentation. Unfermented rooibos tea contains higher levels of polyphenolic antioxidants whereas fermented rooibos loses some antioxidants during the fermentation process (Bramati *et al.*, 2002). The unfermented type was developed into products to maximize antioxidant levels in response to recent interest in the health benefits associated with the antioxidants found in *Camellia sinensis* beverages. Unfermented rooibos tea is tan/yellow in colour rather than the rich reddish color of fermented rooibos tea (Erickson, 2003).

#### 1.5.1.2 Rooibos and antioxidants

The rooibos plant is recognized as one of the relatively few commercial plants that has made the transition from a local wild resource to a cultivated crop in the 20<sup>th</sup> century (Bramati *et al.*, 2002).



Figure 1.3: Photographs of unfermented (a) and red or fermented (b) rooibos (Gadow *et al.*, 1997)

Its leaves and fine stems are used for the production of rooibos: the leaves and stems are cut to 3-4 mm lengths, rolled, fermented by leaf enzymes, and dried in the sun (Bramati *et al.*, 2002). Some of the active components of medicinal products are polyphenolic

compounds (Moure *et al.*, 2001). Phenolic compounds are simply classified as phenols or polyphenols based on the number of phenol units in the molecule. Phenols, in organic chemistry, sometimes called phenolics, are a class of chemical compounds consisting of a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon group. The simplest of the class is phenol also called as carbolic acid  $C_6H_5OH$ . The best and largest studied natural phenols are the flavonoids, which include several thousand compounds, among them the flavonols, flavones, flavan-3ol (*catechins*), flavonones, anthocyanidins and isoflavonoids. It has been proved that both fermented and unfermented rooibos exhibits antimutagenic properties (Erickson, 2003), since flavones that possess antimutagenic activity (Nakasugi & Komai, 1998), flavanones and xanthenes, that exhibit antiviral, antimicrobial and anti-inflammatory activities (Baba *et al.*, 2009) and isoflavones and coumestans that present important physiological effects in humans, have antioxidant action (Moure *et al.*, 2001). The polyphenol antioxidants found in rooibos include the monomeric flavonoids aspalathin, nothofagin, quercetin, rutin, isoquercitrin, orientin, isoorientin, luteolin, vitexin, isovitexin and chrysoeriol (Joubert, 1996; Gadow *et al.*, 1997; Krafczyk *et al.*, 2009).

Currently, rooibos tea is the only known natural source of aspalathin. It also contains phenolic acids with antioxidant activity. The phenolic acids identified in rooibos tea, include caffeic acid, protocatechuic acid, syringic acid, ferulic acid, vanillic acid, *p*-hydroxybenzoic acid, and *p*-coumaric acid. Caffeic acid is an antioxidant as potent as the flavonoids quercetin, isoquercitrin, and aspalathin. The total polyphenol content of an average 150 to 200 ml serving of rooibos tea infusion can be as much as 60 to 80 mg, depending on factors such as the brewing time and amount of leaves used (Erickson, 2003).

With its many positive attributes, rooibos tea is a choice of drink for many health conscious people. It contains no colours, additives or preservatives, making it a natural beverage, rich in volatile compounds polyphenols, and minerals (Bramati *et al.*, 2002). Different from *Camellia sinensis*, rooibos tea is a caffeine-free and has a low tannin content (as gallic acid) (Marnewick *et al.*, 2003) and also antimutagenic (Marnewick *et al.*, 2000). Because of these characteristics, rooibos tea extracts is significantly gaining in popularity as a healthy beverage (Bramati *et al.*, 2002).

### **1.5.1.3 Trace elements and rooibos tea**

Trace elements are fundamental metal and mineral substances, essential to the biochemical processes of metabolism (krebs cycle and respiratory chain). Without this harmonious correlation, interaction between enzymes and vitamins in the body would not be possible.

Until a few decades ago, knowledge of trace elements in food and tissues and their effects on health was only rudimentary (Davidson *et al.*, 1979).

There are a number of elements essential to the enzyme functions in the rats. These include Fe, Se, Cu, Zn, Ni, and Cr. Since these elements are present in amounts in the parts per million ranges (ppm), they are referred to as trace elements. These elements play a most important role in a variety of metabolic pathways and have been studied in many diseases including auto-immune, neurological, and psychiatric disorders (Yanik *et al.*, 2004). Accurate assessment of trace elements concentrations in organs continues to be refined.

Nonetheless, the necessity and the function of certain elements continue to be debated because dietary deficiency models in animals sometimes suggest that they are essential, yet their specific functions at the molecular level have not been established. Moreover, the role of minerals in enzyme functions has been studied extensively in nutrition and biochemistry. For example, magnesium is a cofactor for glucose-6-phosphogluconate dehydrogenase, two pentose-cycle enzymes catalyzing the production of NADPH from NADP<sup>+</sup> (Lippard & Berg, 1994; Chaudiere & Ferrari-Illiou, 1999).

It has been reported that the insufficiency of dietary magnesium reduces glutathione reductase activity which results in radical-induced protein oxidation (indicated by the generation of protein carbonyl) and marked lesions in tissues such as skeletal muscle, brain, and kidney (Rock *et al.*, 1995; Fang *et al.*, 2002).

It is now accepted knowledge that the micro elements Ca, Mg, P and S are important compounds of bones and other supporting tissues. Sulphur forms part of the vitamins biotin and thiamine. However, once bound to proteins, it stabilizes their structures as found in the hormone insulin (Rolfes *et al.*, 2006).

Fe, the most abundant trace element in the body, forms part of haemoglobin and myoglobin, which carries oxygen in the blood and helps release energy while others trace minerals such as Zn, Cu, and Mn are essential for a healthy immune system and are indispensable metals for the activities of Cu-Zn-SOD and Mn-SOD, respectively (Aruoma, 1998; Fang *et al.*, 2002). These minerals form an integral part of the body's antioxidant enzyme systems and act as cofactors to a number of enzymes (Fang *et al.*, 2002). They play a role in growth, healing and formation of protein (Rolfes *et al.*, 2006). Therefore, dietary deficiencies of these minerals markedly decrease tissue Cu-Zn-SOD and Mn-SOD activities and result in

peroxidative damage and mitochondrial dysfunction (Aruoma, 1998; Fang *et al.*, 2002) and the excess in Cu, Mn, Zn, respectively, lead to an hyperactivity, an increase in blood pressure and an impaired in coordination. Cl, Na and K maintain normal fluid and electrolyte balance while Na and K also assist in nerve impulse transmission and muscle contraction (Rolfes *et al.*, 2006).

There is a rich and significant history of selenium nutrition and biochemistry. Since the discovery of glutathione peroxidase as a selenium-dependant enzyme in 1973 (Chaudiere & Ferrari-Illiou, 1999), selenium has been identified as an essential cofactor for selenoprotein P (is the most common selenoprotein found in the plasma) and other selenoproteins (Aruoma, 1998; Fang *et al.*, 2002). A dietary deficiency of selenium noticeably decreases tissue glutathione peroxidase activity by 90% and results in peroxidative damage and mitochondrial dysfunction (Xia *et al.*, 1985; Fang *et al.*, 2002). The essential role of selenium in the removal of free radicals and the maintenance of normal human health is epitomized by the etiology of Keshen disease in China (Yang *et al.*, 1983; Fang *et al.*, 2002). This disease was found by Chinese scientists to result from a severe deficiency of dietary selenium owing to a deficiency of soil selenium (Yang *et al.*, 1983; Fang *et al.*, 2002).

While it is easy to recognize severe deficiencies of some minerals, it is relatively more difficult to detect it for other minerals and these may easily be overlooked (Rolfes *et al.*, 2006). However, it is not always appreciated that the beneficial effects of minerals are confined to tolerable upper intake levels (Nookabkaew *et al.*, 2006) and can be toxic at concentrations beyond those necessary for biological functioning (Fraga, 2005). Moreover, minerals that were previously thought to be non-toxic, such as Pb and Al, have been shown to cause irreparable brain damage in infants and aged, respectively (Campbell, 2001; Hayacibara *et al.*, 2004; Bradberry & Vale, 2007; Mehra & Baker, 2007; Kadir *et al.*, 2008). Even a commonly consumed mineral such as sodium (mainly in the form of sodium chloride) has been correlated with hypertension and may be harmful if taken in excessive amounts (Karppanen & Mervaala, 2006). Both the type and the amount of minerals consumed play an important role in the health of an individual.

Some studies showed that main factors influencing the metal content are horticulture conditions and plant species. Drinking rooibos tea infusions could be a source of mineral substances (Manteiga *et al.*, 1997; Gramza *et al.*, 2005).

A study was carried out by Hesseling *et al.* (1979) to determine if rooibos has a deleterious effect on iron absorption similar to that of ordinary tea (*Camellia sinensis*). Hesseling *et al.* (1979) proved that rooibos tea did not affect iron absorption significantly. Rooibos tea contains high levels of 37 known substances which act to limit the effects of superoxide molecules or oxygen free radicals (Hesseling *et al.*, 1979). Numerous other pharmacological and medicinal benefits, such as its anti-carcinogenic and antimutagenic effects, have been attributed to rooibos tea (Moure *et al.*, 2001). By their antioxidant functions of protecting organisms against free radical damage, essential trace elements such as (Cu, Zn, Mn and Se) and some vitamins (A, C, E and beta-carotene) have been considered as useful in the prevention of disease initiated or promoted by oxygen radical (Hercberg *et al.*, 1998).

Taking the above into account, it is important to determine the mineral compositions of foods and related products in order to understand their nutritive importance.

### 1.5.2 Green tea

The tea plant, *Camellia sinensis*, is a member of the Theaceae family, and black, oolong, and green tea are produced from its leaves (Mukhtar & Ahmad, 2000). Unlike black and oolong tea, green tea production does not involve oxidation of young tea leaves. Green tea is produced from steaming fresh leaves at high temperatures; thereby inactivating the oxidizing enzymes and leaving the polyphenol content intact (Nkubana *et al.*, 2008).

Jankun *et al.* (1997), using molecular modeling, and subsequently demonstrated that one of the major ingredients of green tea, epigallocatechin-3-gallate, inhibits urokinase, an enzyme crucial for cancer growth found in large amounts in human cancers.

Epigallocatechin gallate (EGCG), also known as epigallocatechin 3-gallate, is the ester of epigallocatechin (EGC) and gallic acid (GA) and is a type of catechin most abundant in green tea and is a potent antioxidant that may have therapeutic applications in the treatment of many disorders (for example cancer) (Singh *et al.*, 2003).

Green tea is consumed primarily in China, Japan, and a few countries in North Africa and the Middle East. Fresh tea leaf is unusually rich in the flavanol group of polyphenols known as catechins which may constitute up to 30% of the dry leaf weight (Graham, 1992). Other polyphenols include flavonols and their glycosides and depsides such as chlorogenic acid, coumarylquinic acid and one unique to tea, theogallin (3-galloylquinic acid). Caffeine is present at an average level of 3% along with very small amounts of the other common methylxanthines, theobromine and theophylline (Graham, 1992). The amino acid theanine

(5-Nethylglutamine) is also unique to tea. Tea accumulates aluminum and manganese. There is no tannic acid in tea (Graham, 1992). The composition of green tea is very similar to that of the fresh leaf except for a few enzymatically catalyzed changes which occur extremely rapidly following plucking. New volatile substances are produced during the drying stage.

### **1.6 Importance of rooibos and green teas in this study**

The reason why these teas were used in this study is firstly because polyphenols in rooibos tea and green tea are potent free radical scavengers due to the hydroxyl groups in their chemical structure (Gramza *et al.*, 2005). Many chronic disease states and inflammatory conditions are a result of oxidative stress and subsequent generation of free radicals (Gutteridge & Halliwell, 1989). Some of these include heart disease (resulting from LDL oxidation), renal disease and failure, several types of cancer, skin exposure damage caused by ultraviolet (A and B) rays, as well as diseases associated with aging. The hydroxyl groups form complexes with free radicals and neutralize them, preventing the progression of the disease process. Secondly, it has been found in epidemiological studies that the consumption of green tea such as in the composition of rooibos tea may help prevent cancer. In addition, breast and prostate cancers are reduced in animal models by green tea. Both tea polyphenols have demonstrated significant antioxidant, anticarcinogenic, anti-inflammatory, thermogenic, probiotic, and antimicrobial properties in numerous animal and *in vitro* studies (Graham, 1992; Alschuler, 1998). Finally, rooibos tea and green tea are some of the most widely consumed beverages in the world and their medicinal properties have been widely explored.

### **1.7 Aims of this study and scope of investigation**

#### **1.7.1 Aims of this study**

Rooibos tea contains a diversity of substances possessing the functional groups required to act as antioxidants such as scavengers of active oxygen species which adversely affect human health. The effects of rooibos tea are generally associated with the effect of its flavonoids, since they are considered as antioxidants. In addition, due to the involvement of reactive oxygen species, epidemiological studies have shown that long-term consumption of a diet rich in antioxidants can protect against many diseases such as cardiovascular disease (Hertog *et al.*, 1993; Hertog *et al.*, 1995; Ness & Powles, 1997; Devasagayam *et al.*, 2004; Carlson *et al.*, 2008; Panti *et al.*, 2011).

Studies have been focused on the possible role of free radical suppression of nutrients and free radical scavenging by explaining the beneficial effect of diet compounds. Since one

explanation of numerous beneficial protection of animal health in the prevention of degenerative diseases, particularly cardiovascular diseases and cancers are the antioxidative effects of plant-derived flavonoids (Scalbert *et al.*, 2005).

The aim of this study was to investigate the effects of consumption of an antioxidant rich beverage, rooibos tea, by male Wistar rats on biochemical parameters and trace elements absorption. In other words, this study aims to evaluate, using statistics, whether feeding male Wistar rats with antioxidants and trace elements contained in rooibos tea will have an effect on the absorption of trace elements and biochemical biomarkers.

### **1.7.2 Scope of Investigation**

In the introductory chapter the fundamental nature of trace elements and antioxidants as to the definition, the two broad categories and examples that are commonly known, are discussed. As rooibos tea is the antioxidant on which the comparative study is concentrate, its origin, its properties and the applications that have been made until present, have been elaborated. Although the whole body can be investigated to ascertain the effect(s) of rooibos tea, the blood of the experimental animals was chosen for evaluation.

Elements pertaining to the organs and enzymes indicative of the health state of the body form the basic part of this study. The techniques of analysis, namely X-ray emission (PIXE), ICP-OES & Backscattering analysis and the standard automated machine for clinical biomarkers that were used in this investigation are discussed under the Materials and Methods section.

## CHAPTER TWO

### MATERIALS AND METHODS

#### 2.1 Materials and methods of analysis

##### 2.1.1 Experimental animals

Ten-week-old male Wistar rats, weighing  $300 \pm 30$  grams, were obtained from the animal husbandry unit of the University of Stellenbosch, Stellenbosch, South Africa. The animals were randomly divided into four treatment groups consisting of 11 animals for the control group, 11 animals for the fermented rooibos group (FR), 9 animals for the unfermented rooibos group (UFR) and 9 animals for the green tea group (GT) as they have been fed with a specific diet for a period of three months according to the dietary supplementation received. They were housed individually in stainless steel wired-bottom cages, fitted with Perspex housed in a close environment, and were maintained under a 12h light/dark cycle with the temperature of 24-25°C and 50% humidity.

**Table 2.1: Design for collection of samples.**

Feeding	Sacrificed animal:	Tests performed
Standard Rat Chow	Blood collection	Centrifugation, PIXE,BS,ICP-OES and Biochemical Analysis
Unfermented rooibos	Blood collection	
Fermented rooibos	Blood collection	
Green Tea	Blood collection	

Group A: received water and standard rats chow;

Group B: were given fermented rooibos extract and standard rats chow;

Group C: received unfermented rooibos extract and standard rats chow; and

Group D: received green tea extract and standard rats chow.

All the diets designed for this study were prepared on a daily basis to prevent it from spoiling. Rats in all experimental groups were given supplements in the morning and after consumption they were allowed ad libitum access to standard rat chow (SRC).

##### 2.1.2 Liquid preparation

Aqueous extracts of fermented and unfermented rooibos were prepared daily by the addition of freshly boiled tap water to a mass of leaves and stem, to yield a concentration of 2g/100ml. The herbal concentrations are customary used for rooibos making purposes in South Africa. The mixture were allowed to stand for 30 min at room temperature, then filtered

through Whatman number 4 filter paper, and after cooling, dispensed into water bottles (Marnewick *et al.*, 2003).

### 2.1.3 Feeding program

The animals in the second group (FR+SRC) and third group (UFR+SRC) had free access to standard rat chow and the various aqueous rooibos tea extracts for a period of 10 weeks. The extracts were the sole source of drinking fluid, whilst the control group (CTRL+SRC) was given ordinary tap water. The administration of the fluid was every 48 hours and feeding was maintained during the period. All the animal groups received intraperitoneal injection of 0.5 ml sodium pentobarbitone progressively at the end of the feeding program and these injections have been made using sterile 1 ml disposable syringes and 26G sterile hypodermic needles according to the method of Kumar & Muralidhara (2007). After the injections, the animals have been sacrificed, hearts were removed for another experimental group and blood samples collected were centrifuged to get serum for the measurement.

**Table 2.2** Approximate energy and macronutrient content (g/100g) of rat diets SFA (g): Saturated fatty acids, MUFA (g): monounsaturated fatty acids. PUFA (g): polyunsaturated fatty acids (Engelbrecht *et al.*, 2006; Kruger *et al.*, 2007).

Nutrients	Standard Rat Chow
Energy (kJ)	272.5
Total nonstructural carbohydrates	8.375
Total protein	4.5
Total fat	0.625
Total SFA	0.139
Total MUFA	0.168
Total PUFA	0.297
Total n-6 PUFA	0.247
Total n-3 PUFA	0.049

The energy and macronutrient content of the rat diets is given in Table 2.2 (Engelbrecht *et al.*, 2006; Kruger *et al.*, 2007). The rat chow was supplied by Atlas Animal Foods, Cape Town, South Africa. The rooibos extract in this study was supplied by the Oxidative Stress Research Centre in the Faculty of Health and Wellness Sciences at Cape Peninsula University of Technology.

#### **2.1.4 Ethical considerations**

All animals received humane care in accordance with the principle of Laboratory Animal Care for the National Society of Medical Research and the Guide for the Care and use of Laboratory Animals of the National Academy of Sciences (National Institutes of Health Publications, no 80-23, revised 1978).

## **2.2 Methods**

### **2.2.1 Trace element analysis**

#### **2.2.1.1 X-ray emission**

According to Johansson (1989), X-rays are emitted when charged particles pass through matter. Each element has a characteristic spectrum and hence the X-rays give information about which elements are present and also their concentrations. The theoretical fundamental physics principles of X-ray emission, which is induced by the bombardment with protons (PIXE) had been illustrated by Johansson & Campbell, (1988). Furthermore, the background determinations and minimum detection limits (MDLs) have been established (Ishii & Morita, 1988; Campbell *et al.*, 1988; Campbell & Goldstein, 2011; Mars, 2004) for various sample matrices. In instances, MDLs of down to 1 ppm were obtained. In addition, applications have been made by Mars *et al.* (2004) and Guambe *et al.* (2011) all attest to the application of PIXE. The Computational software (GeoPIXE) was used for the quantification of X-ray emission data (Ryan *et al.*, 1995). It should be noted that pile-up peaks might occur when two or more X-rays enter the detector window simultaneously. Since the detector is a lithium-drifted silicon, Si (Li) detector, the escape peaks of Si are present in the X-ray data spectrum.

#### **2.2.1.2 Backscattering (BS) analysis**

The fundamental physics principles of backscattering spectrometry have been detailed by Chu *et al.* (1978). Applications for determining minimum detection limits MDLs for various matrices have also been extensively applied (Mars, 2004). Also computational software is presently available (Mayer, 1999). Because of the versatility of the Nuclear Microprobe (NMP) chamber, the PIXE and BS determinations were done simultaneously. The detector used for BS analyses was a solid surface barrier detector. The detector was mounted at an angle of 176° to the incoming particle beam.

The NMP houses both the PIXE and BS instrumentation and is versatile in that specimens can be analyzed with the two techniques simultaneously. However, backscattered particles from the BS analyses can damage the detector window of the detector used to detect X-ray emission data. For this reason and to amplify the X-ray emission data, an absorber (also

termed a filter) is placed in front of the PIXE detector. Filters are absorbing foils normally placed in front of a detector to attenuate the bremsstrahlung background and the dominant low energy X-rays peaks found in biological materials. They are also important in preventing recoiling protons from entering the detector. Filters could be constructed from several materials. Low atomic number filters ( $Z < 10$ ). The filter absorbs completely energies below 1.26 keV (Ryan, 1995; Churms, 1993).

PIXE and BS analytical instrumentation are linked to the Nuclear Microprobe (NMP) facility at Material Research Department (MRD), National Research Foundation (NRF)-iThemba LABS, Somerset West, South Africa. The facility is fundamentally based on a 6 MV single ended Van de Graaff accelerator for generating proton, alpha and other beams. The Oxford Microprobe triplet lenses are used for beam focusing (Churms, 1993; Prozesky, 1995).

### **2.2.1.3 Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)**

Since low MDLs were desired for the trace elements, the PIXE and BS analyses were performed at a beam-energy of 3 MeV. At this energy the X-rays emitted for Mg are absorbed in the window of the detector and cannot be determined with these analytical techniques. Mg was therefore determined by ICP-OES.

ICP optical emission spectroscopy (ICP-OES) is the measurement of the light emitted by the elements in a sample introduced into an ICP source. The measured emission intensities are then compared to the intensities of standards of known concentration to obtain the elemental concentrations in the unknown sample. There are two ways of viewing the light emitted from an ICP. In the classical ICP-OES configuration, the light across the plasma is viewed radially, resulting in the highest upper linear ranges. By viewing the light emitted by the sample looking down the center of the torch or axially, the continuum background from the ICP itself is reduced and the sample path is maximized. Axial viewing provides better detection limits, by as much as a factor of 10, than those obtained via radial viewing. Most PerkinElmer ICP-OES systems use a patented dual-viewing (DV) system, allowing the plasma to be viewed in either orientation in a single analysis by simply moving a mirror, providing the best detection capabilities and widest working ranges of any system (Nölte, 2003).

### **2.2.2 Sample preparation and analysis for PIXE and BS**

The serum density was considered as 1.025 g/mL and that it contained a minimum of 90% m/m water (Sniegowski & Moody, 1979). A volume of 100  $\mu$ L of each of the serum samples in the group was placed in a 2ml pre-weighed specimen tube and freeze-dried in a Vitris Genesis freeze-drier (Millrock Technology, Kingston, USA) at  $-40^{\circ}\text{C}$  for 24 hours. The

specimen and tube was reweighed and the mass difference calculated. The dried specimen was pressed into a pellet of maximum 3 mm diameter and coated with a thin layer of carbon and then analyzed. The elemental concentration was calculated using the PIXE result and the mass difference.

The specimen was bombarded with a 3 MeV beam of protons until a minimum charge of 1  $\mu\text{C}$  has been collected. The current was maintained at 100 pA. For PIXE a Si(Li) detector of 140 keV and a 125 $\mu\text{m}$  Be absorber were used. For BS analysis, a solid-state surface barrier detector was used

### **2.2.3 Sample preparation and analysis for ICP-OES**

All glassware was soaked overnight in chromic acid solution and then rinsed with copious amounts of double distilled water. Of each sample in a group, 50 $\mu\text{L}$  was added to a 50 mL glass beaker. 20 mL of a 5%v/v aqueous solution of  $10\text{HNO}_3:1\text{H}_2\text{O}_2$  was then added and digested until a clear solution was obtained. The prepared sample was cooled to room temperature and diluted to 25 mL. The acids were all AR grade and was sourced locally through B&M Scientific, Cape Town, South Africa. An ICP-OES (GBC Company, Australia) was used for analysis of magnesium. The argon gas flow was 12 L/min and a concentric nebulizer was used. The nebulizer Flow = 0.45 L/min, the RF wattage = 1500, the Pump Flow Rate = 1.50 mL/min. The read delay = 15 seconds. The plasma condition was set to wet and the background correction was set continuously. The wavelength was 285.213 nm.

### **2.2.4 Serum analysis**

Blood samples were taken at the end of the feeding period by cardiac puncture and placed into containers (tubes without anticoagulant) and centrifuged at 4000 rpm for 10 min to obtain serum to be used for analyses. (The 100  $\mu\text{L}$  serum for X-ray emission analysis was taken from this sample.) For serum chemistry analysis, a volume of 100  $\mu\text{L}$  was used. The activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatine phosphate kinase (CPK) as well as the concentrations of albumin, uric acid, blood urea nitrogen, total proteins, total cholesterol, urea, creatinine, total bilirubin and creatinine in serum were all determined by standard automated techniques using reagents strips and the appropriate kits from Arkray, Kyoto, Japan (John *et al*, 1997; EBI, 1998; Yamada, 2011). The reagent strips provided method for the determination of each item in the blood sample. These features make it useful for a wide range of clinical applications from routine diagnostics to bedside or emergency testing and are composed of multi-layered test field containing reagents necessary to generate a color that is quantified by reflectance

spectrophotometry. The layers consist of a sample-retention layer, a layer containing the reagents and a support layer. A fixed amount of serum is placed on the test field of the reagent strip and spread in a uniform fashion across the entire surface of the sample retention and then permeates into the reagent layer where the reaction is initiated.

The chemistry principles, on which the clinical analyses were based, have been widely discussed (Tietz, 1983; Vassault, 1986; Thomas, 1998; Kiehlbauch *et al.*, 2000). These principles are briefly explained for ease of reference.

#### Total Protein test:

Total protein in serum reacts with copper ion at high pH to form a blue-purple color (Zaia *et al.*, 2005). The intensity of the blue-purple color as determined by reflectance spectrometry is proportional to the concentration of total protein in serum.

#### Albumin test:

Albumin in serum is combined with bromocresol green (BCG) in acidic conditions to form a blue green complex (Doumas & Peters, 2009). The intensity of the blue green color as determined by reflectance spectrometry is proportional to the concentration of albumin in serum.

#### Creatinine:

In serum, creatinine reacts with 3,5-dinitrobenzoic acid under alkaline conditions to form a red color (Blass, 1995). The intensity of the red color as determined by reflectance spectrometry is proportional to the concentration of creatinine in serum.

#### Uric acid:

This measurement is specifically oxidized to form allantoin and hydrogen peroxide by the catalytic action of uricase (Busi *et al.*, 2007). The hydrogen peroxide oxidizes and condenses 4-aminoantipyrine (4AAP) and N-Ethyl-N (2-hydroxy-3-sulfopropyl)-m-toluidine sodium salt (TOOS) by the catalytic action of peroxidase to form a reddish purple color. The intensity of the reddish-purple color as determined by reflectance spectrometry is proportional to the concentration of uric acid in serum.

Blood Urea Nitrogen (BUN) test:

In serum, BUN reacts with *o*-phthalaldehyde to produce 1,3- dihydroxyisoindoline (DHI). The carbonium ion of DHI reacts with N-1-naphthyl-N'-diethylethylenediamineoxalic acid under strong acidic conditions (pH 0-0.5) to form a blue purple color (Jung *et al.*, 1975). The rate at which the blue purple color is generated in this detection layer is proportional to the concentration of blood urea nitrogen in serum.

Lactate dehydrogenase (LDH):

LDH, in serum, reacts with lithium L-lactate to produce pyruvic acid and NADH (Smith & Olson, 1974; Zhu *et al.*, 2010). The NADH reduces tetrazolium violet to a purple chromogen by diaphorase. The rate at which the purple colour is generated in this detection layer is proportional to the LDH activity in serum.

Alanine Aminotransferase (ALT):

ALT transfers amino group of L-alanine to alpha-ketoglutaric acid and produces L-glutamic acid and pyruvic acid. The pyruvic acid, in the presence of magnesium ion and thiamine pyroline, is oxidized by the catalytic action of pyruvic acid oxidase, to produce hydrogen peroxide (Szasz, 1976). The hydrogen peroxide oxidizes and condenses 4AAP and DAOS by the catalytic action of peroxide to form a blue color. The rate at which the blue color is generated in this detection layer is proportional to the GPT activity in serum.

Aspartate Aminotransferase (AST):

AST transfers amino group of L-alanine to alpha-ketoglutaric acid and produces L-glutamic acid and oxalacetic acid. The oxalacetic acid generates pyruvic acid by oxalacetic acid decarboxylase. Pyruvic acid, in the presence of magnesium ion and thiamine pyrophosphoric acid, is oxidized by the catalytic action of pyruvate oxidase, to produce hydrogen peroxide (Szasz, 1976). The hydrogen peroxide oxidizes and condenses 4AAP and DAOS by the catalytic action of peroxidase to form a blue color. The rate at which the blue color is generated in this detection layer is proportional to the AST activity in serum.

Total Bilirubin:

Both direct and indirect bilirubin reacts with diazonium salt in the presence of dyphylline and an acidic buffer to form azobilirubin as a final product (Blanckaert *et al.*, 1986). The intensity of the red color as determined by reflectance spectrometry is proportional to the concentration of bilirubin in serum.

#### Creatinine Phosphokinase:

This measurement catalyzes a reaction with creatine phosphate and ADP to produce creatine and ATP. ATP and glucose produce glucose-6-phosphate and ADP in the presence of hexokinase. Glucose-6-phosphate is dehydrogenated by G6PDH with NADPH<sup>+</sup> as a coenzyme to produce 6-phosphogluconic acid and NADPH (Ota *et al.*, 1979; Miclet, 2001). NADPH reduces tetrazolium violet via diaphorase to form a purple color. The rate at which the purple color is generated in this detection layer is proportional to the CPK activity in serum.

#### Total Cholesterol:

Lipoprotein in serum is denatured to free esterified cholesterol by surfactant. The cholesterol ester is then hydrolyzed to cholesterol by the action of cholesterol esterase. This free cholesterol is then specifically oxidized to form cholest-4-en-3-one and hydrogen peroxide by the catalytic action of the cholesterol oxidase. The hydrogen peroxide oxidizes and condenses 4AAP and DAOS by the catalytic action of peroxidase to form a blue color. The intensity of the blue color as determined by reflectance spectrometry is proportional to the concentration of total cholesterol in serum (Allain *et al.*, 1974).

### 2.2.5 Statistical Analysis

GaphPad™ PRISM 5 was used for all statistical evaluations and graphical representations. This study employed the students t-test (McGrath & Meyer, 2006) and the one-way analysis of variance (ANOVA) (Moore & McCabe, 2003) with the Bonferroni multiple comparison test (Benjamini, 2010). Differences were regarded statistically significant if  $p < 0.05$ . One-way ANOVA analyses were predominantly used to assess the reproducibility of measurements when ascertaining optimal measurement. Student's t-test was used to test for significant differences between sets of measurements. Results are given as means with their standard deviations (SD) (mean  $\pm$  SD).

Analysis of variance (often referred to as ANOVA) is a technique for analyzing the way in which the mean of a variable is affected by different types and combinations of factors. One-way analysis of variance is the simplest form. It is an extension of the independent samples t-test (see statistics review) and can be used to compare any number of groups or treatments. This method could be used, for example, in the analysis of the effect of three different diets on total serum cholesterol or in the investigation into the extent to which severity of illness is related to the occurrence of infection.

Analysis of variance gives a single overall test of whether there are differences between groups or treatments. Why is it not appropriate to use independent sample t-tests to test all possible pairs of treatments and to identify differences between treatments? To answer this, it is necessary to look more closely at the meaning of a  $p$  value.

## CHAPTER THREE

### RESULTS AND DISCUSSIONS

#### 3.1 Effect of tea extracts on the elemental absorption (X-ray emission analysis)

The X-rays that emit at energies lower than 1.5 keV are not shown in the spectra of the X-ray data since these are absorbed in the window of the detector and therefore not detected. X-ray peaks of the elements such as K, V, Ni, Cr, Rb and Sr, not discussed in this work, are indicated for completion of the X-ray peak identification. The spectra of the PIXE data of the freeze-dried serum sample from the animals in the control group, fermented rooibos group, unfermented rooibos group and green tea group is shown in figure 3.1.

Although the carbonaceous and nitrogenous matter cannot be determined from these spectra, the concentrations can be deduced from the background of the spectra. There is a slight decrease in the carbonaceous and nitrogenous matter between those of the serum from the animals that were fed with water and those that were fed with the fermented rooibos extract. However, the serum sample obtained from the control animals has a higher phosphorous and sulphurous matter. The carbonaceous and nitrogenous matter in the serum of the control experimental animals is approximately an order of magnitude lower than the carbonaceous and nitrogenous matter of the animals fed with unfermented rooibos extract. There are however a marked decrease in phosphorous and sulphurous matter and trace element content. The serum of the animals fed with green tea extract has a slightly higher carbonaceous and nitrogenous matter than the serum of the animals in the control group. There are however corresponding decreases in the phosphorous and sulphurous matter and a depletion of the trace element content.

With respect to the trace element absorption, there is a marginal increase in the absorption of the elements when comparing the X-ray data of the serum of the control animals and those fed with fermented rooibos extract. The Se and Mo concentrations are half of those present in the control group. Comparing the trace element content of the X-ray data of the serum of the animals in the control group to those fed with unfermented rooibos extract, it is evident that the trace element content is markedly higher which indicate an increase in absorption of the trace element content in the serum of the animals fed with unfermented rooibos extract. Both the Se and Mo contents are significantly lower.

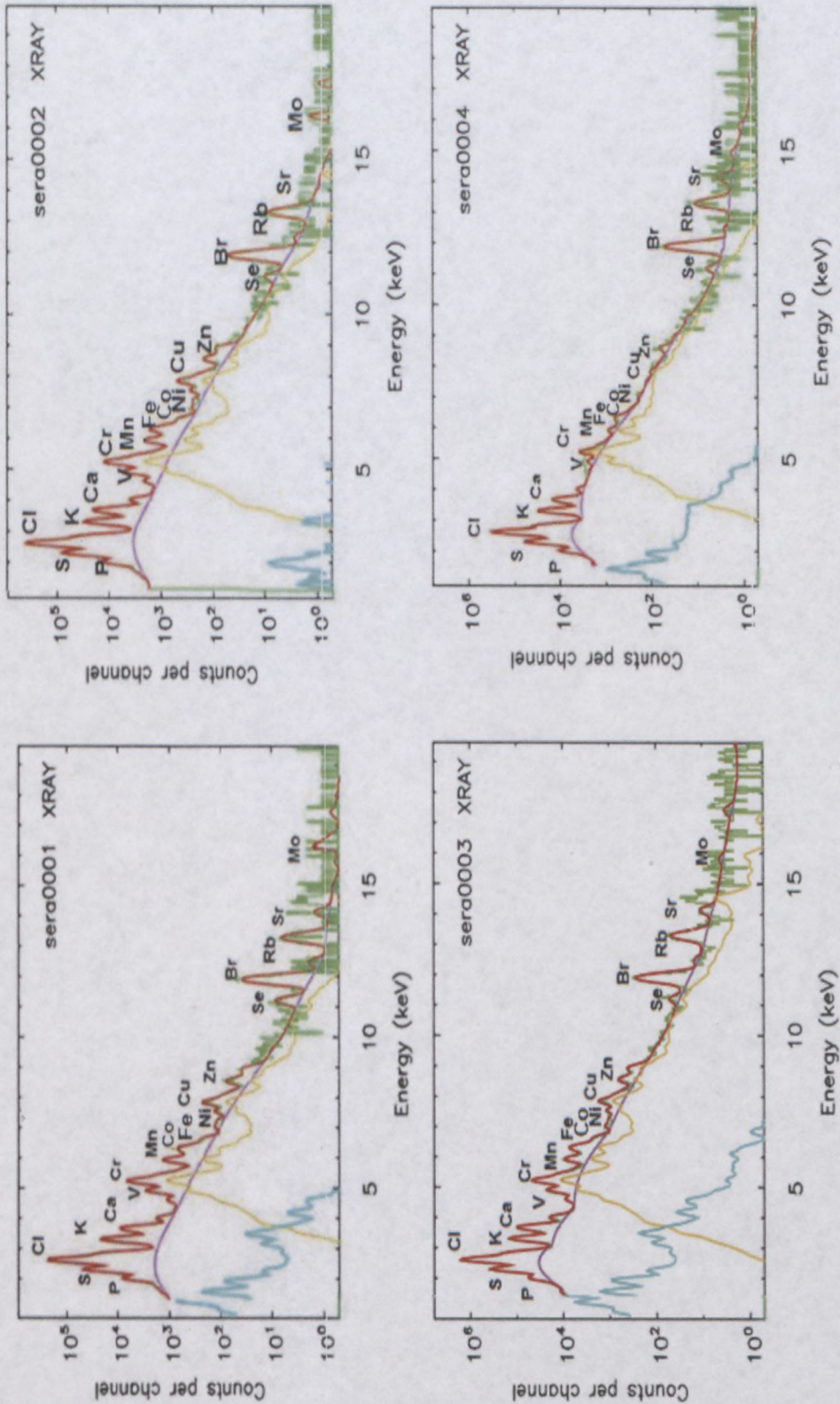


Figure 3.1 Spectra of the X-ray data, as the concentration in counts per channel and the energy in keV, of the freeze-dried serum specimen obtained from the animals in the control group (top left), the animals fed with fermented rooibos extract (top right), animals fed with unfermented rooibos extract (bottom left) and of the animals fed with green tea extract (bottom right). The green line is the X-ray data, the red line, the computational fit to the X-ray data and the purple line is the background. The yellow line is the pile-up from the spectrum and the blue line, the Si-escape peaks. The position of the element symbol coincides with the energy at which the K X-ray line of the element is emitted.

In comparison with the serum of the animals fed with green tea extract, virtually all trace element content was absorbed. The same holds for the Se and Mo content.

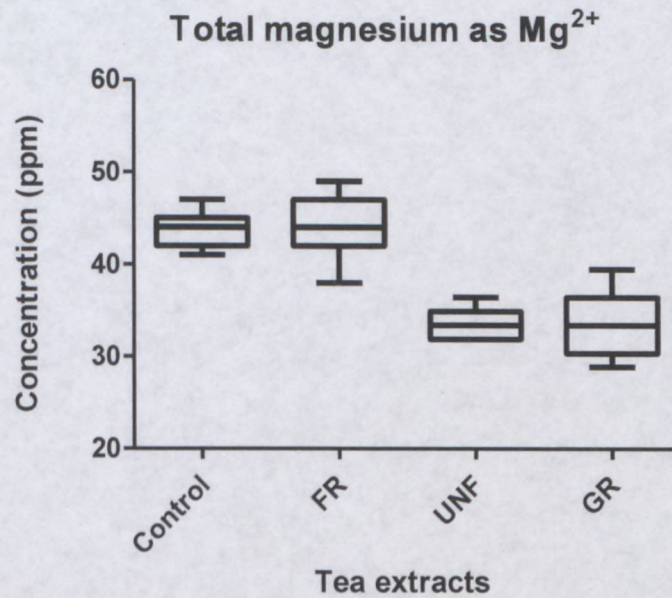


Figure 3.2 Graphic representation of the box and whisker statistical evaluation of the elemental concentration of magnesium as Mg<sup>2+</sup> in the rat sera samples. The maximum, 75th percentile, the average (mean), the 25th percentile and the minimum values are indicated. FR is serum of the rats fed with extract of fermented rooibos, UNF is the serum of the rats fed with the extract of unfermented rooibos and GR is that of the green tea.

The graphic representation of the box and whisker statistical evaluation of the elemental concentration of magnesium as Mg<sup>2+</sup> in the rat serum samples, are shown in figure 3.2. There is no significant statistical difference between magnesium content of the control and the magnesium content of the sera obtained from the rats fed with the extract of fermented rooibos. However, there is a statistical difference between the magnesium content of the serum of the control animals and the magnesium content of the serum of the rats fed the unfermented rooibos extract. Considering the mean concentration, there is an increase of approximately 10 ppm in the absorption. Also, there is a statistical difference between the magnesium content of the control and the magnesium content of the serum of the rats fed with the green tea extract. The increase in absorption is about 10 ppm.

The graphic representation of the box and whisker statistical evaluation of the elemental concentration of zinc as Zn<sup>2+</sup> in the rat serum samples, are shown in figure 3.3.

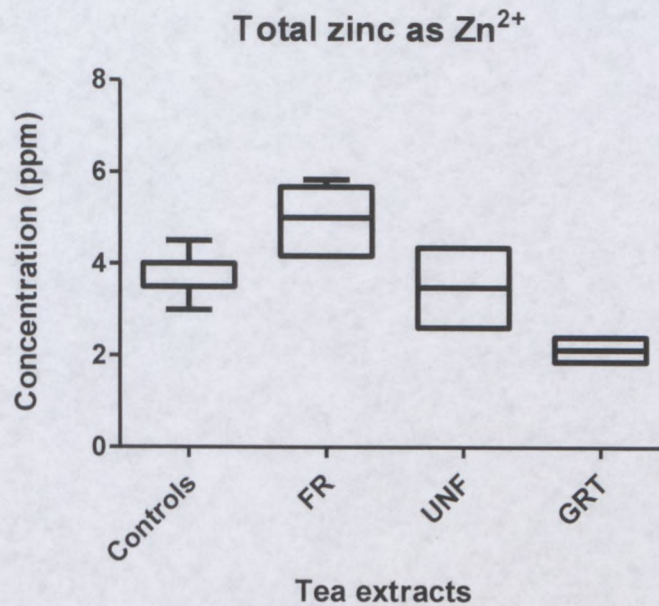


Figure 3.3 Graphic representation of the box and whisker statistical evaluation of the elemental concentration of zinc as Zn<sup>2+</sup> in the rat sera samples. The maximum, 75th percentile, the average (mean), the 25th percentile and the minimum values are indicated. FR is serum of the rats fed with extract of fermented rooibos, UNF is the serum of the rats fed with the extract of unfermented rooibos and GRT is that of the green tea.

There is a statistical difference between the zinc content of the serum of the control animals and the zinc content of the animals fed fermented rooibos extract. There is, however, no significant statistical difference between zinc content of the serum of the control animals and the animals fed with unfermented rooibos extract. There is a statistical difference between the zinc content of the serum of the control and the zinc content of the serum of the animals that have been fed with green tea extract. There is a difference of approximately 1 ppm in concentration of zinc between that of the control group and the animals fed with fermented rooibos extract, but a difference of 2 ppm in the case of animals fed with green tea extract.

The graphic representation of the box and whisker statistical evaluation of the elemental concentration of iron, both Fe<sup>2+</sup> and Fe<sup>3+</sup>, as Fe<sup>3+</sup> in the rat sera samples are shown in figure 3.4. There is a statistical difference between the content of the Fe<sup>3+</sup> in the serum of the control group and the serum of the animals fed with fermented rooibos extract. There is also a statistical difference between the total iron content as Fe<sup>3+</sup> in the serum of the control animals and the serum of the animals fed with unfermented rooibos extract. In addition, there is a statistical difference between the total iron content of the serum of the control animals and those fed with green tea extract.

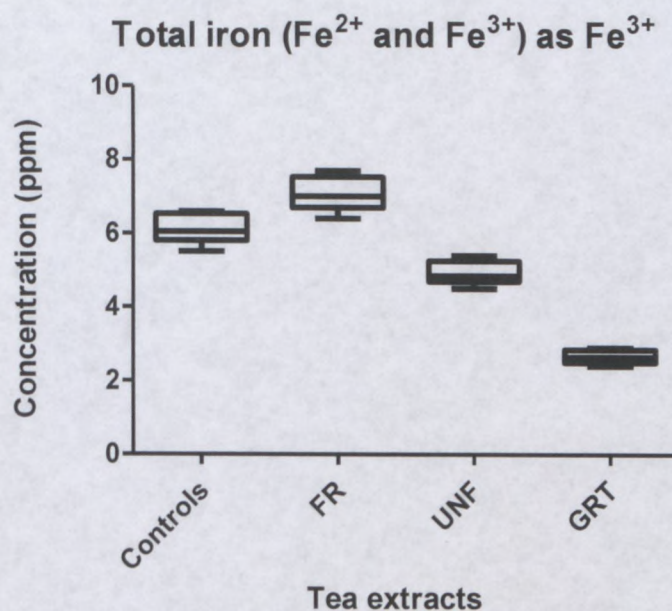


Figure 3.4 Graphic representation of the box and whisker statistical evaluation of the elemental concentration of iron, both  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  as  $\text{Fe}^{3+}$  in the rat sera samples. The maximum, 75th percentile, the average (mean), the 25th percentile and the minimum values are indicated. FR is serum of the rats fed with extract of fermented rooibos, UNF is the serum of the rats fed with the extract of unfermented rooibos and GRT is that of the green tea.

The difference, a decrease in absorption, in iron content in the case of the animals fed with fermented rooibos extract is approximately 1 ppm when considering only the mean value. However, in the case of the animals fed with unfermented rooibos extract there is an increase of approximately 1 ppm in the iron absorption. The increase in absorption of the total iron in the case of the animals fed with green tea extract is approximately 3 ppm.

The graphic representation of the box and whisker statistical evaluation of the elemental concentration of manganese as  $\text{Mn}^{2+}$  in the rat sera samples are shown in figure 3.5. There is no significant statistical difference between the manganese content of the serum of the animals in the control group and the serum of the animals fed with fermented rooibos extract. However, there is a statistical difference between the manganese content of the serum of the animals in the control group and those fed with unfermented rooibos extract. Also, there is a statistical difference between the manganese content of the serum of the control group animals and the serum of the animals fed with green tea extract. For both the unfermented rooibos extract and the green tea extract, the absorption of manganese was increased by 0.3 ppm.

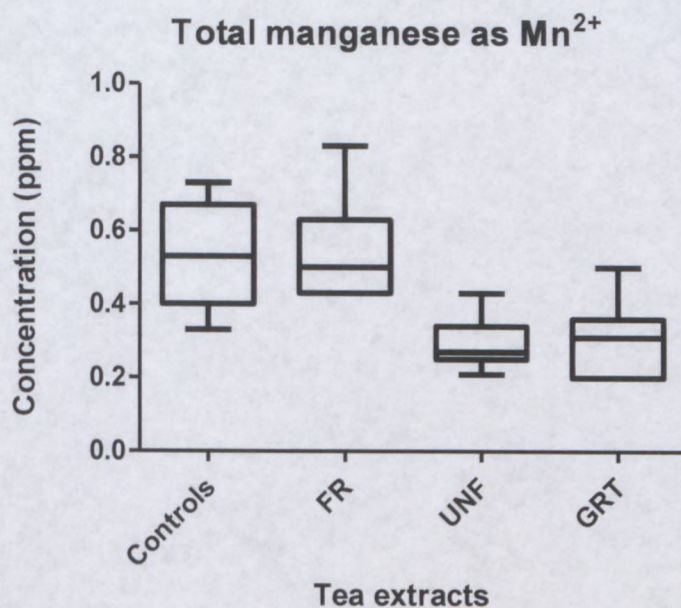


Figure 3.5 Graphic representation of the box and whisker statistical evaluation of the elemental concentration of manganese as Mn<sup>2+</sup> in the rat sera samples. The maximum, 75th percentile, the average (mean), the 25th percentile and the minimum values are indicated. FR is serum of the rats fed with extract of fermented rooibos, UNF is the serum of the rats fed with the extract of unfermented rooibos and GRT is that of the green tea.

The graphic representation of the box and whisker statistical evaluation of the elemental concentration of the total copper content, both Cu<sup>+</sup> and Cu<sup>2+</sup> as Cu<sup>2+</sup> in the rat sera samples are shown in figure 3.6.

There is a statistical difference between the total copper as Cu<sup>2+</sup> of the serum of the control group animals and that of the serum of the animals fed with fermented rooibos extract. There is however no significant statistical difference between total copper content of the serum of the control group animals and that of those fed with unfermented rooibos extract. Furthermore, there is a statistical difference between the total copper content of the serum of the control animals and the total copper content of the serum of the animals fed with green tea extract.

When considering the mean values, the absorption of the copper content of the serum of the animals fed with fermented rooibos extract decreased by a concentration of 2 ppm. In contrast, the absorption of the total copper content of the animals fed with green tea extract increased by more than 2 ppm. The statistical non-significance between the content of the serum of the animals of the control group and the animals fed with unfermented rooibos extract is regarded as tentative since the standard deviation in the data is relatively large

when compared to the standard deviation of the total copper content data of the other groups.

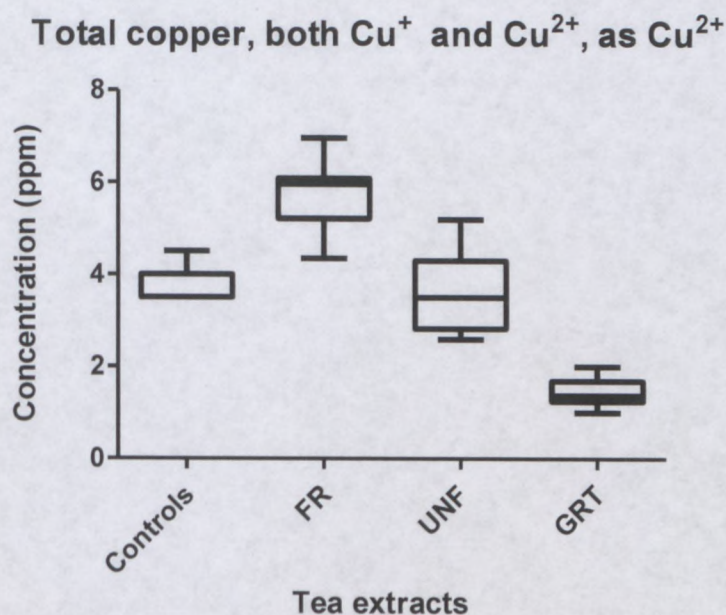


Figure 3.6 Graphic representation of the box and whisker statistical evaluation of the elemental concentration of copper, both  $\text{Cu}^+$  and  $\text{Cu}^{2+}$  as  $\text{Cu}^{2+}$  in the rat sera samples. The maximum, 75th percentile, the average (mean), the 25th percentile and the minimum values are indicated. FR is serum of the rats fed with extract of fermented rooibos, UNF is the serum of the rats fed with the extract of unfermented rooibos and GRT is that of the green tea.

It should be noted that the concentration of  $\text{Cu}^+$  in the human body is at least an order of magnitude smaller than the concentration of  $\text{Cu}^{2+}$  (Mader, 2004; Adreini *et al.*, 2008; Adreini *et al.*, 2009). This consideration is necessary should the effects of the tea extracts be converted to applicable values for clinical use. For this reason the concentration of the total copper may be regarded as that of  $\text{Cu}^{2+}$ . The graphic representation of the box and whisker statistical evaluation of the elemental concentration of cobalt as  $\text{Co}^{2+}$  in the rat sera samples are shown in figure 3.7.

There are no statistical differences between the cobalt, as  $\text{Co}^{2+}$  contents of the serum of the control group animals and those of the animals fed with fermented rooibos extract and unfermented rooibos extract. There is a statistical difference between the cobalt content of the serum of the control group animals and the cobalt content of the serum of the animals fed with green tea extract. The increase in the absorption of cobalt in the serum of the animals fed with green tea extract is approximately 0.1 ppm.

The graphic representation of the box and whisker statistical evaluation of the elemental concentration of molybdenum as  $\text{Mo}^{4+}$  in the rat sera samples are shown in figure 3.8.

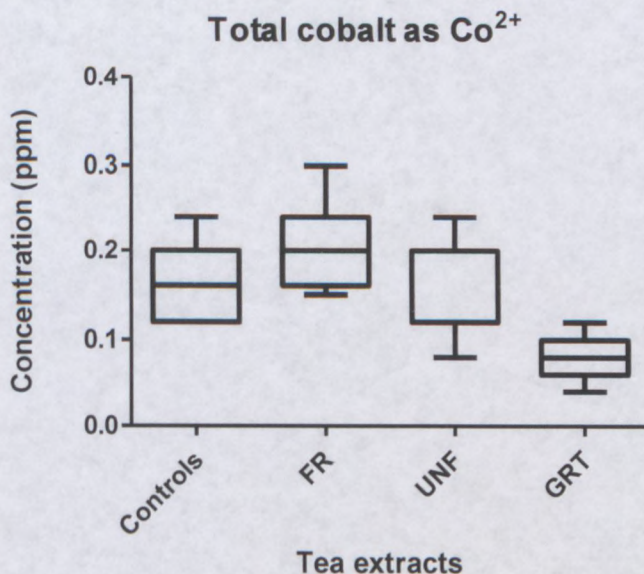


Figure 3.7. Graphic representation of the box and whisker statistical evaluation of the elemental concentration of cobalt as  $\text{Co}^{2+}$  in the rat sera samples. The maximum, 75th percentile, the average (mean), the 25th percentile and the minimum values are indicated. FR is serum of the rats fed with extract of fermented rooibos, UNF is the serum of the rats fed with the extract of unfermented rooibos and GRT is that of the green tea.

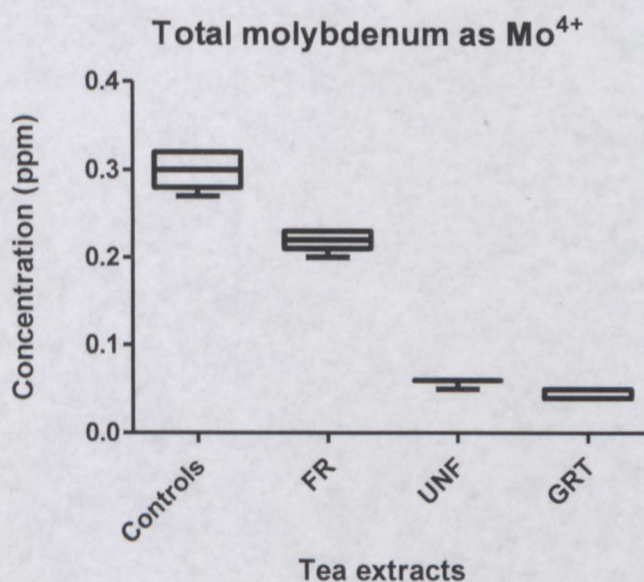


Figure 3.8. Graphic representation of the box and whisker statistical evaluation of the elemental concentration of molybdenum as  $\text{Mo}^{4+}$  in the rat sera samples. The maximum, 75th percentile, the average (mean), the 25th percentile and the minimum values are indicated. FR is serum of the rats fed with extract of fermented rooibos, UNF is the serum of the rats fed with the extract of unfermented rooibos and GRT is that of the green tea.

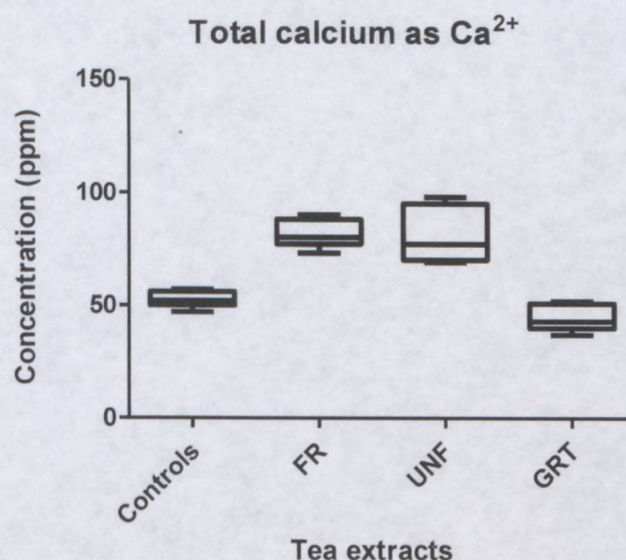


Figure 3.9 Graphic representation of the box and whisker statistical evaluation of the elemental concentration of calcium as Ca<sup>2+</sup> in the rat sera samples. The maximum, 75th percentile, the average (mean), the 25th percentile and the minimum values are indicated. FR is serum of the rats fed with extract of fermented rooibos, UNF is the serum of the rats fed with the extract of unfermented rooibos and GRT is that of the green tea.

There are statistical differences between the molybdenum content of the serum of the control group animals and the molybdenum content of the animals fed with fermented rooibos extract, unfermented rooibos extract and those fed with green tea extract. The increase in absorptions varies from 0.1 ppm to 0.25 ppm respectively.

The graphic representation of the box and whisker statistical evaluation of the elemental concentration of calcium as Ca<sup>2+</sup> in the rat sera samples are shown in figure 3.9. There is a statistical difference between the calcium content of the serum of the control group animals and the calcium content of the serum of the animals fed with fermented rooibos extract. There is also a statistical difference between calcium content of the serum of the control group animals and that of the animals fed with unfermented rooibos extract. There is however no significant statistical difference between calcium content of the serum of the control group animals and the calcium content of the serum of the animals fed with green tea extract. The calcium content of both the serum samples of the animals fed with fermented rooibos extract and unfermented rooibos extract indicates a decrease in calcium absorption; 25 and 20 ppm respectively.

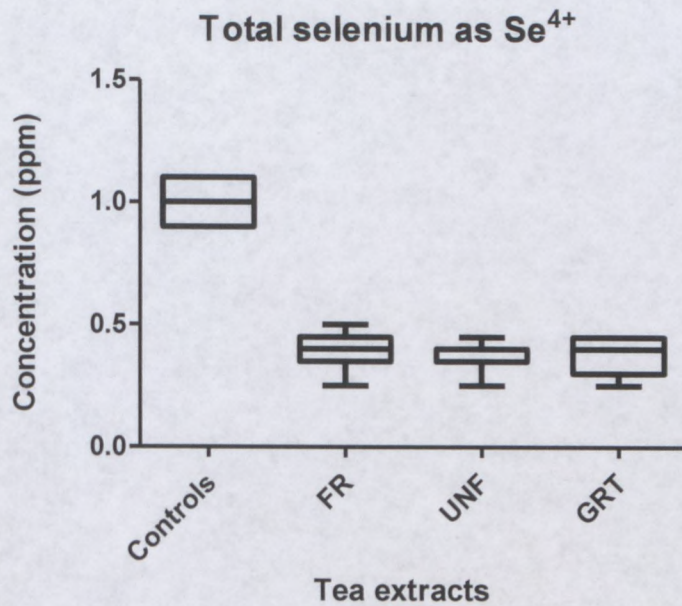


Figure 3.10 Graphic representation of the box and whisker statistical evaluation of the elemental concentration of selenium as  $\text{Se}^{4+}$  in the rat sera samples. The maximum, 75th percentile, the average (mean), the 25th percentile and the minimum values are indicated. FR is serum of the rats fed with extract of fermented rooibos, UNF is the serum of the rats fed with the extract of unfermented rooibos and GRT is that of the green tea.

The graphic representation of the box and whisker statistical evaluation of the elemental concentration of calcium as  $\text{Se}^{4+}$  in the rat sera samples are shown in figure 3.10. There are statistical differences between the selenium content of the serum of the control group animals and that of the animals fed with fermented rooibos extract, that of the animals fed with unfermented rooibos extract and that of the animals fed with green tea extract. The increase in absorption is approximately 0.5 ppm in each instance.

### 3.2 Effect of tea extracts on the elemental absorption (backscattering analysis (BS))

Spectra of the backscattered data of the freeze-dried serum specimens obtained from the animals in the control group, the animals fed with fermented rooibos extract, animals fed with unfermented rooibos extract and of the animals fed with green tea extract are shown in figure 3.10. The height of the front end of each of the spectra indicates the total content of the sample matrix. The MDLs for BS is approximately 1% m/m depending on the matrix composition (Mars, 2004).

For this reason the concentrations of the trace elements cannot be determined with BS. Moreover, the statistical significance is also dependent on the degree of resolution that can be obtained in BS analysis. The differences in total contents are not significant ( $p>0.05$ ) and hence the total content of the combined four specimens are the same. This significance also attests to the proper preparation of the specimens.

The carbonaceous content is equal to the height between C and O, that of oxygen is equal to the height between O and N, that of P between N and P and the sulphurous content that between P and the background. It should be noted that in biological matrices that the carbonaceous content includes oxygen, but not necessarily nitrogen, phosphorus or sulphur. More so, the carbonaceous content must include O. The nitrogenous content must include O and C, but not necessarily P and S. Also, both the P and S content must include both C and O, but not necessarily nitrogen.

When comparing the matrix compositions of the serum obtained animals in the control group and that of the animals fed with fermented rooibos extract, it was found that there was:

- an increase in carbon concentration;
- a decrease in oxygen;
- a decrease in phosphorous concentrations;
- no difference in the sulphur concentration and
- no difference in the nitrogen concentration.

For comparison of the serum obtained from the animals in the control group with the serum of the animals fed with unfermented rooibos extract there were no statistical differences in the concentrations of the matrix elements.

In the comparison of the serum obtained from the animals in the control group and the serum obtained from the animals fed with unfermented rooibos extract it was found that:

- there was an increase in the carbonaceous content

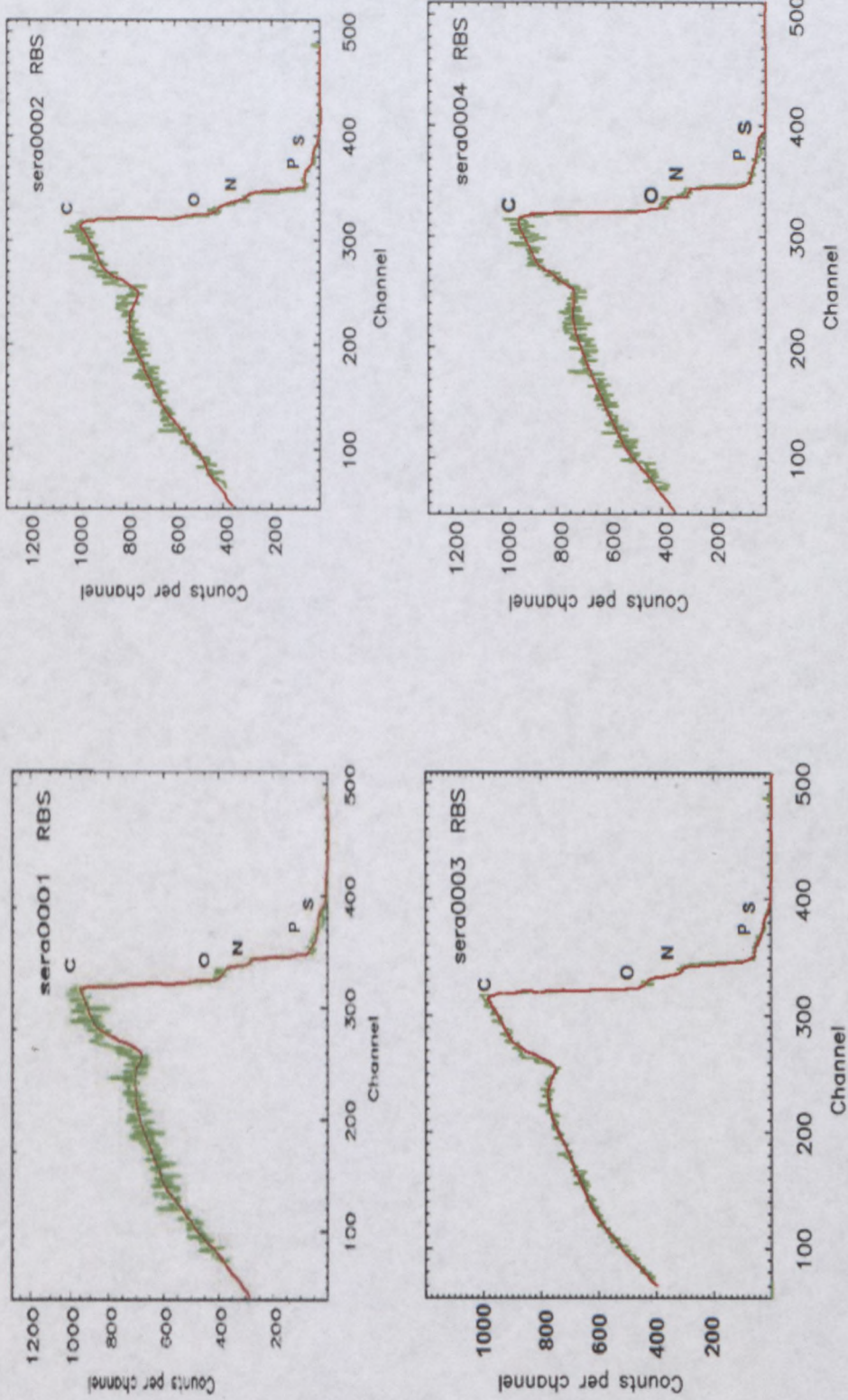


Figure 3.11 Spectra of the backscattered data, as the concentration in counts per channel to the energy in channels of the freeze-dried serum specimen obtained from the animals in the control group (top left), the animals fed with fermented rooibos extract (top right), animals fed with unfermented rooibos extract (bottom left) and of the animals fed with green tea extract (bottom right). Of the channels, 480 channels is equivalent to 3 MeV, the energy of the beam of protons. The green line is the backscattered data and the red line the computational fit to the backscattered data. The positions of the symbols indicate the energies of the respective elements when located at the surface of the samples.

- the oxygen concentration remained constant;
- there was a significant increase in nitrogen content;
- the phosphorous content remained the same and
- there was a decrease in the sulphurous content.

In the comparison of the serum obtained from the animals in the control group to the serum obtained from the animals fed with green tea extract it was found that:

- there was an increase in carbonaceous content;
- the oxygen content remained constant;
- there was a significant increase in nitrogenous content;
- the phosphorous content remained constant and
- the sulphurous content remained constant.

### 3.3 Effect of tea extracts on clinical parameters

The graphic representation of the box and whisker statistical evaluation of the total protein content in the rat sera samples are shown in figure 3.12.

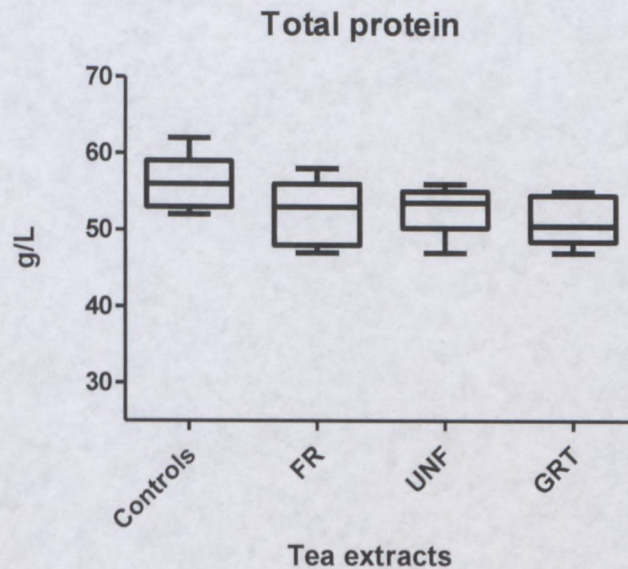


Figure 3.12 Graphic representation of the box and whisker statistical evaluation of the concentration of the total protein in the rat sera samples. The maximum, 75th percentile, the average (mean), the 25th percentile and the minimum values are indicated. FR is serum of the rats fed with extract of fermented rooibos, UNF is the serum of the rats fed with the extract of unfermented rooibos and GRT is that of the green tea.

There is no significant statistical difference between the total protein content in the serum of the control group animals and the total protein content in the serum of both the animals fed with fermented rooibos extract and the serum of the animals fed with unfermented rooibos extract. There is however a statistical difference between total protein content of the serum of the control animals and the total protein content of the animals fed with green tea extract.

The graphic representation of the box and whisker statistical evaluation of the albumin content in the rat sera samples are shown in figure 3.13. There are no significant statistical differences between the serum of the control group animals and the serum of the animals fed with fermented rooibos extract, those fed with unfermented rooibos extract and those fed with green tea extract.

The graphic representation of the box and whisker statistical evaluation of the globulin content (concentration of the total protein less the concentration of the albumin) in the rat sera samples are shown in figure 3.14.

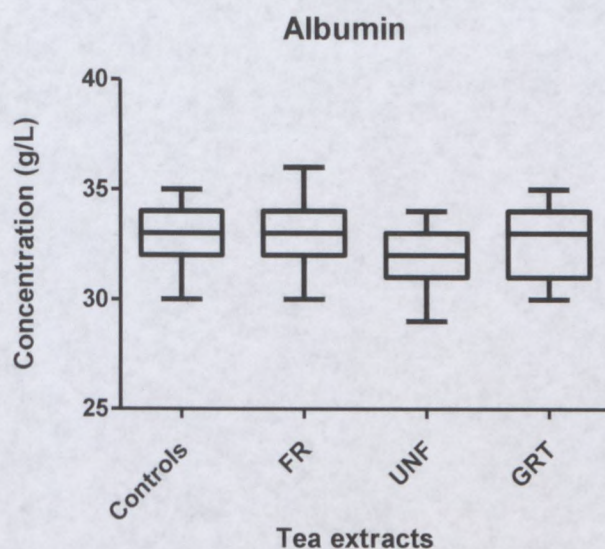


Figure 3.13 Graphic representation of the box and whisker statistical evaluation of the content of the albumin in the sera of rats fed with different tea extracts. The maximum, 75<sup>th</sup> percentile, the average (mean), the 25<sup>th</sup> percentile and the minimum values are indicated. FR is the extract of fermented rooibos, UNF the extract of the unfermented rooibos and GRT is extract of green tea.

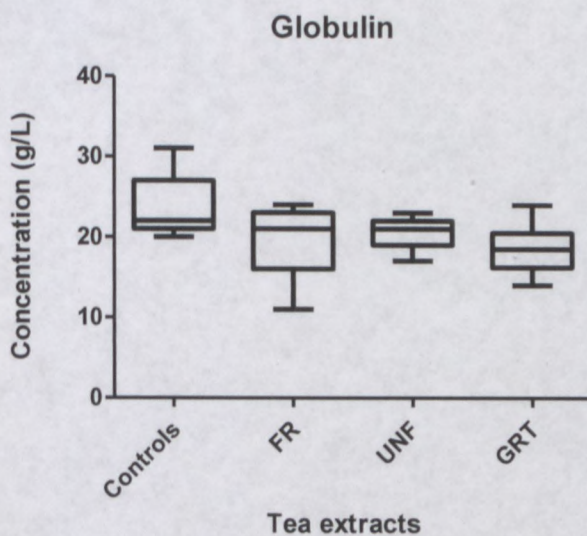


Figure 3.14 Graphic representation of the box and whisker statistical evaluation of the content of the globulin in the sera of rats fed with different tea extracts. The maximum, 75<sup>th</sup> percentile, the average (mean), the 25<sup>th</sup> percentile and the minimum values are indicated. FR is the extract of fermented rooibos, UNF the extract of the unfermented rooibos and GRT is extract of green tea.

There is no significant statistical difference between the globulin content in the serum of the control group animals and that of the animals fed with fermented rooibos extract. There is

however a statistical difference between the globulin content in the serum of the control animals and that of the serum of the animals fed with green tea extract.

The globulin content has however been determined by difference between the total protein content and the albumin content. Since there is a statistical difference between the content of the total protein in the serum of the control animals and not a significant difference between the albumin content in the serum of the control animals and that in the serum of the animals fed with green tea extract, it is expected that the globulin content would differ. Which necessitates further evaluation since the variances in the total protein content and in the albumin content are now included in the globulin content. For this reason the linear correlation between the albumin content and the total protein content was determined. These linear correlations are shown in figure 3.15. The intercepts of the linear correlations were set to zero since a zero value for total protein content implies a corresponding zero value for the albumin content.

From the total protein to the albumin correlation equations for the control animals and animals fed with fermented rooibos extract and unfermented rooibos extract, it is deduced that the albumin content constitutes approximately 60% of the total protein content. However, the correlation coefficient for the albumin content in the serum of the animals fed with green tea extract is extremely weak. This weak correlation may also contribute to the non-significance of the globulin content.

The graphic representation of the box and whisker statistical evaluation of the bilirubin content in the rat sera samples are shown in figure 3.16. There is a statistical difference between the bilirubin content in the serum of the control group animals and the bilirubin content in the serum of the animals fed with fermented rooibos extract. There are however no significant statistical differences between the bilirubin content in the serum of the control animals and the bilirubin content in the serum of both the animals fed with unfermented rooibos extract and in the serum of those fed with green tea extract.

The graphic representation of the box and whisker statistical evaluation of the lactate dehydrogenase content in the rat sera samples are shown in figure 3.17. There are no significant statistical differences between the lactate dehydrogenase content in the serum of the control animals and in the serum of the animals fed with fermented rooibos extract or unfermented rooibos extract or green tea extract.

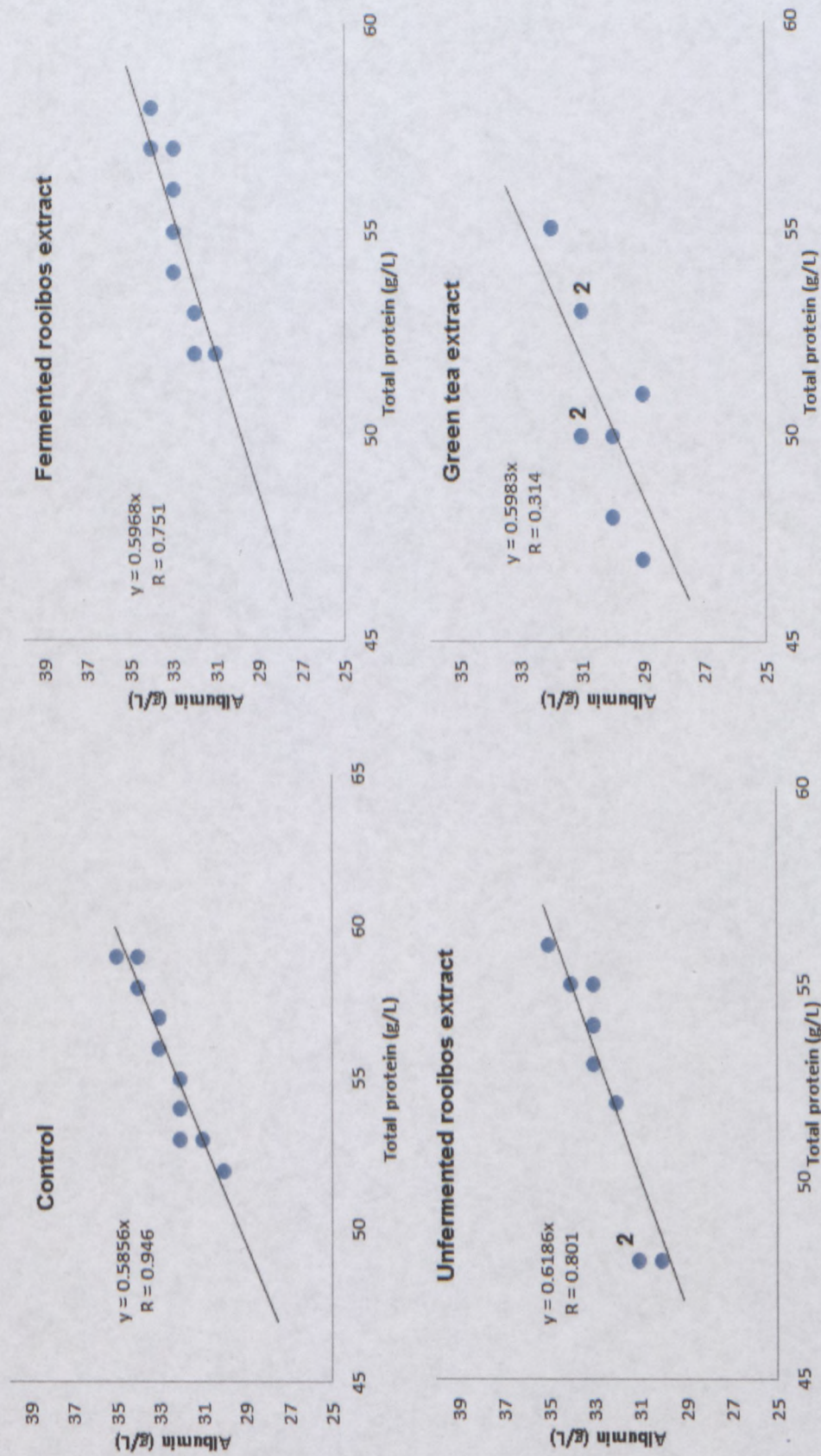


Figure 3.15 Graphic representation of the linear regression of the albumin content versus the total protein content in the serum samples. The intercept was set at zero. The correlation coefficient R is indicated. The numbers indicate coincident data.

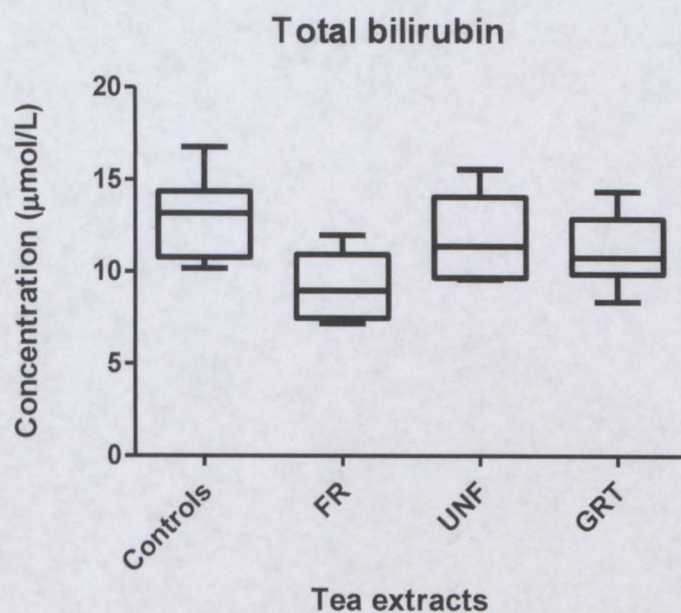


Figure 3.16 Graphic representation of the box and whisker statistical evaluation of the total bilirubin content in the rat sera samples. The maximum, 75th percentile, the average (mean), the 25th percentile and the minimum values are indicated. FR is serum of the rats fed with extract of fermented rooibos, UNF is the serum of the rats fed with the extract of unfermented rooibos and GRT is that of the green tea.

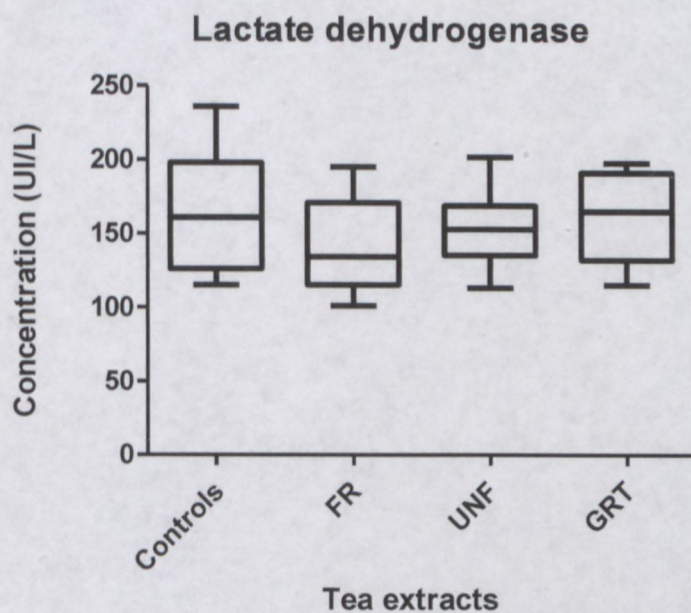


Figure 3.17 Graphic representation of the box and whisker statistical evaluation of the lactate dehydrogenase content in the rat sera samples. The maximum, 75th percentile, the average (mean), the 25th percentile and the minimum values are indicated. FR is serum of the rats fed with extract of fermented rooibos, UNF is the serum of the rats fed with the extract of unfermented rooibos and GRT is that of the green tea.

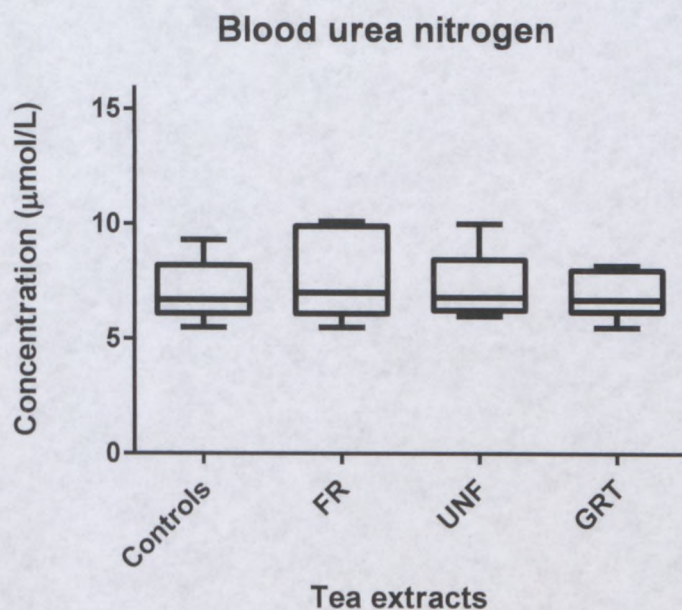


Figure 3.18 Graphic representation of the box and whisker statistical evaluation of the blood urea nitrogen content in the rat sera samples. The maximum, 75th percentile, the average (mean), the 25th percentile and the minimum values are indicated. FR is serum of the rats fed with extract of fermented rooibos, UNF is the serum of the rats fed with the extract of unfermented rooibos and GRT is that of the green tea.

The graphic representation of the box and whisker statistical evaluation of the blood urea nitrogen content in the rat sera samples are shown in figure 3.18. There are no significant statistical differences in the blood urea nitrogen content in the serum of the control animals and that in the serum of the animals fed with fermented rooibos extract or unfermented rooibos extract or green tea extract.

The graphic representation of the box and whisker statistical evaluation of the uric acid content in the rat sera samples are shown in figure 3.19. There is no significant statistical difference between the uric acid content in the serum of the control animals and the uric acid content in the serum of the animals fed with fermented rooibos extract and the serum of the animals fed with unfermented rooibos extract. There is a statistical difference between the uric acid content in the serum of the animals in the control group and in the serum of the animals fed with green tea extract. There is an increase in the concentration of the uric acid of approximately 0.02  $\mu\text{mol/L}$ .

The graphic representation of the box and whisker statistical evaluation of the total cholesterol content in the rat sera samples are shown in figure 3.20.

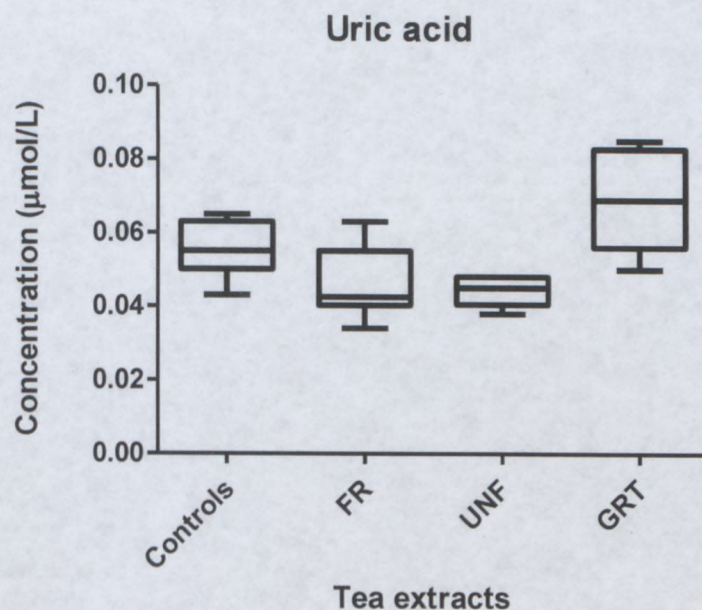


Figure 3.19 Graphic representation of the box and whisker statistical evaluation of the uric acid content in the rat sera samples. The maximum, 75th percentile, the average (mean), the 25th percentile and the minimum values are indicated. FR is serum of the rats fed with extract of fermented rooibos, UNF is the serum of the rats fed with the extract of unfermented rooibos and GRT is that of the green tea.

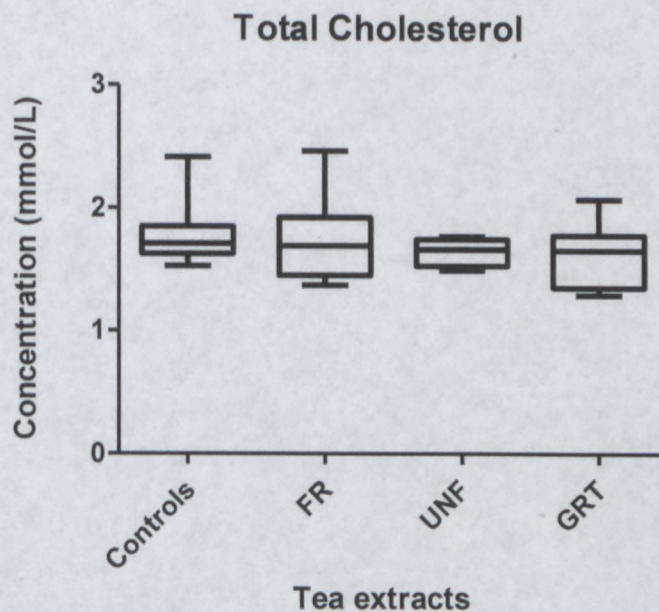


Figure 3.20 Graphic representation of the box and whisker statistical evaluation of the total cholesterol content in the rat sera samples. The maximum, 75th percentile, the average (mean), the 25th percentile and the minimum values are indicated. FR is serum of the rats fed with extract of fermented rooibos, UNF is the serum of the rats fed with the extract of unfermented rooibos and GRT is that of the green tea.

There are no significant statistical differences between the total cholesterol content in the serum of the control animals and the total cholesterol content in the serum of the animals fed with fermented rooibos extract, that of the animals fed with unfermented rooibos extract and that of the animals fed with green tea extract.

The graphic representation of the box and whisker statistical evaluation of the alanine aminotransferase content in the rat sera samples are shown in figure 3.21. There are no significant statistical differences between the alanine aminotransferase content in the serum of the control group animals and the alanine aminotransferase content in the serum of the animals fed with fermented rooibos extract, unfermented rooibos extract and green tea extract.

The graphic representation of the box and whisker statistical evaluation of the aspartate aminotransferase content in the rat sera samples are shown in figure 3.22. There are no significant statistical differences between the aspartate aminotransferase content in the serum of the control group animals and the aspartate aminotransferase content in the serum of the animals fed with fermented rooibos extract, unfermented rooibos extract and green tea extract.

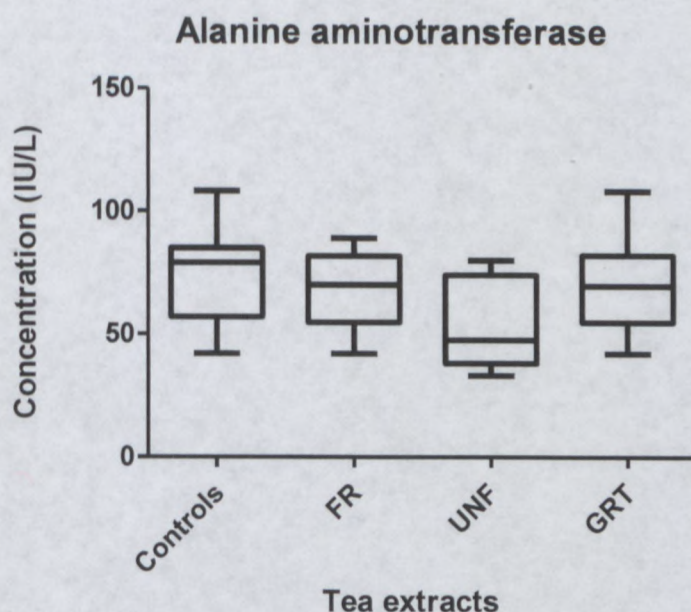


Figure 3.21 Graphic representation of the box and whisker statistical evaluation of the alanine aminotransferase content in the rat sera samples. The maximum, 75th percentile, the average (mean), the 25th percentile and the minimum values are indicated. FR is serum of the rats fed with extract of fermented rooibos, UNF is the serum of the rats fed with the extract of unfermented rooibos and GRT is that of the green tea.

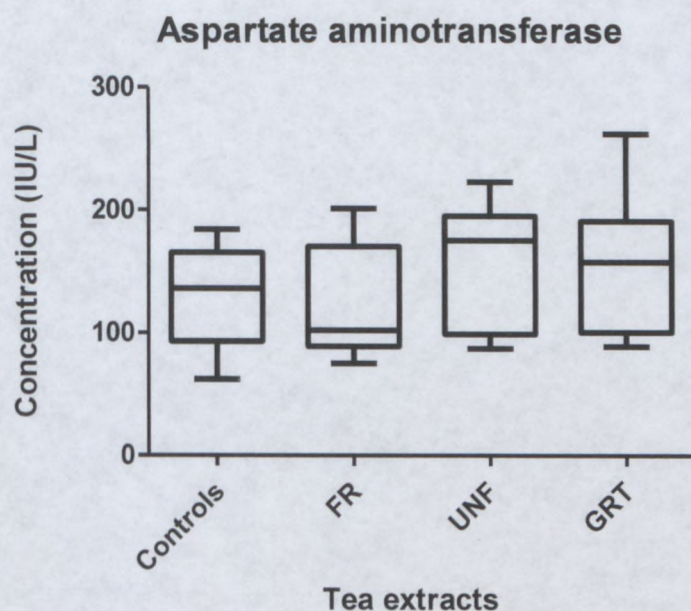


Figure 3.22 Graphic representation of the box and whisker statistical evaluation of the aspartate aminotransferase content in the rat sera samples. The maximum, 75th percentile, the average (mean), the 25th percentile and the minimum values are indicated. FR is serum of the rats fed with extract of fermented rooibos, UNF is the serum of the rats fed with the extract of unfermented rooibos and GRT is that of the green tea.

The graphic representation of the box and whisker statistical evaluation of the creatine phosphokinase content in the rat sera samples are shown in figure 3.23. There are no statistical differences between the creatine phosphokinase content in the serum of the control group animals and the creatine phosphokinase content in the serum of the animals fed with fermented rooibos extract and green tea extract but there are statistical differences between the creatine phosphokinase content of the control group and the unfermented rooibos extract group.

The graphic representation of the box and whisker statistical evaluation of the creatinine content in the rat sera samples are shown in figure 3.24. There are no significant statistical differences between the creatinine content in the serum of the control group animals and the creatinine content in the serum of the animals fed with fermented rooibos extract and green tea extract. But there are significant statistical differences between the creatinine content in the serum of the control group and animals fed with unfermented rooibos extract.

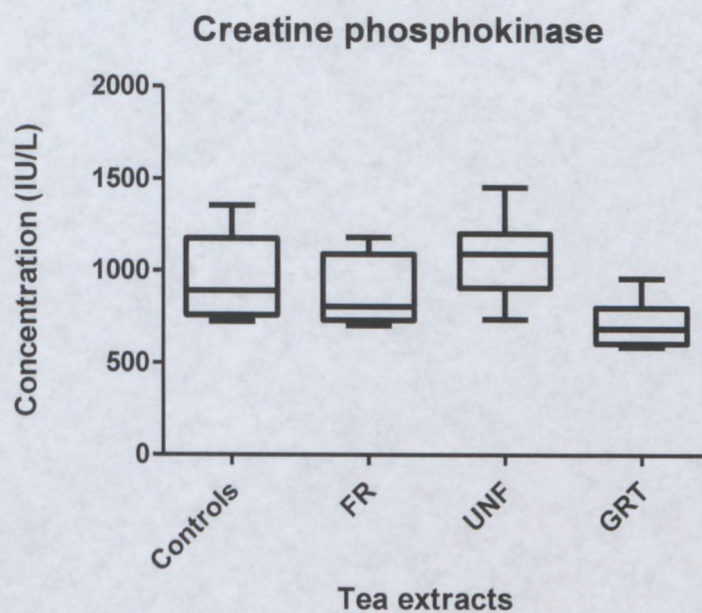


Figure 3.23 Graphic representation of the box and whisker statistical evaluation of the creatine phosphokinase content in the rat sera samples. The maximum, 75th percentile, the average (mean), the 25th percentile and the minimum values are indicated. FR is serum of the rats fed with extract of fermented rooibos, UNF is the serum of the rats fed with the extract of unfermented rooibos and GRT is that of the green tea.

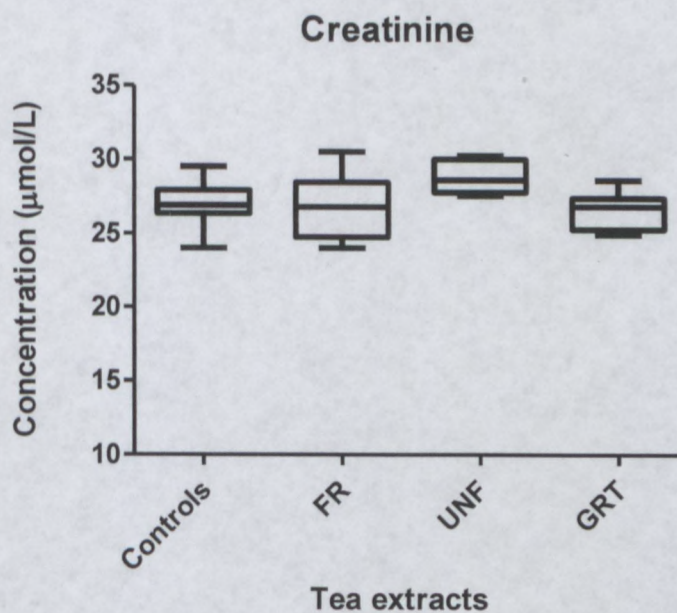


Figure 3.24 Graphic representation of the box and whisker statistical evaluation of the creatinine content in the rat sera samples. The maximum, 75th percentile, the average (mean), the 25th percentile and the minimum values are indicated. FR is serum of the rats fed with extract of fermented rooibos, UNF is the serum of the rats fed with the extract of unfermented rooibos and GRT is that of the green tea.

## SUMMARY

Seeing that the total Mg level in the serum of animal treated with UFR and GRT decreased, it shows that there was an increase in absorption of magnesium in the UFR and GRT groups. However, this study has shown, for the first time that unfermented rooibos tea as well as green tea can modulate magnesium concentration in the rat serum.

The concentration of Zn increased in the serum of the rats fed with FR extract and decreased in the serum of those fed with extract GRT. In terms of absorption, the concentration of zinc decreased of about 1ppm in the FR and increased in about 2ppm in the GRT.

The Fe concentration in the serum of the animals fed with the extract of FR increased and the Fe concentration in the serum of the animals fed with the extracts of UFR and GRT decreased. The increase and decrease in absorptions of Fe were respectively about 1ppm and about 3ppm.

The Mn concentration in the serum of the animals decreased in both UFR and GRT groups. However, the decrease in concentration means that there was an increase in the absorption of Mn in the serum of about 3ppm. At this point, this study has shown, once more again, for the first time the effect of the unfermented rooibos tea as well as green tea when compared with controls.

Cu concentration increased in the FR and decreased in the GRT groups. The increase in concentration shows that there was a decrease in the absorption of Cu by 2ppm in the FR group and an increase in the absorption of Cu in the GRT group by 2ppm. However, rooibos tea has shown to be modulating Cu the same way that green tea affected the concentration of Cu in the serum of the animals.

There was a slight decrease in concentration of Co in the GRT group. Therefore, the Co absorption increased in the GRT group. According to the result, rooibos tea didn't affect Co.

Total Ca concentration increased in the FR and the UFR groups. This result shows that there was a decrease in Ca absorption in the serum in the FR and the UFR groups. In other words, total Ca absorption was affected by rooibos tea by about 50ppm.

Total Se concentration in serum of the treated animals was affected by decreasing in all the groups (FR, UFR and GRT) when compared to the controls. This decrease in concentration means that there was an increase in the absorption of selenium in all groups. However, as Se is a very important element for human being and animal, they will be increase in selenium activity in the serum of rats.

Mo showed a decrease in concentration in the FR, UFR and GRT groups, meaning that the absorption of Mo in theses 3 groups increased. The increase in absorptions varies from 0.1

ppm to 0.25 ppm respectively. This result shows again, how rooibos tea as well as green tea affected the absorption of Mo in the serum.

P concentration decreased in the serum of animal treated with fermented rooibos tea whereas S concentration decreased in the UFR group. These two results suggest that both unfermented and fermented rooibos extract affected the concentrations of P and S in the serum of the animals. It means P and S increased in absorption.

Total Protein concentration decreased in the GRT group. This explains how GRT affected the total protein concentration. This result is in line with the previous study done on green tea aqueous extract reduces visceral fat and decreases protein availability in rats fed with a high-fat diet (Bajerska *et al.*, 2011).

Uric acid concentration in the serum of the animals was not affected by rooibos tea but was affected by green tea extracts as the result showed an increase in concentration of 0.02  $\mu\text{mol/L}$ .

Aqueous extracts of rooibos and green teas slightly lowered plasma concentrations of globulin in the GRT group and bilirubin, in the FR group when compared with controls group. This might be due to hepatoprotective effects of these antioxidants (Ulicna *et al.*, 2006).

Kidney function indicator, such as creatinine, was slightly increased in the UFR group when compared with the control group; concentrations of other indicators were not affected as shown in the previous study. This result is in line with previous study done in human (Marnewick *et al.*, 2011).

It can be also concluded from our results that rooibos tea, containing a large amount of flavonoids (Rabe *et al.*, 1994), produced from legume *Aspalathus linearis*, particularly its non-fermented form (green), seems to have an effect on Creatine Phosphokinase and Creatinine by increasing their concentrations in the serum of animals.

## CHAPTER FOUR

### CONCLUSIONS AND RECOMMENDATIONS

#### 4.1 Conclusions

The present research study investigated *in vivo*, 4 groups of experimental animals, the effects of rooibos extract consumption on trace elements and biochemical parameters absorption in a strictly controlled diet when compared with the green tea extract without inducing any oxidative stress using Wistar rats. This study showed interest in trace elements and biochemical parameters because they are essential triggers for many biological mechanisms in the digestive, muscular, circulatory and cerebral systems. They are vital if the organism is to function properly and maintain a healthy balance.

Referring to the table 4.1, our results show that P content was really affected by the fermented rooibos extract. Moreover, the absorption of the element such as Ca was affected by increasing in concentration and decreasing in the absorption when using the fermented and the unfermented rooibos extracts. The concentration of S increased only with the unfermented rooibos extract. We also observed from our result that Fe was affected by decreasing with the fermented rooibos extract and increasing with unfermented rooibos and the green tea extract. Trace element such as Co didn't show any effect by any of the rooibos teas similar situation with the biochemical parameters like albumin, lactate dehydrogenase, blood urea nitrogen, total cholesterol, aspartate aminotransferase and alanine aminotransferase.

In addition, results from table 4.1 show that among the biochemical parameters the creatine phosphokinase and the creatinine contents were affected with the unfermented rooibos extracts. The rest of the trace elements and clinical parameters are not really affected by any of the three teas.

This study raised questions concerning clinical effect of these abnormalities on the absorption of these metals and on biochemical parameters which require further investigation.

In this research project, we are discussing the results from a statistical point of view. Translation of the outcomes of this study from statistical significance to clinical relevance necessitates the use of animal to human correlations such as the comparison of the body surface areas (BSA) and the basic metabolism rates (BMR). In this regard it should be noted that even if the BSA correlation does not necessarily implies BMR correlation. This translation is however not in the scope of this investigation.

The biochemical parameters measured, are the indicators or biomarkers for the kidney, heart and the liver. They inform about how healthy these organs are.

The abnormalities on the absorption of metals and biochemical parameters are given in table 4.1.

**Table 4.1** Tabulation, as an overview, of the abnormalities in the absorption of the metals (elements and trace elements) and the clinical parameters caused by the extracts of the three different teas when compared to the control values. 0 indicates that the concentration was not affected. The numbers 1 and 2 indicate the extent of the significance,  $0.01 < p < 0.05$  and  $p < 0.01$  respectively. The negative and positive signs indicate a decrease and an increase in absorption for the elements and an increase and decrease in the content of the clinical parameters respectively.

	Controls	Fermented rooibos extract	Unfermented rooibos extract	Green tea extract
Phosphorus (P)	0	1	0	0
Sulphur (S)	0	0	1	0
Calcium (Ca)	0	-1	-1	0
Manganese (Mn)	0	0	1	1
Iron (Fe)	0	-2	2	1
Copper (Cu)	0	-1	0	1
Cobalt (Co)	0	0	0	1
Zinc (Zn)	0	-1	0	2
Selenium (Se)	0	1	1	1
Molybdenum (Mo)	0	1	2	2
Magnesium (Mg)	0	0	2	2
Total protein	0	0	0	-1
Albumin	0	0	0	0
Total bilirubin	0	-1	0	0
Lactate dehydrogenase	0	0	0	0
Blood urea nitrogen	0	0	0	0
Uric acid	0	0	0	1
Total cholesterol	0	0	0	0
Aspartate aminotransferase	0	0	0	0
Alanine aminotransferase	0	0	0	0
Creatine phosphokinase	0	0	1	0
Creatinine	0	0	1	0

## 4.2 Recommendation

### *Statistical significance and clinical relevance or importance*

The evaluation of the effects on trace element absorption and on the biochemical parameters of the various tea extracts is based solely on the statistical analysis assuming a p value less than 0.05. It should be considered that the p value is inherently dependent on the minimum detection limits incorporated in each analysis. Hence in the instances where p values equal to 0.5, it is assumed that the effects of the specific extract on both the elemental and clinical parameters are not (statistically) significant. More so, in linear correlations of less than 0.4 (when any outcome is only represented by 40% of the data), a p value of at times less than 0.01 can be found.

In addition, the outcomes are of statistical significance and not of clinical importance or at least clinical relevance. The principal aim in many research works, in the final outcome, is normally desired for these outcomes to be applicable clinically. In this regard, various theoretical considerations need to be made which would translate the outcomes of an animal-based experiment to clinical importance. This exercise would entail the conversion of animal physiology and biochemistry to that of the human being. Various mathematical equations for such translation do exist but only as an overall translation and not specific to particular organ functions such as those of the liver, kidney and heart, in which the biochemistry should be included.

For the clinical analyses, the data presently available in the literature leave much to be desired as the statistical evaluation of the literature data implies heteroscedacity. The result is that comparative analysis is compromised in that in using only the data within the 25th and 75th percentiles of these previous studies should significantly alter the outcomes of any animal study. However such statistical evaluations have not yet been research and in addition such a venture would be the basis of a new study and therefore is not within the scope of this work.

### *Ionic forms of the elements*

The analysis of trace elements performed in this study was exclusively for the total elemental concentrations. It was observed that some of these elemental concentrations do differ, such as the amount of Fe absorbed. Hence it cannot be deduced from this study which of these ions had been absorbed. Similarly, the abnormalities in the Cu concentration can be ascribed

to any of the  $\text{Cu}^+$  and  $\text{Cu}^{2+}$  ions. It is therefore recommended that the ionic form of the ions be determined by using analytical methods such as polarography and voltammetry. The data obtained from these instrumental techniques can then be correlated to the known orientation of these ions in the moiety of the enzymes. This further necessitates that an in-depth literature search be done into the coordination of the ions in these moieties. This is however not in the scope of this study.

Notable exceptions to these instrumental analyses are ions such as Mg which exists only as  $\text{Mg}^{2+}$ , Ca which exists as only  $\text{Ca}^{2+}$  and also Zn, as  $\text{Zn}^{2+}$ .

**CHAPTER FIVE**  
**SCIENTIFIC RESEARCH OUTPUT**

**INTERNATIONAL CONFERENCE HOSTED IN PORTUGAL**

**Authors:** C. Kunsevi-Kilola, J.A. Mars, J.S. Esterhuyse, D. Gihwala

**Title:** PIXE and RBS analysis of serum specimens of rats fed with tea extracts to ascertain trace element absorption

**Conference:** 13th International Conference on Nuclear Microprobe Technology & Applications 2012 The conference was held at ESTeSL (Escola Superior de Tecnologias da Saúde) Auditorium. The conference room is located in the *University Campus* at the Expo area of Lisbon by the majestic Tagus riverside.

**Location:** Lisbon, Portugal

**Date:** 22 - 27 July 2012

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