MODULATORY ROLE OF ROOIBOS ON EXERCISE-INDUCED OXIDATIVE STRESS AND PERFORMANCE WHILE EXPLORING INFLUENCES ON THE HUMAN METABOLOME AND EFFECT OF GENETIC VARIATIONS

BY

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NOT TO US,
Lord
BUT TO YOU BE THE
glory
PSALM 115:1

DECLARATION

I, Oiva Viety Kamati, declare that the contents of this dissertation/thesis represent my own single-handed 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 and funders.

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ABSTRACT

Phase I - Establishment of an exercise-induced oxidative stress model for testing herbal interventions, such as Rooibos

Background: Despite general clarity on the importance of oxidative stress to human health, it is widely acknowledged that this phenomenon is not yet well enough understood to allow for effective clinical manipulation. Oxidative stress was conceptualised several decades ago and was first defined by Sies and co-workers, as the imbalance of pro-oxidative molecules and antioxidants within a system. In recent years, it has been redefined as a cellular condition caused by an imbalance between the production and accumulation of reactive species in the cells/tissues and the ability of a biological system to detoxify these reactive species/products. Many *in vitro* and animal models have been developed over the years to conduct oxidative stress-based research, but to date, there is to our knowledge, no standardised human intervention models. This may in part be because, beyond biological variation, it is much more difficult to control lifestyle factors in a human population than in an experimental animal population. Furthermore, it remains challenging to generate oxidative stress in a predictable and controllable fashion in a human subject without undue risk of further harm. It is, however, extremely important that this phenomenon be further studied to understand the role of free radicals and antioxidants in physiology, but also for possible development of preventative and/or therapeutic interventions. Especially, after many years of research studies, only a few therapeutic interventions could be directly attributed to the research done on oxidative stress, with one of the major factors frustrating these efforts being the lack of standardised human models. Even though the *in vitro* and animal models are helpful, they do not generally translate well to humans in the field of oxidative stress. **Methods:** This study, therefore, aimed to standardise an exercise model to induce oxidative stress which may serve as a repeatable and controllable non-pathology-related method of inducing oxidative stress. In order to use this as a research model, it would, also require that other factors impacting oxidative stress be controlled. This was achieved by recruiting thirty young apparently healthy adult male participants and assessing their physical activity, dietary intake, medicinal or recreational drug use and screening for general pathological conditions which may contribute to oxidative stress. To induce oxidative stress in a controlled fashion, the exercise intervention was designed to include sprints to exhaustion. In addition to this, the trial was designed as a crossover trial, meaning that participants would take part in both the placebo and Rooibos intervention sessions at different times with a washout period in between. These dietary interventions (Rooibos and placebo) were taken before commencement of the exercise regimes, while markers of oxidative stress were measured after consumption with a standardised snack, during exercise and after exercise. Exercise performance parameters (time, distance, power, work, heart rate and oxygen uptake) were captured and measured by the Wattbike monitor,

while exercise-induced oxidative stress damage blood biomarkers for lipid peroxidation (TBARS and CDs), protein oxidation (protein carbonyl), DNA damage, fatigue and muscle damage (lactate, creatine kinase and lactate dehydrogenase), blood redox status (GSH, GSSG, GSH/GSSG ratio) and biochemical analytes were analysed using respective assays and analyser. **Results and Conclusion:** In our proposed model, a controlled Wattbike exercise regime reliably and repeatably induced oxidative stress in the study population in a manner that has a low likelihood of causing an adverse event. The exercise regime caused an increase in oxidised glutathione, an important endogenous antioxidant and induced lipid peroxidation in study participants irrespective of the dietary oral intervention. It was also established that herbal interventions do not complicate the model and thus can be successfully introduced along with the model. Rooibos modulated the oxidative stress response, by allowing relevant oxidative stress markers to return to baseline levels more rapidly than placebo. As such we suggest that our proposed model demonstrates that exercise can be used to induce oxidative stress reliably and controllably in a selected human population. In addition, our results demonstrate that oral interventions do not complicate the onset of oxidative stress. This, therefore, opens the way for further studies to refine the model and standardise a human oxidative stress model for clinical testing within the context of sport and exercise.

Phase II – The modulatory role of a standardised fermented Rooibos beverage to serve as an ergogenic aid using a sub-maximal exercise regime in adult males

Background: Excessive production and/or accumulation of reactive species, particularly reactive oxygen and nitrogen species during exercise may result in or cause oxidative stress, exercise-induced ailments and/or overall poor exercise performances and recovery. Many athletes and sport/fitness professionals have been and still use non-specific treatments and preventative approaches to mitigate and alleviate exercise-induced ailments. These approaches may include synthetic drugs, which can be very costly and have various side effects. However, recent studies have shown that the use of natural products such as plant-derived polyphenolic constituents could be the game changer in an attempt to minimise the effects of oxidative stress and exercise-induced ailments experienced during certain physical activities. As a result, many athletes and sport professionals are increasingly ingesting these naturally derived phytochemicals and including them in their daily diet as a means to modulate exercise-induced ailments and also as health boosting and/or disease prevention strategies. Rooibos is a well-known indigenous herbal tea in South Africa with a growing demand worldwide because of its reported health promoting bioactive properties. **Aim:** The current study aimed to assess if a standardised fermented Rooibos beverage could modulate exercise-induced oxidative stress and improve exercise performance outcomes. **Methods:** Using a blinded randomised, cross-over placebo-controlled design, thirty healthy adult males consumed 375 mL of the standardised Rooibos or placebo beverages before completing an

exercise regime. The regime included a modified sub-maximal exercise test followed by repeated sprints (up to 10 sets) on a Wattbike. Blood samples were collected at various time points and analysed for serum total polyphenolic content and antioxidant capacity, oxidative lipid and -protein damage markers, muscle damage markers, while various exercise performance outputs were recorded. **Results:** Study results indicated an increasing trend in plasma total polyphenol content and a reduction trend in oxidative lipid and -protein damage in participants who consumed the Rooibos. Study results also showed an improved total and reduced glutathione level ($p<0.05$) and decreased ($p<0.05$) levels of creatine kinase, aspartate aminotransferase and alanine aminotransferase when Rooibos was consumed before embarking on the exercise regime. Exercise sub maximal (75 – 80%) test outcomes indicated participants exercised for longer (14.05% increase) enabling them to cover a greater distance (13.60% increase) when they consumed Rooibos compared to placebo. Those participants also achieved a higher work rate and cycled with more (3.65% increase) power (W) and greater (2.35%) work (Kj) during the Rooibos intervention trial, while their heart rate and VO_2 relative oxygen consumption differences between the Rooibos and placebo trial was higher by 2.40% and 5.53%, respectively. this was less than overall improvement in endurance performance measures in terms of mean gain of 14.05% in time to withdrawal and 13.60% increased distance completed. After a 24 h recovery, serum lactate levels showed a decreasing trend, although not significant, when Rooibos was consumed. **Conclusion**: These study findings not only support Rooibos' known bioactivities but also make novel contributions to the field of sport and exercise and Rooibos as potential ergogenic aid to the relevant communities.

Phase III – The influence of exercise-induced oxidative stress and Rooibos consumption on the human serum metabolome

Background: Changes in the human metabolome because of different stimuli have been observed for many decades. Exercise is an external stimulus which causes disturbance to the metabolome and the whole body's homeostasis which often requires an immediate response that subsequently alters the metabolomic profile. Application of metabolomics to investigate the altered metabolome offers novel opportunities for a better understanding and evaluating an organisms' response to several challenges such as drugs/therapeutics, intense stress or exercise, food nutrients or any other stimulus to the metabolome. However, research on the metabolite shift and metabolic processes that occur and possibly underscore positive effects/outcomes after consumption of phytochemical polyphenolic-rich compounds such as Rooibos, remains unknown. **Aims:** To evaluate the influence of a standardised fermented Rooibos and placebo beverages on plasma metabolites or metabolic pathways in thirty healthy adult males following a submaximal exercise regime. **Methods:** Collected blood samples at the study time points, 0 h (baseline), immediately after exercise (IAE) and 24 h post-exercise,

were analysed and quantified using nuclear magnetic resonance (NMR) and liquid chromatography mass spectrometry (LC-MS) platforms. The study data were analysed as placebo vs Rooibos at the different study time points (0 h, IAE and 24 h post-exercise). The study participant group was also further separated into two subgroups, i.e. average sprint performers (ASP) and high sprint performers (HSP). **Results:** Metabolites did not differ significantly at the time points (0 h, IAE and 24 h post-exercise) when comparing the placebo vs Rooibos interventions for the participant group. However, when comparing the data from the subgroups, specifically the ASP, consumption of fermented Rooibos significantly increased plasma tetradecanoylcarnitine and acetylcarnitine at time points IAE and 24 h post-exercise, respectively. In the HSP group, aspartic acid was significantly reduced at time point IAE, while pipecolic acid was significantly increased 24 h post-exercise when Rooibos was consumed. **Conclusion:** In this study, fermented Rooibos consumption positively influenced the study subgroups' metabolic process, particularly the energy metabolic pathways as indicated by a significant increase in the mentioned plasma metabolites which all play key roles in the energy production process and the recovery time of exercised muscles.

Phase IV – The influence of genetic variations in genes related to oxidative stress and injury on exercise outputs following the consumption of an acute dose of a standardised fermented Rooibos beverage

Introduction: Antioxidant enzymes help to mitigate exercise-induced damage, however, genetic variations in genes encoding these antioxidant enzymes, do affect the effectiveness of these enzymes. During exercise or physical activity, this may subsequently influence the extent of the induced oxidative stress damage and/or other exercise-induced ailments in individuals. **Aim:** To determine a possible link between selected genetic variants in genes involved in oxidative stress, inflammation and exercise injury risk with oxidative stress status and muscle damage markers in a healthy, active male population. **Methods:** Blood samples were collected directly after completion of the exercise test regime for the application of pathology-supported genetic testing (PSGT). The real-time polymerase chain reaction (PCR) using TaqMan endpoint genotyping assays was used to detect genetic variants associated with inflammation (TNF-α -308 G>A), oxidative stress (MnSOD 47 T > C, V16Al), and exercise injury risks [COL1A1(G > T), GDF5 (T > C), CASP8 (CTTACT > del), MIR608 (C>G) and COL5A1 genes genetic variants (i) C > T, (ii) del > AGGG, (iii) ATCT > del, and (iv) A > T)] in thirty physically active healthy males. **Results:** When compared the baseline level and 24 h post-exercise biochemical biomarker results, participants with the mutant homozygote genotype of most analysed genes' genetic variants had increased plasma oxidative lipid (TBARS, CDs) and -protein (PC) damage, and increased serum muscle damage (CK, LDH and lactate) levels. Those with the wild-type genotype and to an extent, the heterozygote genotype, had decreased levels of the analysed damage biomarkers. **Conclusion:** Study results suggest

wild-type genotypes may have positive influences and/or protect against exercise-induced muscle and oxidative stress damage, while the mutant homozygous may negatively influence the extent of muscle and oxidative damage. Hence, it is reasonable to hypothesise that individuals with the mutated allele (mutant homozygote genotypes) may be more susceptible to muscle and oxidative damage, than those with wild-type and to an extent, heterozygote genotypes. The use of PSGT in combination with blood biochemistry markers has the potential to improve the quality of suggested dietary interventions through a comprehensive approach that involves genetic and biochemical analyses.

Graphical abstract

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DEDICATION

To the Lord my God, to you be all the Glory!!!

And

To my family, God bless you all !!!

TABLE OF CONTENTS

LIST OF TABLES

Chapter 2: Literature Review

Chapter 5: Results and discussion

LIST OF FIGURES

Chapter 2: Literature Review

Chapter 4: Materials and methods

Chapter 5: Results and discussion

Chapter 6: Summary and conclusion

ABBREVIATIONS

Abbreviation/Acronyms/Term	Definition/Explanation
$_1O_2$•	Singlet oxygen
3-PBA	3-phenoxybenzonic acid
4-HNE	4-hydroxy-2-nonenal
5-OH-Cyt	5-hydroxycytosine
5-OH-Ura	5-hydroxyuracil
8-OHdG	8-hydroxyl-2'deoxyguanosine
A	
AAE	Ascorbic acid equivalents
AAPH	2,2'-Azo-bis (2-methylpropionamidine) dihydrochloride
ABTS	2,2'-azino-di-3-ethylbenzthialozine sulphonate
ACE	Angiotensin-converting enzyme
ACSM	American College of Sports Medicine
ADP	Adenosine diphosphate
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AOX	Antioxidant
ASP	Average sprint performer
AST	Aspartate transaminase
ATP	Adenosine triphosphate
ACL	Anterior cruciate ligament
B	
BCA	Bicinchoninic acid
BHT	Butylated hydroxytoluene
BMI	Body mass index
BPM	Beats per minute
BSA	Bovine serum albumin
C	
C2	Acetylcarnitine
C14	Tetradecanoylcarnitine

CASP8	Caspase 8
CAT	Catalase
CCl4	Carbon tetra chloride
CD	Conjugated diene
CE	Catechin equivalents
CK	Creatine kinase
CONSORT	Consolidated Standards of Reporting Trials
CPUT	Cape Peninsula University of Technology
CRP	C-reactive protein
CVD	Cardiovascular disease

D

D_2O	Deuterium oxide
ddH_2O	Double distilled water
DHA	Dehydroascorbic acid
DHEA	Dehydroepiandrosterone
DHODH	Dihydroorotate dehydrogenase
DHP	Dihydropyridine
DMACA	4-dimethylaminocinnamaldehyde
DMPA	Dimethylphenylalanine
DNA	Deoxyribonucleic acid
DNP	Dinitrophenylhydrazone
DNPH	Dinitrophenylhydrazine
DoEA	Department of Environmental Affairs

E

ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ETC	Electron transport cascade/chain
ETF-QO	Electron transfer flavoprotein-ubiquinol oxidoreductase
EtOH	Absolute ethanol
EU	European Union

F

F2-IsoP	F2-isoprostanes
FC	Folin-Ciocalteu

FCR	Folin Ciocalteu reagent
FDR	False discovery rate
$FeCl_3$	Iron (III) chloride
FI	Fluorescein
FRAP	Ferric reducing antioxidant power
FRs	Free radicals

G

GAE	Gallic acid equivalents
GC-MS	Gas chromatography-mass spectrometry
GDF5	Growth Differentiation Factor 5
GGT	Gamma-glutamyl transferase
GI	Geographical indications
GPx	Glutathione peroxidation
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GST	Glutathione–S-transferase
GTE	Green tea extract
GTP	Green tea polyphenol

H

H_2O	Water
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HDL	High density lipoprotein
H_3PO4	Ortho-phosphoric acid
HNa_2PO_4	Sodium phosphate dibasic
HNO_2•	Nitric dioxide radical
HOCl	Hypochlorous acid
HPLC	High-performance liquid chromatography
HSP	High sprint performer
HRmax	Maximum heart rate

I

IL-1β	Interleukin 1β
IL-6	Interleukin 6

IsoP	Isoprostanes

K

KGDH	ketoglutarate dehydrogenase

L

LC-MS	Liquid chromatography mass spectrometry
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LFT	Liver function test
LMWA	Low molecular weight antioxidants
LOOH	Lipid peroxide radical
LPO	Lipid peroxidation

M

M2VP	1-methyl-2-vinylpyridinium
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MDA-TBA	Malondialdehyde- thiobarbituric acid
MeOH	Absolute methanol
MnSOD	Mitochondrial superoxide dismutase
MPA	Meta-phosphoric acid
MS	Mass spectrometry

N

N_2O_2	Dinitrogen trioxide
Na_2CO_3	sodium carbonate
NADPH	Nicotinamide adenine dinucleotide phosphate
NaF	Sodium fluoride
NaH_2PO_4	Sodium dihydrogen monophosphate
NaN_3	Sodium azide
NFκ B	Nuclear factor kappa B
NaOH	sodium hydroxide
NFκ B	Nuclear factor-kappa B
NMR	Nuclear magnetic resonance

NO	Nitric oxide
NO•	Nitric oxide radical
NOS	Nitric oxide synthase
Nrf2	Nuclear factor erythroid-related factor-2
NSAID	Non-steroidal anti-inflammatory drug

O

O_2•	Superoxide radical
O_3	Ozone
OH•	Hydroxyl radical
ONOO•	Peroxynitrite
OS	Oxidative stress
OSRC	Oxidative Stress Research Centre
oxLDL	Oxidised LDL

P

PBS	Phosphate buffer saline
PCA	principal component analysis
PC	Protein carbonyls
PCR	Polymerase chain reaction
PDO	Protected designations of origin
PSGT	pathology-support genetic testing
PUFA	Polyunsaturated fatty acid

Q

QC	Quality control

R

RNA	Ribonucleic acid
RNS	Reactive nitrogen species
RO•	Alkoxyl radical
RONS	Reactive oxygen nitrogen species
ROO•	Peroxyl radical
ROOH•	Hydroperoxyl
ROS	Reactive oxygen species
RPE	Rate of perceived exertion

RPM	Revolution per minute
RS	Reactive species
RT	Room temperature
RT-PCR	Reverse transcript Polymerase chain reaction

S

SD	Standard deviation
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
SST	Serum separate tube

T

TAC	Total antioxidant capacity
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
tGSH	total Glutathione
TNB	5-thionitrobenzoic acid
TNF- α	Tumour necrosis factor- Alpha
TPC	Total phenolic content
TPTZ	2,4,6-tri [2-pyridyl]-s-triazine
TRX	Thioredoxin
TSP	Trimethylsilyl-2,2,3,3-tetradeuteropropionic acid

U

UDP-GT	Uridine diphosphate glucuronyltransferase
UV	Ultraviolet light

W

WHO	World Health Organisation

X

XO	Xanthine oxidase

GLOSSARY

Anti-inflammatory cytokines	Cytokines which inhibit inflammation
Antioxidant	Substance or reducing agents that delay, detoxify, attenuate and/or prevent or remove oxidative damage to a substrate
Antioxidant network	A mechanism whereby antioxidants interact with each other to regenerate their antioxidant properties
Biomarker	A measurable product that can be used to objectively measure normal biological processes, pathological processes or pharmacological and/or biochemical responses to therapeutic/nutritional treatment
Cytokines	Cell signalling proteins/molecules that regulate immune and inflammatory responses
Direct antioxidant	Short-lived, redox-active antioxidants which often consumed or chemically modified in the process of their antioxidant activity and are likely to evoke pro-oxidant effects
Endogenous	Originating within an organism, tissue or cell
Exogenous	Originating from outside of an organism, supplied externally
Fenton reaction	The non-enzymatic reaction of hydrogen peroxide with ferrous iron produces hydroxyl radicals and ferric iron
Flavonoids	Plant secondary metabolites which are a subclass of polyphenols
Free radical	A highly reactive molecule capable of independent existence and possess one or more unpaired electron in its outer shell
Genome	A complete set of DNA present within an organism, cell, or tissue
Genomics	An analysis aimed at identifying/measuring or comparing genome features such as DNA sequence, gene expression or structural variations
In vitro	Experimentation on isolated cell components e.g., in a petri dish
In vivo	Experimentation in/on the intact or whole organism
Indirect antioxidants	Redox-inactive antioxidants, which are unlikely to evoke pro-oxidant effects, but can activate and boost the endogenous antioxidant system
Inflammation	Protective tissue responds to harmful stimuli, such as pathogens, damaged cells or irritants
Interleukins	The family of cytokines produced by white blood cells to modulate inflammation and immune response

Lipidomics	A branch of metabolomics that involves the comprehensive analysis of all lipids, fatty acids, and lipid-like molecules in a biological or environmental sample
Metabolic fingerprinting	A metabolomic analysis process that makes use of biofluids (blood, serum and plasma) critically needed to sustain a living cell or organism. This analysis process method is commonly used in whole organism metabolomic studies as well as in cell, cell culture, or microbial studies.
Metabolic foot printing or exo-metabolomics	A metabolomic analysis that makes use of waste biofluids (cell media excreta, urine, saliva, sweat, faecal water) that are discarded or secreted by a living organism or found in cellular growth media. This method is commonly used in microbiology and biotechnology.
Metabolic profiling	It is a term that is normally reserved for metabolomic studies with a smaller, more defined set of metabolites that may have common physiochemical properties (e.g. carbohydrates, amino acids, organic acids, and nucleotides) or are involved in specific metabolic pathways (such as glycolysis, gluconeogenesis, beta-oxidation, beta-oxidation, and the Krebs cycle)
Metabolites	Low molecular weight substrates/ intermediate/ end products of cellular metabolism that play a crucial role in energy production, storage, cell signalling, apoptosis and provide information on the physiological state of an organism
Metabolome	The complete set of low molecular weight metabolites (primary metabolites, secondary metabolites, endogenous and exogenous compounds) that can be found in a cell, a tissue, a biofluid, or an organism and it indicates the physiological or pathological status of any organism
Metabolomics	The comprehensive characterization of metabolites (small molecules with molecular weight < 1500 Da) and other chemical species (both exogenous and endogenous) in biological specimens in response to different perturbations or interventions
Metabonomics	Often synonymous with metabolomics. Metabolomics is generally focused on the application of metabolomic methods to study metabolic responses to therapeutic interventions or genetic modifications
Nutrigenomics	A genomic analysis that focuses on an individual's response and/or sensitivity to nutrient intake or supplementation
Oxidant	A species or chemical derivatives that cause or promote oxidation
Oxidation	Loss of electron from a molecule
Oxidative stress	A cellular condition caused by an imbalance between the production and accumulation of reactive species in the cells/tissues and the ability of a biological system to detoxify these reactive species/products

Phenolic compound	Compound that has an aromatic ring bearing one hydroxyl group only
Phytochemical	Any biologically active compound occurring naturally in plants
Polyphenol	A compound containing one or more phenol rings
Polyphenolic compound	Compounds that possess more than one aromatic ring bearing more than one hydroxyl group
Primary metabolites	Metabolites produced by endogenous catabolism and anabolism and are essential for living organism growth, development and other physiological functions
Pro-inflammatory cytokines	Cytokines which promote inflammation
Reactive oxygen and nitrogen species	Molecules produced by the incomplete reduction of oxygen or nitrogen
Reactive species	A collective name describing both reactive oxygen species and non-radical reactive derivatives
Recovery	A rate at which muscle function returns to normal/baseline after becoming fatigued
Secondary metabolites	Metabolites from exogenous sources (diet/food, microbial products, pollutants, pesticides or other environmental factors) and could be essential or sometimes detrimental to an organism's growth and development. These metabolites are also sometimes referred to as xenometabolites
Spectra deconvolution	A manually or semi-automated process used to determine which peak (molecule/metabolite) present in biofluid NMR or MS spectra matches a compound in the database of the referential NMR or MS spectra of pure compounds
Synergistic	The interaction between two compounds such that the total effect is interaction greater than the sum of individual effects
Targeted metabolomics	A metabolomic analysis approach aimed at analysing specific groups of known metabolites of similar properties or those belonging to a certain group or metabolic pathways of interest and is considered ideal for hypothesis testing (answering a hypothesis which has already been formulated) and biomarker detection
Untargeted metabolomics	A metabolomic analysis approach aimed at analysing all detectable known and unknown metabolites in biological samples, and is considered ideal for hypothesis generation and biomarker discovery

CHAPTER ONE

INTRODUCTION

The health benefits of exercise are undeniable, however, under certain conditions, it induces an increase in oxygen consumption which leads to the continuous formation of reactive species (RS) such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Elejalde et al., 2021). Depending on the concentration, RS may be harmful or beneficial to living organisms. At low and/or moderate concentrations, RS are involved in physiological roles such as cell signalling, defence against infectious agents and upregulation of endogenous enzymatic antioxidant (AOX) activity (Steinbacher & Eckl, 2015). However, at high concentrations, RS may result in oxidative stress (OS) and subsequently damage of important cellular macromolecules (lipid, proteins, and DNA) and consequently alteration of their functions (Gupta et al., 2014; Elejalde et al., 2021) and decline of the antioxidant capacity of defence systems. To mitigate against RS damaging effects, living organisms are equipped with a complex network of an antioxidant defence system that can neutralise and/or stabilise these RS (Poljsak et al., 2013; Elejalde et al., 2021). However, once the defence system fails or gets compromised due to a depletion of endogenous AOX enzymes, diseases development, ageing or inadequate dietary antioxidant intake this leads to the occurrence of OS in the system develops (Powers et al., 2004). Thus, it is of utmost importance to continuously support the endogenous antioxidant system and replace the AOX sources in the body, through the intake of naturally occurring AOX, so that when the body is subjected to a redox imbalance, it will be in a better position to defend itself (Elejalde et al., 2021).

Many studies have showed that high oxygen intake due to high energy demand during exercise increases the generation of reactive oxygen and nitrogen species (RONS) resulting in exercise-induced OS, fatigue, muscle damage and overall poor exercise performances and recovery. Surprisingly, to date, there is still no official protocol/approach/practice to prevent or mitigate exercise-induced ailments (Wan et al., 2017). Nonetheless, many athletes opt to use some nonspecific treatment and preventative approaches such as the use of synthetic drugs. The safety and efficacy of these drugs have been questioned by some studies (Nieman et al., 2006; Wan et al., 2017). Moreover, these drugs are often very expensive, and normally can only be obtained through a medical doctor's prescription and some have been made known to cause serious side effects and lack tolerance in certain individuals. Therefore, novel interventions are needed to supplement or substitute the use of synthetic drugs. Therefore, there is a need to identify and develop safer, more effective, and well-tolerated non-prescription prophylaxis procedures or preventative approaches for these athletes. On this basis, scientific research into natural products such as phytochemicals, polyphenol-rich compounds has increased dramatically in the last two to three decades due to their numerous bioactive properties, including positive health impacts. However, to date, literature and/or understanding of metabolite shift and genetic variations influences on OS and exercise

performance upon consumption of polyphenolic compounds is limited. Guest et al. (2019) pointed out that, there is strong research-based evidence linking both metabolites and gene variations influence on OS damage and exercise outcomes. Thus, it is important to know and understand the modulatory effect of polyphenolic compounds as well as their influence on metabolite shifts and genetic variability in relation to OS-induced damage and exercise performance outcomes. Historically, plants' natural products were used to treat and alleviate a wide range of ill-health conditions (Che & Zhang, 2019). Currently, there is an increasing trend worldwide in consumers shifting to botanical dietary supplements and phytopharmaceuticals (natural remedies) as preventative strategies for the maintenance of health (Calixto, 2019). The use of natural plant products as a means of modulating exercise-induced OS pathologies and other related exercise ailments emerge to have several advantages over synthetic drugs, because many natural plant products are: **a)** easily accessible, **b)** contain unique compositions of various bio-active, **c)** typically have low or no toxicity, **d)** react with most or all types of reactive species, and **e)** are more compatible to normal human physiology (Anon, 2002; Sen et al., 2010; Hong et al., 2014; Calixto, 2019; Jantan et al., 2021; Davies et al., 2023). Rooibos (*Aspalathus linearis*) is a popular indigenous South African herbal tea enjoyed for its taste and aroma. For generations, it has been grown and harvested in the Cederberg mountainous region of South Africa, Western Cape province. It has traditionally been used as a therapeutic remedy, to alleviate colic disorders, eczema, nausea, asthma, headache, allergies etc (McKay & Blumberg, 2007). Recently, it gained many researchers' attention due to its rich and unique phytochemical content with potent bio-activities including antioxidant, anti-inflammatory and anti-fatigue properties which are of potential health use to prevent, detoxify and attenuate exercise-induced OS effects, and inflammatory responses as well as improving exercise performance and recovery (Chen et al., 2013; Hong et al., 2014; Watanabe et al., 2014), hence its consideration for the current study.

1.1 Research focus

The present study is based on the fact that, despite the widely acknowledged impacts of OS on human health, this phenomenon is not yet well enough understood to allow for effective clinical manipulation and potential development of therapeutic interventions and/or modulators (Sharifi-Rad et al., 2020; Ji & Yeo, 2021). After many years of research studies focused on oxidative stress, there is little therapeutic interventions directly attributed to the research into oxidative stress mainly due to the lack of standardised human models. Despite, many developed *in vitro* and animal models of oxidative stress-based research to date there is no reliable human intervention model. Even though the *in vitro* and animal models are helpful, they do not generally translate well to humans in the field of oxidative stress (Oteiza et al., 2021). Furthermore, it is challenging to induce oxidative stress in a predictable and controllable manner in a human without undue risk of further harm (Alberto De La Riva et al., 2023).

Nonetheless, this phenomenon must be further studied to understand the role of radicals and AOXs in physiology, but also for possible development of modulatory and/or therapeutic interventions.

Generally, if OS is not effectively and properly managed, it may lead to damage of important cellular macromolecules including DNA, lipid and protein (Kurutas, 2015; Luna & Estévez, 2018) and eventually cellular dysfunctions, impairments and/or ultimate development of several diseases (Pingitore et al., 2015). However, genes encoding for the endogenous AOX enzymes that help to mitigate potential oxidative stress damage, unfortunately, might possess genetic variations that may affect/ impact the effectiveness of these AOX enzymes and subsequently influence the extent of OS damage or other exercise-induced ailments in individuals. To date, there is a lack of therapeutic/ interventional approaches for mitigating exercise-induced cell stress. The synthetic drugs that are commonly used to counteract the harmful effects of exercise-induced cell stress are expensive and often have serious side effects as most of them are stimulants (Nieman et al., 2006). Therefore, novel, safer and relatively inexpensive approaches are needed to supplement, substitute and/or support current existing approaches. It has been observed that consumption of polyphenolic-rich plant phytochemical may offer some help. For centuries, people have consumed plants and their products for food, nutritional support and/or as a source of medicines (Che & Zhang, 2019). However, current literature or knowledge on metabolite shift and molecular mechanisms that occur upon consumption of phytochemical flavonoid compounds is very limited (Oteiza et al., 2021). Hence, the application of metabolomics to analyse metabolites are sensitive to both external and internal stimuli and possess the ability to reflect what is going on within the organism's metabolome in response to perturbations or interventions (Duft et al., 2017; Wishart, 2019). Furthermore, metabolomic provides a platform to understand the cellular mechanisms from a metabolite point of view, which may also help in the design and/or development of a potential modulator or interventional therapy to mitigate exercise-induced oxidative stress ailments (Miao et al., 2018). In the past two to three decades, the identification of plant products and their ingestion by individuals and sports professionals in an attempt to minimise the effects of OS experienced during certain physical activities has significantly increased. Furthermore, there is an increasing trend worldwide by consumers including natural products in their daily diet to serve as health boosting, disease prevention strategies and to maintain their health (Calixto, 2019). It has been well reported that various plants and natural products have long been used to treat almost all kinds of ill-health conditions (Che & Zhang, 2019). Hence, it is not surprising that athletes use of natural plant products as a means to modulate exercise-induced ailments or pathologies emerging to have several advantages over synthetic drugs. Rooibos, an indigenous South African herbal tea and a dietary source of unique bioactive including AOXs, may complement and assist in alleviating and/or mitigate

exercise-induced ailments or deleterious effects caused by excessive RONS. The research into the health benefits of bioactive appears to have started in the 1980's driven by the importance of antioxidants to human health, but more recently research has highlighted, additional health benefits associated with fruit and vegetable consumption (Oteiza et al., 2021). A detailed explanation on exercise and OS, the antioxidant defence systems, exercise-induced alterations of the human metabolome, genetic variations, phytochemical polyphenols, and lastly the promising Rooibos as a potential polyphenolic-rich phytochemical source, and the role(s) it may play to alleviate and mitigate exercise-induced OS and related ailments are reviewed in chapter 2.

The study aim was motivated by the identification of gaps in the current body of knowledge (Chapter 3) related to exercise-induced oxidative stress in humans, effect on the metabolome, the role of genetic variability and exercise performance outcomes and potential dietary interventions, with focus on using the indigenous herbal tea, Rooibos. These gaps are addressed in the **four phases** of our study, with **phase I**, establishing an exercise-induced oxidative stress human model suitable for testing any herbal intervention, **Phase II**, using the proposed model (phase I) to assess the modulatory role and potential of a standardised fermented Rooibos beverage to serve as an ergogenic aid using a dietary intervention design, **Phase III**, evaluating the effect of a standardised fermented Rooibos beverage on the study participants' plasma metabolome with emphasis on possible metabolite(s) shift and/or metabolic pathways and lastly **phase IV**, evaluating the influence of genetic variability in the human antioxidant system on exercise-induced oxidative stress and muscle damage biomarkers. A description of the study participant cohort and methods used in the various study phases is detailed in Chapter 4 while the findings obtained for each of the study phases are presented in the results and discussion section (Chapter 5). The study summary and conclusion reached based on the new knowledge and results of this study is presented in Chapter 6. The references is provided in Chapter 7, followed by additional results pertaining to each of the study phases, while the thesis is concluded with Chapter 8, appendices of relevant study support documents such as consent forms, questionnaires and the ethical clearance certificate issued before commencement of this research study.

1.2 Scientific outputs from this study

Published:

1. Simeon E.H. Davies, Jeanine L. Marnewick, Dirk J. Bester, **Oiva V. Kamati**. 2024. Positioning research and scholarly innovation within a "transdisciplinary" approach: the case of the south African Indigenous plant rooibos (*Aspalathus linearis*) and its potential As an ergogenic aid. RISUS - Journal on Innovation and Sustainability. Volume 4 (14): 84 – 103.

https://revistas.pucsp.br/index.php/risus/article/view/62867.DOI:org/10.23925/2179-3565.2023v14i4p84-103.

2. Simeon E.H. Davies, Jeanine L. Marnewick, **Oiva Kamati**, Dirk J. Bester. 2023. The power of transdisciplinary research for business innovation: the case of rooibos as a potential ergogenic sport drink. Chapter 2 in the Book" Current Aspects in Business, Economics and Finance, ed: Dr chun-Chien Kuo, Publisher: BP International, online publication. Print ISBN: 978-81-19039-04-3, eBook ISBN: 978-81-19039-06-7; DOI: 10.9734/bpi/cabef/v8Co-author.

Submitted:

1. Oiva Viety Kamati, Simeon Davies, Roan Louw, Jeanine L Marnewick. Rooibos polyphenolic constituents as dietary supplement for physical performance: evidence to date. (Under review: Heliyon, manuscript number Heliyon-D-23-37552).

2. **Oiva V. Kamati**, Simeon Davies, Roan Louw, Laura Bragagna, Karl-Heinz Wagner, Dirk Bester, Jeanine L Marnewick. The modulatory role of fermented Rooibos on oxidative stress and its impact on submaximal exercise performance in adult males. (submitted to the South African Journal for Research in Sport, Physical Education and Recreation (SAJRSPER).

Manuscripts in draft:

1. Oiva Viety Kamati, Dirk Bester, Laura Bragagna, Karl-Heinz Wagner, Simeon Davies, Roan Louw, Jeanine L Marnewick. Establishment of an exercise-induced oxidative stress model for testing of Rooibos and other herbal interventions.

2. **Oiva Viety Kamati**, Simeon Davies, Laura Bragagna, Karl-Heinz Wagner, Dirk Bester, Roan Louw, Jeanine L Marnewick. Fermented Rooibos protects against oxidative stress and improves performances and hemodynamics in sprinters differently after aerobic and anaerobic exercise regimes.

3. **Oiva Viety Kamati**, Simeon Davies, Laura Bragagna, Karl-Heinz Wagner, Dirk Bester, Roan Louw, Jeanine L Marnewick. The influence of Rooibos ingestion on human metabolites, metabolic pathways, and cardiorespiratory functions.

4. **Oiva Viety Kamati**, Simeon Davies, Kelebongile E Moremi, Maritha J Kotze, Roan Louw, Jeanine L Marnewick. The influence of genetic variations in genes related to oxidative stress and muscle damage susceptibility on blood biochemical biomarkers of adult males following an exercise test regime.

***Conference presentations*:**

1. Oiva Viety Kamati, Simeon Davies, Roan Louw, Jeanine L Marnewick. Rooibos, a potential oxidative stress modulator and ergogenic aid. Oral presentation at the CPUT research festival, postgraduate conference, (1 March 2023).

2. Simeon EH Davies, Jeanine L Marnewick, **Oiva Kamati** and Dirk Bester. The power of transdisciplinary research for business innovation: the case of rooibos as a potential ergogenic sport drink. International Conference on Business and Management Dynamics, Hybrid meeting, online presentation, 7-8 September 2022. (Co-author, oral presentation).

3. Oiva Viety Kamati, Simeon Davies, Kelebongile E Moremi, Maritha J Kotze, Roan Louw, Jeanine L Marnewick. The association between exercise-induced pro-inflammatory response and genetic individuality: A proposal, at the CPUT Biotechnology Research Symposium, online oral presentation (7 September 2022).

4. Oiva V. Kamati; Simeon E.H. Davies; Jeanine L. Marnewick, Roan Louw. Effects of rooibos intake on the human metabolome and gene expression: a proposal, at the CPUT postgraduate conference, online presentation (November 2021), published abstract in CPUT postgraduate conference 2021 booklet.

5. Oiva V. Kamati; Naeem S. Abdul; Dirk J. Bester; Simeon E.H. Davies; Jeanine L. Marnewick. Effect of a dietary rooibos beverage on exercise-induced oxidative stress, performance and recovery at the 1st joint Conference of the Pan African Environmental Mutagen and Genomics Society and Society for Free Radical Research: Africa, online meeting held 15-16 September 2021.

6. Oiva V. Kamati; Naeem S. Abdul; Simeon E.H. Davies; Jeanine L. Marnewick. Effect of Rooibos on exercise-induced oxidative stress, performance, and recovery at the CPUT postgraduate conference, online presentation (November 2020), published abstract in CPUT postgraduate conference 2020 booklet.

Research Award

1. Young investigator: Best e-poster prize to **Oiva V. Kamati;** Naeem S. Abdul; Dirk J. Bester; Simeon E.H. Davies; Jeanine L. Marnewick. Effect of a dietary rooibos beverage on exercise-induced oxidative stress, performance and recovery at the 1st joint Conference of the Pan African Environmental Mutagen and Genomics Society and Society for Free Radical Research: Africa, online meeting held 15-16 September 2021.

1.3 Other scientific outputs

1. Olawande Daramola, Tatenda Duncan Kavu, Maritha J Kotze, **Oiva Kamati**, Zaakiyah Emjedi, Boniface Kabaso, Thomas Moser, Karl Stroetmann, Isaac Fwemba, Fisayo Daramola, Martha Nyirenda, Susan J van Rensburg, Peter S Nyasulu and Jeanine L Marnewick. 2023. Detecting the most critical clinical variables of COVID-19 breakthrough infection in vaccinated persons using machine learning. Digital Health Volume 9: 1–23. DOI: 10.1177/20552076231207593 journals.sagepub.com/home/dhj.

CHAPTER TWO

LITERATURE REVIEW

2.1 What is oxidative stress?

The term oxidative stress (OS) was coined and conceptualised by Sies and Cadenas (1985) as a “disturbance in the prooxidant-antioxidant balance in favour of the former”. However, as the knowledge grows and the field of redox biology advances, OS has been redefined as a cellular condition caused by an imbalance between the production and accumulation of reactive species in the cells/tissues and the ability of a biological system to detoxify these reactive species/products (Jones, 2006). Basically, OS is the consequence of an imbalance between the generation and removal of reactive species which are generally generated during either normal physiological conditions (cellular metabolism) or due to other various factors mainly diseases, ageing, poor diet, exhaustive and prolonged training, deficiency of antioxidant compounds (Sies, 2000; Urso & Clarkson, 2003; Powers & Jackson, 2008; Liguori et al., 2018; Powers et al., 2020). Excessive generation and/or accumulation of different reactive species (RS) particularly reactive nitrogen species (RNS) or reactive oxygen species (ROS) (Table 2.1) may overwhelm the cellular antioxidants defence system, and this is believed to be the primary cause of OS in humans and other living organisms. Furthermore, countless exposures to exogenous (environmental and synthetic) and endogenous (natural) toxicant agents (Table 2.2) also contribute to the formation and/or exacerbate the generation of RS in humans and other living organisms (Davis et al., 2010; Gupta et al., 2014; Phaniendra et al., 2015), which may subsequently lead to OS condition if no intervention made.

Human and animal studies demonstrated that accumulation of reactive oxygen and nitrogen species (RONS) leads to damage of macromolecules (lipid peroxidation, cell membrane damage, glutathione depletion, DNA strand breaks, protein denaturation and activation) resulting in development of cellular dysfunctions and/or several pathologies (Mastaloudis et al., 2004; Awoniyi et al., 2011; Pingitore et al., 2015; Demirci-Çekiç et al., 2022). Furthermore, these RONS are regularly generated as by-products of normal cellular metabolism, therefore, human, and other living organism exposure to RONS is unavoidable (Ji & Yeo, 2021). For instance, when adenosine triphosphate (ATP) energy is generated in the cell, oxygen is used, and free radicals such as superoxide and hydroxyl radicals are produced in the process as by-products (Prior & Wu, 2013). When RONS such as nitric oxide (NO) reacts with superoxide radical form peroxynitrite (ONOO•) free radical which is most RS responsible for damage of macromolecules (Figure 2.1) (Dröge, 2002; Wang & Hai, 2016). Free radical (FR) refer to a highly reactive molecule capable of independent existence and possessing one or more unpaired electron(s) on its outer shell, which makes it not stable enough, hence, it becomes reactive towards other molecules to gain stability. While non-free radicals are molecules formed when two FRs share their unpaired electrons (Morales-Alamo & Calbet, 2014; Kurutas, 2015; Demirci-Çekiç et al., 2022).

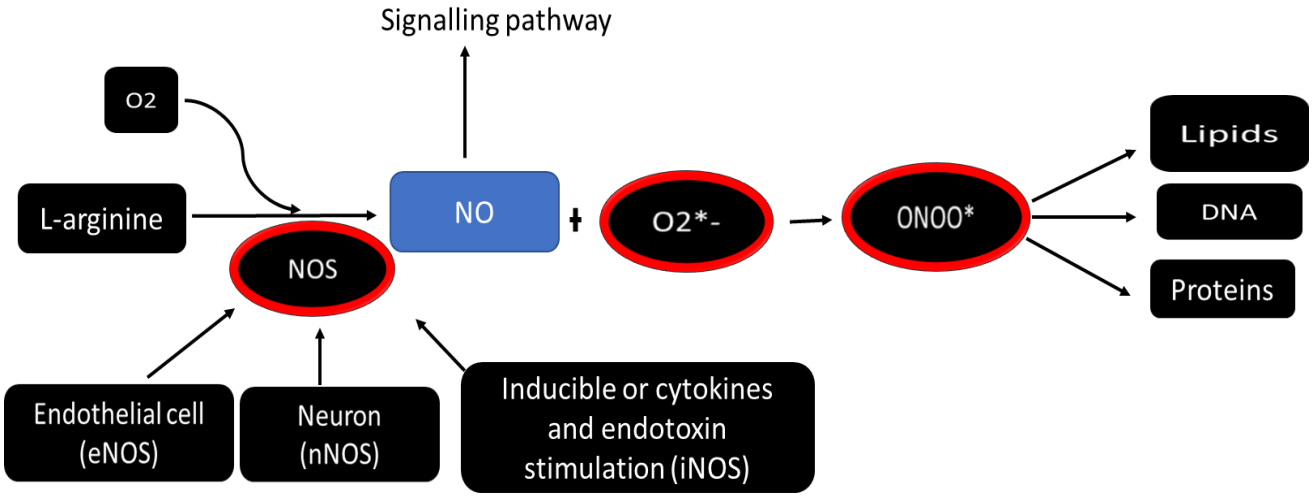


Figure 2.1: Source of Nitric oxide synthases (NOS) and interaction with reactive oxygen species (ROS).

(NOS) catalyse the reaction between L-arginine and oxygen, to generate nitric oxide (NO) which then reacts with superoxide to produce peroxynitrite (ONOO•), radical which can damage macromolecules severely.

Table 2.1: Reactive species and their effects on macromolecules

Reactive species	**Formula**	**Main effects**
Reactive oxygen species (ROS)		
Free radicals		
Superoxide	O_2•	Lipid peroxidation Protein oxidation DNA damage
Hydroxyl	HO•	
Peroxyl	ROO•	
Alkoxyl	RO•	
Non-free radical		
Hydrogen peroxide	H_2O_2	
Hydroperoxyl	ROOH•	
Hypochlorous acid	HOCl	
Ozone	O_3	
Singlet oxygen	$_1O_2$	
Lipid peroxide	LOOH	
Reactive nitrogen species (RNS)		
Dinitrogen trioxide	N_2O_3	Lipid peroxidation Protein oxidation
Peroxynitrite	ONOO•	

Nitrogen dioxide	NO_2•	DNA damage
Nitric oxide	NO•	

Table 2.2: Sources of exogenous and endogenous reactive species

Exogenous sources (environmental and synthetic)	Endogenous sources (natural)
Water and air pollution	Aerobic metabolism
Cigarette smoke	Physical activity (exercise),
Alcohol	Microbial infection involves the activation of phagocytes
Heavy or transition metal (e.g., lead, silver, iron)	Inflammations
Medication (e.g., bleomycin, gentamycin, tacrolimus etc.)	Metal stress
Industrial solvents	Ischemia
Cooking (e.g., smoked meat, used oil and fat)	Cancer
Radiations	Ageing

However, despite RONS being causative agents of OS damage and cellular dysfunction, RONS are also essential to living organisms' well-being. For instance, ROS generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases play a vital role in the insulin signalling pathway (Mahadev et al., 2004). While RONS generated by immune cells acts as a defence mechanism against numerous strains of bacterial and fungal infections (Freitas et al., 2010; Poljsak et al., 2013). Additionally, RONS also play regulatory roles in signalling pathways, upregulating gene expression of transcription factors, differentiation, and development (Steinbacher & Eckl, 2015; Thirupathi & Pinho, 2018), however, these roles are only possible when RONS is generated at moderate concentration.

2.2 Oxidative stress and macromolecule damage

As mentioned earlier, living organism exposure to RONS which may lead to damage of macromolecules (lipid, protein, and DNA) is a continuous and unavoidable phenomenon (Demirci-Çekiç et al., 2022). However, the extent of damage suffered often depends on various factors such as the nature of the targeted macromolecule, concentration of the targeted macromolecular, location of targeted macromolecule and occurrence of secondary damaging events such as chain reactions (Davies, 2016). The damage of macromolecules can be assessed or quantified by measuring the stable end product (biomarker) of OS damage processes. Biomarker refers to an indicator that can be used to objectively measure normal physiological or pathological processes (changes that occur in the body during health, or diseases) or in response to pharmacological or nutritional treatment. Biomarkers can be also used to diagnose diseases, monitor treatment and/or prognosis, or evaluate the effects of drugs, food, beverages, or supplements (Niki, 2014). However, for a product to be considered a reliable biomarker of OS, it should be **(i)** a stable oxidation product that can accumulate to a

detectable level, **(ii)** the source and oxidative pathway should be explicit/unambiguous, **(iii)** its concentration should reflect the severity of that specific sickness/ailment and **(iv)** it should not show diurnal variations (Isabella et al., 2006; Margonis et al., 2007).

2.2.1.1 Lipid peroxidation and biomarkers

Lipids are macromolecules, primarily involved in energy storage, cell membrane structural components, cellular metabolism, and signal transductions (Demirci-Çekiç et al., 2022). Most lipids are made up of fatty acids composed of two or more carbon double bonds which make them more prone to ROS oxidation, resulting in the lipid peroxidation (LPO) process. The LPO sometimes referred to as lipid auto-oxidation occurs when the hydrogen atom on the methylene group next to the carbon double bond is removed by RS such as peroxynitrite. This result into formation of a carbon-centred radical that stabilised through rearrangement of double bonds and subsequently form conjugated dienes (Demirci-Çekiç et al., 2022). When the carbon-centred radical is formed, it reacts with oxygen molecules in the cell membrane and generates a peroxyl radical in the process which starts a chain reaction and transforms the polyunsaturated fatty acids (PUFA) into lipid hydroperoxides (Birben et al., 2012). Lipid hydroperoxides are very volatile and easily decompose into other relatively reactive stable aldehyde products such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE), hydrocarbons (pentane and ethane), isoprostanes and trans conjugated dienes, which can be measured in plasma, serum, or urine as a biomarker of OS damage LPO (Wood et al., 2000; Niki, 2014; Kurutas, 2015). This OS damage lipids cause disturbance of cell membrane integrity and impair its functions due to loss of membrane fluidity and inactivation of membrane-bound enzymes which may lead to the development of various pathological disorders (Kurutas, 2015). To assess lipids damage, colourimetric assays such as thiobarbituric acid-reactive substances (TBARS) have been utilised over the years mainly due to their simplicity and convenience/user-friendly (Draper et al., 1993; Marnett, 1999; Tsikas, 2017), however, these assays lack specificity and sensitivity hence, their usage has now declined. But, as technology advances, more and better techniques such as chromatography, fluorescence, mass spectrometry (MS), immunochemistry and high-performance liquid chromatography (HPLC) have been developed to improve detection and quantification of LPO biomarkers such as malondialdehyde (MDA), conjugated dienes (CDs), isoprostanes, lipid hydroperoxides and oxidised low-density lipoprotein in biological samples (Hwang & Kim, 2007; Cháfer-Pericás et al., 2016).

1*. Thiobarbituric acid-reactive substances (TBARS) -* ***the*** common assay used to determine MDA. Under an acidic condition, MDA reacts with thiobarbituric acid (TBA) and produces a stable, pinkish-coloured product (MDA-TBARS) which is quantified spectrophotometrically at 532 nm (Hwang & Kim, 2007; Tsikas, 2017). Despite the assay's simplicity, affordability and

user-friendly, it has some limitations and has been criticised by many researchers (Birben et al., 2012; Tsikas, 2017). Firstly, the TBARS assay lacks specificity, as many other reactions end products other than MDA (e.g., bilirubin, amino acids, ketones, and other aldehydes) can react with TBA and produce similar products to the MDA-TBA adduct. Secondly, artefactual production of MDA during analysis (heating step during analysis can induce fatty acids degradation) gives no assurance that measured MDA is from oxidative processes itself. Thirdly, discrepancies in data variability (MDA levels) even in blood samples from the same person, and finally, lack of standardised procedure/protocol, hence, one could not even compare current literature results since different laboratories use different TBA and the colour intensity formed during the reaction depends on the type and strength of the TBA used. Due to such difficulties, the assay's utility has been declining and/or increasingly modified to include the HPLC procedure so that MDA-TBA adducts/products are separated from interfering chromophores which helps to improve the assay's specificity and data validity (Hwang & Kim, 2007; Birben et al., 2012).

***2**. Conjugated dienes (CDs)* are first products of LPO, formed from the rearrangement of carbon double bonds after the abstraction of hydrogen atom (Demirci-Çekiç et al., 2022). Spectrophotometrically, CDs can be detected and quantified at 230 – 235 nm which itself could be a possible assays' limitation, because many other biological substances (e.g., PUFA) can be detected in the same UV ranges as well. Additionally, CDs serum concentration can be easily influenced by diet, hence, for the assay and study validity, diet should be always strictly controlled (Romieu et al., 2008).

***3**. Isoprostanes (IsoP)* are stereoisomers of prostaglandin F, mostly formed during the LPO of arachidonic acids (Ďuračková, 2010). Isoprostanes are generally formed *in situ*, esterified to phospholipids, cleaved, and released into the bloodstream by phospholipase (Roberts & Morrow, 2000). Once IsoP released into bloodstream, they only stay for about 18 minutes and then excreted which then posed an analytical dilemma because of their short plasma half-life (Roberts & Morrow, 2000). However, to analyse IsoP formed over a given period, urine is considered a suitable sample (Milne et al., 2015). Up to 64 IsoP can be formed, but F_2-IsoP (8-iso-PGF_2) is the more abundant one and is widely used or accepted as the reliable biomarker of LPO *in vivo* (Kadiiska et al., 2005; Niki, 2014). Several analytical procedures such as gas chromatography (GC), enzyme-linked immunosorbent assay (ELISA) and MS are used to analyse F_2-IsoP in biological samples (Roberts & Morrow, 2000), but ELISA is found to be less accurate due to cross-reactivity (Roberts & Morrow, 2000; Niki, 2014). Same to CDs, IsoP serum/plasma concentration can be also easily influenced by diet, hence, the diet should be strictly and firmly monitored (Gopaul et al., 2000; Ďuračková, 2010), however, diet doesn't influence urinary IsoP level (Roberts & Morrow, 2000).

***4**. Lipid hydroperoxides* are primary end products of cholesterol and PUFA oxidation, mostly caused by singlet oxygen and/or lipoxygenase. Lipid hydroperoxides are very unstable and easily react with metal ions (copper and iron) and form other RS (Cháfer-Pericás et al., 2016). For the detection and quantification of lipid hydroperoxides in biological samples, HPLC can be used to separate different kinds of lipid hydroperoxides and quantify them with either chemiluminescence, fluorescence or MS procedure. However, these procedures require a high level of expertise and costly equipment (Dalle-Donne et al., 2003; Niki, 2014; Peña-Bautista et al., 2018), thus, their usage is rare and analysis of lipid hydroperoxides in research and clinical labs is not quite common.

***5**. Oxidised low-density lipoprotein (ox-LDL)* is a cholesterol particle, also known as bad cholesterol. Low-density lipoproteins are mostly oxidised by lipoxygenase, cytochrome P450, myeloperoxidase and by some FRs and non-free radical oxidants (Itabe, 2012). The oxLDL level in plasma/serum samples has been used as a diagnostic biomarker of atherosclerosis and CVD, with sandwich ELISA analyses frequently used as the procedure of choice (Itabe, 2012).

2.2.1.2 Protein oxidation and biomarkers

Protein oxidation refers to chemical damage or modification of protein caused directly or indirectly by RONS. Protein oxidation occurs *in vivo* mostly during ageing or as a result of certain diseases, but it can also be induced *in vitro* by a wide range of oxidants and/or toxicant agents (Shacter, 2000; Luna & Estévez, 2018). Modifications/damages of proteins could severely affect protein functions such as their enzymatic and binding activities, which maximise proteins' susceptibility to degradation, proteolysis, and aggregation as well as increase or decrease their uptake by the cells (Shacter, 2000; Birben et al., 2012). It's believed that RONS, particularly hydroxyl radicals play a crucial role in the initiation and propagation of protein oxidation. Basically, hydroxyl radicals initiate protein oxidation through the abstraction of a hydrogen atom, and amino acids with a thiol group and aromatic side chains such as lysine, cysteine, methionine, and arginine are most susceptible to oxidation (Luna & Estévez, 2018; Demirci-Çekiç et al., 2022). This results in protein carbonylation - an integration/binding of reactive ketones and aldehydic LPO products to protein structure. Protein carbonylation refers to irreversible protein damage/modification resulting in proteins losing their activity due to a change in the peptide chain structure as a result of RONS direct attack on amino acids, whereas protein carbonyl (product of RONS direct attack on amino acid side chains) widely accepted as a biomarker of protein oxidation (Weber et al., 2015; Demirci-Çekiç et al., 2022).

Immunodetection assays and analytical procedures have been utilised by many researchers to evaluate protein carbonyl (PC) in biological samples (Himmelfarb et al., 2000; Winterbourn et al., 2003; Morillas-Ruiz et al., 2006; Neubauer et al., 2008; Debevec et al., 2015). The dinitrophenylhydrazine (DNPH) is the most common assay used, whereby PC in a sample is firstly coupled with DNPH reagent to form a stable 2,4-dinitrophenylhydrazone (DNP) product which is measured with either chromatography, spectrophotometric or fluorometric method (Levine et al., 1990; Uchiyama et al., 2011). Spectrophotometric procedure has been used by many researchers since it is more affordable and easier to perform, though it lacks specificity, time consuming and has high sample throughput. The use of MS analytical technique in recent years provides a powerful analysis approach since it provides more details; and can recognise specific carbonylated amino acid residues (Colombo et al., 2016). However, MS practice requires highly expensive instruments, hence, to date, its usage and distribution in clinical and research laboratories is very limited (Fedorova et al., 2014; Colombo et al., 2016).

2.2.1.3 DNA damage and biomarkers

Deoxyribonucleic acid (DNA) is a molecule composed of two strands around each other forming a double helix. The DNA contains genetic instructions used in the organism's growth, and development as well as to manage its functioning, behaviour, and reproduction. On a daily basis, despite relative stability, DNA is subjected to RS attack, especially hydroxyl radicals (Soares et al., 2015). This often results in DNA damage, particularly, base modifications, which may lead to mutagenesis, if not efficiently cleared or repaired (Halliwell & Whiteman, 2004; Kryston et al., 2011; Wojtczyk-Miaskowska & Schlichtholz, 2018). Generally, DNA damage involves any modification to the DNA structure that alters its coding properties or interferes with cell processes (replication, transcription, and metabolism) (Gonzalez-Hunt et al., 2018). Oxidative stress DNA damage occurs when a hydrogen atom is either removed or added to the methyl group of thymine and the C-H bond of 2-deoxyribose by the hydroxyl radical (Halliwell & Whiteman, 2004). As a result, DNA damage may cause variety of DNA lesions, mostly strand breaks, abasic sites and base modifications, which may easily breach the genome integrity (Gonzalez-Hunt et al., 2018). Despite the DNA's ability to repair cell damage before the cell reaches the replication phase, defects in DNA repair capability influence the amount and extent of oxidative DNA damage. This has been observed in several human pathologies (xeroderma ataxia telangiectasia and Fanconi's anaemia) characterised by 8-oxo-dihydro guanine (8-oxo-dG – most commonly assessed oxidative stress DNA damage biomarker, formed as results of guanine oxidation) and DNA repair mechanisms defects (Jaruga, 1996; Møller & Loft, 2010; Ermakov et al., 2013). To measure oxidative stress DNA damages, different analytical procedures such as HPLC, PCR, gas chromatography-mass spectrometry [GC-MS], comet and immunological can be utilised, using either whole blood, plasma, urine, or homogenate tissue as a suitable sample for analysis (Gedik et al., 2002;

Gonzalez-Hunt et al., 2018). However, due to the costly reagents and equipment, the routine practice of DNA damage analysis is limited in most clinical and research laboratories thus far (Phillips et al., 2000).

2.3 The antioxidant defence system and oxidative stress

Living organisms naturally possess a complex network of antioxidant defence systems that counteracts the damaging effects of RONS (Powers & Jackson, 2008; Kawamura & Muraoka, 2018). The antioxidant defence system of living organisms is divided into two groups, endogenous and exogenous antioxidant defence system (Figure 2.2). The endogenous antioxidant defence system produces antioxidant naturally (endogenously), while exogenous antioxidant defence system supplies antioxidants through diet or pharmaceutical supplements to overcome the deleterious effects of OS (Ďuračková, 2010). By definition, antioxidant is referred to as any substance or reducing agent that delays, detoxifies, attenuates and/or prevents oxidative damage to a substrate and it can be found either intra-or extra-cellularly or both. The potency of an antioxidant substance is determined by its antioxidant mechanisms of action, site of action (extra - or intra-cellular or both) and required concentration/strength for its activity (Halliwell, 2007). Numerous substances tend to show antioxidant properties *in vitro*, but most fail to convey such capacity *in vivo* as they may have lost it as a result of metabolism processes (Brglez Mojzer et al., 2016). Generally, the two antioxidant defence systems work synergistically, to protect cells and body organ systems against RONS damage through stabilising, neutralising, and scavenging excess RONS (Pham-Huy et al., 2008; Kurutas, 2015; Collins et al., 2022). Hence, according to their defence mechanisms of action, antioxidants are mainly divided into three subgroups (Demirci-Çekiç et al., 2022).

(i) Preventive AOXs - They terminate or prevent the production of FRs (Preventive AOXs) by suppressing the formation of RS or transforming them into other stable components.

(ii) Radical scavenger AOXs - They inhibit/interceptor chain initiations and/or proliferation reactions by acting against FRs before they damage cell molecules

(iii) Enzyme repairing AOXs – They are responsible for repairing damaged macromolecules e.g., transferases, lipases, proteases, and DNA repair enzymes.

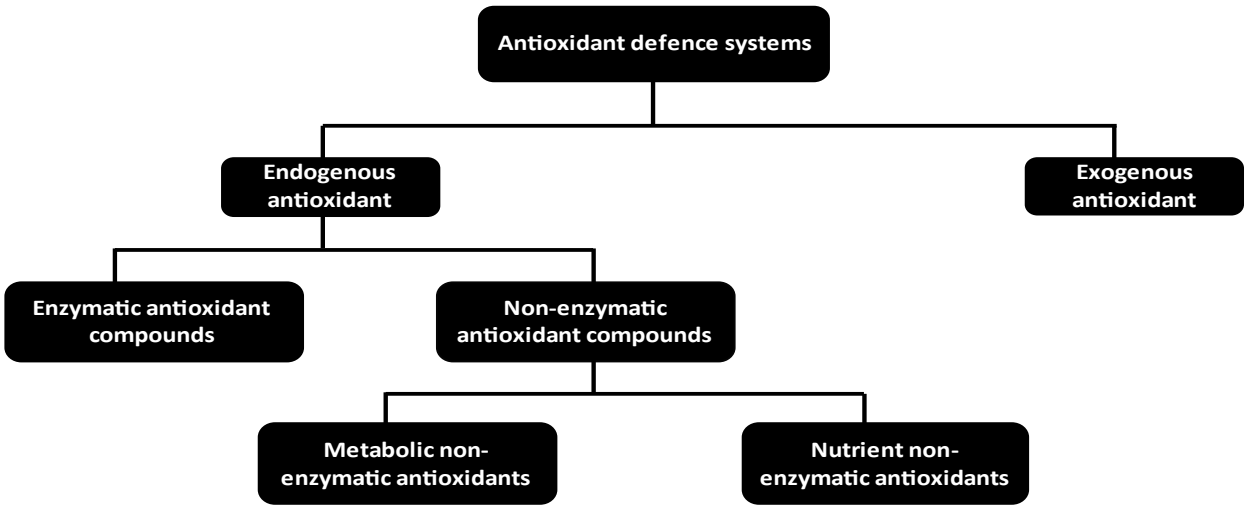


Figure 2.2: The antioxidant defence systems

2.3.1 Endogenous antioxidant defence system

As mentioned previously, the endogenous AOX defence system includes AOXs that are produced naturally in *situ.* These AOXs mainly function as preventive, interceptors or scavengers of RS and are further divided into enzymatic and non-enzymatic AOX compounds (Pham-Huy et al., 2008; Kawamura & Muraoka, 2018; Collins et al., 2022).

2.3.1.1 Enzymatic antioxidant compounds

As the name suggests, enzymatic AOX compounds are AOX enzymes that often work synergistically to detoxify, attenuate and/or convert RS into more stable molecules such as water and oxygen (Wang & Hai, 2016; Powers et al., 2022). These antioxidant enzymes are mostly found within cell cytoplasm and other cell organelles (Table 2.3) and include enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxide (GPx) and glutathione reductase (GR) (Wang & Hai, 2016; Elejalde et al., 2021).

Table 2.3: Localisation and actions of endogenous enzymatic antioxidants

Antioxidant enzymes	Cofactor	Cellular localisation
Mitochondrial manganese SOD (Mn-SOD)	Manganese (Mn)	Mitochondria
Cytoplasmic copper and zinc SOD (Cu-Zn-SOD)	Copper (Cu) and Zinc (Zn)	Cytosol – mitochondria (membrane)
Extracellular Cu/ZnSOD	Cu and Zn	Muscles
Catalase	Iron	Peroxysome, cytosol and mitochondria
Glutathione peroxide	Selenium	Cytosol and mitochondria

Abbreviations: Cu = Copper; Mn = Manganese; SOD = superoxide dismutase; Zn = Zinc

The SOD enzyme acts as the first line of defence and has three isoforms, two are found intracellular while the third one is found extracellular (Kurutas, 2015; Powers et al., 2022). The mitochondrial manganese SOD (Mn-SOD) mainly catalyses the conversion of O_2• to H_2O_2, while copper and zinc SOD (Cu-Zn-SOD) is the main cytoplasmic FRs scavenger (Mirończuk-Chodakowska et al., 2018). The CAT is an intracellular antioxidant enzyme that accelerates the conversion of H_2O_2 into water and oxygen with the help of iron as a cofactor. The GPx is also intracellular, and it ensures the complete removal of H_2O_2 from the cell. Both CAT and GPx have a similar action on H_2O_2, but CAT does better with H_2O_2 concentration, whereas GPx is more efficient with ROS levels, and it requires other secondary enzymes such as GR, G6PDH and cofactor GSH and NADPH to remove H_2O_2 from the cell (Elejalde et al., 2021). The threshold of CAT and GPx induction is different (Demirci-Çekiç et al., 2022), When only a low level of H_2O_2 is produced GPx activity is often good enough to handle (scavenge) the H_2O_2 level, but in the case of excessive production of H_2O_2, CAT is often required to compensate for the insufficient clearance of H_2O_2 by GPx (Vecchio et al., 2017).

2.3.1.2 Non-enzymatic antioxidant compounds

Non-enzymatic antioxidant compounds are also endogenous antioxidants, but they are not enzymes. They are divided into metabolic non-enzymatic antioxidants and nutrient non-enzymatic antioxidants (Marquardt & Watson, 2014). Metabolic non-enzymatic mostly include low molecular weight antioxidants (LMWA) such as lipoic acid, glutathione (GSH), thioredoxin (TRX), coenzymeQ10, melatonin, uric acid, bilirubin, as well as metal-binding proteins (myoglobin, ferritin, transferrin, ceruloplasmin, lactoferrin and albumin) (Mirończuk-Chodakowska et al., 2018). Nutrient non-enzymatic antioxidants include likes of vitamin E, vitamin C, carotenoids, trace metals (selenium, manganese, zinc), flavonoids, omega-3 and omega-6 fatty acids, etc (Willcox et al., 2004; Pham-Huy et al., 2008; Poljsak et al., 2013). Based on their protective role against RONS, non-enzymatic antioxidants are further divided into direct antioxidants and indirect antioxidants (Marquardt & Watson, 2014). Direct non-enzymatic antioxidants refer to short-lived, redox-active LMWA compounds, that can scavenge RONS and other reactive molecules, and also get consumed or chemically modified in the process of their antioxidant action. Hence, they often need to be replaced or regenerated which may subsequently induce pro-oxidant effects (Marquardt & Watson, 2014). While indirect non-enzymatic antioxidants are redox inactive antioxidants, unlikely to induce pro-oxidant effects, they mainly activate and boost the body's endogenous antioxidant system via recycling of direct antioxidants, boosting the body's detox enzymes (phase II enzymes) and play a vigorous role in the production of enzymatic antioxidants (Marquardt & Watson, 2014). Like enzymatic antioxidants, non-enzymatic antioxidants are also found within different cellular components

(Table 2.4), but they elicit distinct antioxidant properties which often maximised their effectiveness (Powers et al., 2004).

Table 2.4: Localisation and actions of endogenous non-enzymatic antioxidants

Direct antioxidants	Localisation	Actions
Vitamin E (tocopherol)	Lipids	Lipid peroxidation inhibition
	Cell/mitochondria membrane	Membrane stability
Vitamin A (retinol)	Lipids	Lipid peroxidation reduction
	Cell/mitochondria membrane	
Vitamin C (ascorbic acid)	Aqueous middle	Vitamin E regeneration
	Cytosol	LDL protection
	Extracellular liquid	
Glutathione	Aqueous middle	Substrate for GPx
		Vitamins E and C regeneration
Cysteine	Intracellular middle	Glutathione precursor
Lipoic acid	Aqueous middle	Lipid peroxidation inhibition
		Vitamins E and C and cystine regeneration
Thioredoxin	Aqueous middle	Mn-SOD synthesis
		Vitamin C regeneration
Glutaredoxin	Aqueous middle	
Flavonoids	Linked with glucids	Pro-oxidant enzyme inhibition
		Pro-oxidant ions trapping
		LDL protection
Uric acid	Aqueous middle	Pro-oxidant ions trapping
		Erythrocytes, haemoglobin, DNA and lipids protection
Coenzyme Q10	Internal membrane of mitochondria	Vitamins E and C and cystine regeneration
		LDL protection
Indirect antioxidants		
HSP	Aqueous middle	Protection of proteins (cells)
Iron	Aqueous middle	Catalase cofactor
Ferritin	Aqueous middle	Free iron trapping

		SOD cofactor (Cu-Zn-SOD)
Zinc	Aqueous middle	LDL and thiols protection
		FRs production inhibition
Selenium	Aqueous middle	GPx cofactor
Manganese	Aqueous middle	SOD cofactor (Mn-SOD)
Albumin	Aqueous middle	Give electron to FR
		Copper ions trapping
Caeruloplasmin	Aqueous middle	Give electron to FR
		Copper and iron ions trapping
Bilirubin	Aqueous middle	Give electron to FR
		Lipid peroxidation inhibition
		Erythrocytes protection

Abbreviations: FR = free radicals, Cu = Copper; DNA = deoxyribonucleic acid; GPx = Glutathione peroxide; LDL = Low density protein; Mn = Manganese; SOD = superoxide dismutase; Zn = Zinc

2.3.2 Exogenous antioxidant defence system

For the antioxidant defence system to function optimally and effectively, exogenous (dietary) antioxidants form an important component of this system because they complement the endogenous members. Exogenous antioxidants are externally supplied to the body mostly through diet and other supplements. Exogenous antioxidants include vitamin E, vitamin C, pro-vitamin A, selenium, and flavonoids, and are often obtained from a variety of dietary sources (Table 2.5). Vitamins (A, C and E) also exist within the cell as direct non-enzymatic antioxidants, but mostly, they are supplied through diet (Tiwari, 2001; de Oliveira et al., 2019).

Table 2.5: Exogenous antioxidants and dietary sources

Antioxidants	Dietary sources
Vitamin C	Tomatoes, green vegetables, acid fruits
Vitamin E	Vegetable oils, wheat germ oil, whole grain, nuts, cereals, fruits, eggs, meat, and poultry
pro-vitamin A (β-carotene)	Carrots, fruits, spinach, grain, tomatoes, and oils
Selenium	Onion, garlic, nuts, seafood, meat, and liver
Flavonoids	Green tea, grapes (red wine), apples, cocoa (chocolate), *Gingko biloba*, soya beans, broccoli, onion, and curcuma.

Exogenous antioxidants and their mechanisms of action against reactive species:

1. *Vitamin E*: It is a lipid-soluble vitamin, also known as α-tocopherol and is associated with a decreased risk of cardiovascular diseases (CVD) as well as slowing down the progression of degenerative diseases (Institute of Medicine, 2000; Niki, 2010). Vitamin E exists in different isomers (α-, β-, γ- and δ- tocopherol), but α -tocopherol is the most bioactive isomer with radical-scavenging capability, henceforth, it gained attention of many researchers (Pham-Huy et al., 2008). As an antioxidant, vitamin E safeguards cell membranes and plasma lipoprotein against lipid peroxidation via scavenging and stabilising of RS especially peroxyl radical (Institute of Medicine, 2000; Kurutas, 2015). However, in the process of stabilising RS, vitamin E also gets oxidised, but it later interacts with vitamin C in the aqueous region and regenerates back to its reducing form (Rietjens et al., 2002). When a vitamin E rich diet is consumed, its circulating level in the blood increases and about 70% is absorbed, with the largest portion found in the heart, liver, adrenal cortex, muscles, and adipose tissues (Niki, 2010). Vitamin E can be obtained from a variety of dietary sources, but sometimes foods' natural vitamin E content is lost or destroyed during the processing/cooking or storage conditions (Pham-Huy et al., 2008).

2. *Vitamin C*: It is a water-soluble vitamin, and is also known as either ascorbic acid, ascorbate, or dehydroascorbic acid (DHA). *In vivo*, vitamin C is found more in ascorbic acid form and is widely distributed in different tissues throughout the body. As an antioxidant, vitamin C works synergistically with vitamin E to quench and inactivate numerous RONS thereby minimising cell/tissue oxidative damage (Institute of Medicine, 2000). Moreover, it can interact with other antioxidants such as vitamin E and glutathione and regenerate them to their original properties – this is referred to as antioxidants network – which further ensures the balance network of antioxidants (Rietjens et al., 2002; Elejalde et al., 2021). Apart from being an antioxidant, Vitamin C plays various vital roles in the body; its deficiency often may cause scurvy - a disease characterised by deterioration of collagen production, fragility of blood vessels, swollen bleeding gyms and wound healing impairment (Paschalis et al., 2016). Vitamin C can be obtained from various dietary sources, and once consumed, it is absorbed either through active transport (if it is available in low concentration) or simple diffusion (if it's already available in high concentration) (Institute of Medicine, 2000). Naidu (2003) cautioned against the use/intake of a high dose of vitamin C (e.g., 2000 mg or more/day) since it could become a prooxidant and/or carcinogenic. Similar to Vitamin E, vitamin C is heat-labile, it can be easily lost/destroyed during cooking (Naidu, 2003), and its plasma concentration also decreases with age (Galan et al., 2005).

3. *Pro-vitamin A*: Pro-vitamin A (also known as carotenoids) is a group of pigmented compounds (over 600) that give a bright colour to the plants leaves, fruits and flowers (Nimse & Pal, 2015). Carotenoids are known to exhibit the potential of effectively quenching singlet oxygen and scavenging peroxyl radicals, generated during lipid peroxidation (Cvetkovic et al., 2016). Hence, carotenoids are considered one of the exogenous antioxidants that play a crucial role in protecting cell membranes against RS via their peroxyl radical scavenging activity (Nimse & Pal, 2015). About 40 carotenoids can be found in human diet, but only about 2500 – 5000 IU/day is recommended (Martin & Appel, 2010). The intake of carotenoids has to be taken with caution because routine intake has raised some concerns, being associated with an increased risk of CVD (Pham-Huy et al., 2008).

4. *Flavonoids:* Flavonoids are a subgroup of polyphenols, consisting of about fifteen carbon atoms, arranged in a C6-C3-C6 skeleton structure (Bellavite, 2023). Many flavonoids occur naturally as glycosides rather than aglycones and their structure contains two aromatic rings (ring A and ring B) connected by a three-carbon bridge, oxygenated heterocyclic (ring C) (Figure 2.3) (Manach et al., 2004; Ignat et al., 2011). In term of antioxidant capacity, flavonoid hydroxyl groups in ring B make them excellent free radical scavengers (Oteiza et al., 2021). And, due to chemical structure and variations in the substitution pattern of ring C, over 6,000 flavonoid compounds have been discovered and classified into six subgroups (Table 2.6) (Malaguti et al., 2013; Bhagwat et al., 2013).

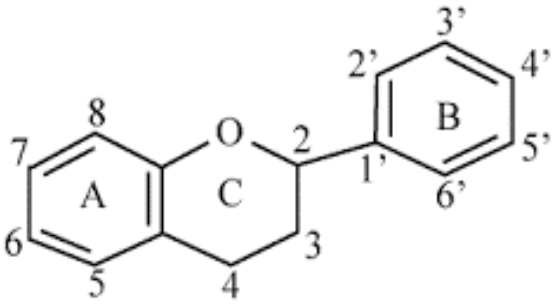


Figure 2.3: The flavan structure upon which flavonoids are based

Table 2.6: Flavonoids classification and dietary sources

Flavonoids subclass	Representative compounds	Dietary source
Flavonols	Quercetin, Kaempferol, Myricetin	Onion, apple, asparagus, berries cawlifower, grape, moringa, nectarines, tomatoes, strawberry, peaches, peepal, spinach, herbal tea
Flavones	Apigenin, Luteolin	Citrus species, lettuce, lemongrass Rooibos
Flavanols	Epicatechin, Gallocatechin	Apple, apricots, barley, blackberries, cherries, *Camellia sinesis,* dark chocolates, grapes, peaches, pistachios, red wine, peanuts, nectarines
Flavanones	Naringenin, Hesperidin	Bananas, lemon, kiwi, Rooibos, tomato, orange juice, grapes
Iso-flavanes	Daidzein, Genistein, Glycitein	Green pear, black beans, kudzu, soya, perennial wine
Anthocyanins	Cyanidin, Malvidin, Delphinin	Cherries, berries, lollo rosso, lettuce, peaches, plums, red grapes, red onion

Flavonoids have many health positive impacts which are often attributed to their FRs scavenging activities (Villaño et al., 2010; Hong et al., 2014). Flavonoids FRs scavenging ability or potential ranges between 300 and 500 mV, which is similar to other FRs scavenging substances that act as such as vitamin A and E, that act as direct antioxidants (Oteiza et al., 2021). Herbal plants such as green tea are rich in flavonoids, and they exhibit antioxidant, anti-carcinogenic, anti-hypercholesterolemic, anti-bacterial and anti-inflammatory properties (Pham-Huy et al., 2008; Marnewick et al., 2011). A study on quercetin (one of the most abundant natural flavonoids, mostly found in fruits and herbs) noted that quercetin was able prevent oxidative damage and cell death by scavenging FRs, quenching singlet oxygen and reduce lipid peroxidation by chelating metal ions (Lee et al., 2010). In addition, some studies concluded that consumption of flavonoid-rich diets and/or beverages improves exercise performance and might prevent or improve OS associated pathologies such as cancer, stroke, ageing, CVD, arthritis, and Alzheimer's disease (Myburgh, 2014; Zhang & Tsao, 2016; Overdevest et al., 2018; Bellavite, 2023).

2.4 Exercise and Oxidative Stress

The relationship between exercise and oxidative stress is quite complex and it often depends on the mode, type, duration, and intensity of exercise (Pingitore et al., 2015). When energy is generated in the cells during exercise RONS are produced within skeletal muscles mostly through different potential sources. In 1978, for the first time, a study by Dillard et al., (1978) reported the relationship between exercise and OS. In the study, Dillard and colleagues

observed an increase in expired pentane (lipid biomarker) after 60 minutes of endurance exercise at 60% of VO_2max. Since then (1978), numerous studies confirmed that both aerobic and anaerobic high intensity or prolonged exercise results in RONS overproduction (Neubauer et al., 2010; Gomes et al., 2012; Steinbacher & Eckl, 2015). Unfortunately, all studies conducted to date could not exactly clarify the main source of RONS during exercise. This challenge has been attributed to limited access to most tissues which makes it difficult to deeply investigate the multi-faced nature of exercise as it involves various organ systems connected through the energy requirement of contracting skeletal muscles (Powers & Jackson, 2008). Generally, at rest, muscles produce RONS such as superoxide anions and nitric oxide at a low rate, but during exercise, the production rate increases drastically due to increased oxygen uptake by the whole body and contracting muscles. A study by Sen, (1995) noted a rise of 10 to 15 folds in the rate of oxygen consumption of the whole body and more than 100 fold or 110 times of oxygen flux in the active muscles during exercise. Also, Richardson et al. (1995) concluded that prolonged or intense muscle contractions alter the physiological environment in the muscle fibre and predispose them to a higher rate of RONS generation. In 2004, Arbogast and Reid, showed that muscle contraction is associated with an increased muscle temperature, increased carbon dioxide tension, and decreased cellular pH that can stimulate and accelerate RONS production within the muscles. This same notion was also reported by Ferreira and Reid (2008), whereby an increased oxygen consumption in muscle fibre during exercise, lowered oxygen intracellular tension which then augmented the generation of RONS. However, despite mounting evidence regarding increased RONS generation during exercise, the exact/primary source is not yet clearly known to date (Kawamura & Muraoka, 2018). Some cellular mechanisms have been identified as potential sources, yet there is still a lack of sufficient knowledge about how much each contributes to the total amount of RONS generated during exercise (Vollaard et al., 2005; di Meo et al., 2016; Perrone et al., 2020; Powers et al., 2022). Such cellular mechanisms often, but not exclusively include the following:

1. *Mitochondria respiration system:* Over 90% of oxygen consumed by a human is utilised in the mitochondria to generate energy (ATP). This process involves the transportation and transfer of electrons via electron transport cascade/chain (ETC) carried out by four enzyme complexes (complex I [NADH dehydrogenase]), complex II [succinate dehydrogenase], complex III [coenzyme Q and cytochrome C oxidoreductase] and complex IV [cytochrome C oxidase]) (Wang & Hai, 2016; Demirci-Çekiç et al., 2022). The ETC use about 85 to 90% of oxygen consumed, while about 1 to 3% is incompletely metabolised resulting in a generation of superoxide radical mostly in complex I and complex III (Wang & Hai, 2016). Furthermore, mitochondrial enzymes such as electron transfer flavoprotein-ubiquinol oxidoreductase (ETF-QO), α-ketoglutarate

dehydrogenase (KGDH), dihydroorotate dehydrogenase (DHODH) and monoamine oxidase (MAO), also produce RS, particularly, ROS (Starkov, 2008; Watmough & Frerman, 2010).

2. *Autooxidation of catecholamines:* Catecholamine is a collective name referring to adrenaline, noradrenaline, and dopamine. During exercise, contraction of skeletal muscles leads to increased plasma catecholamines concentration, and their oxidation results in the production of RS such as superoxide and hydrogen peroxides (Gomes et al., 2012).

3. *Xanthine oxidase and Ischemia-reperfusion:* Xanthine oxidase (XO) is a cytosolic molybdoflavo enzyme that catalyse purine catabolism (hydroxylation of hypoxanthine to xanthine and then xanthine to uric acid) a key source of RS (Agarwal et al., 2011; Hussain et al., 2016). Additionally, due to active muscles' high oxygen demand during exercise, blood flow is shunted from many organs and tissues and redirected to active muscles. This ischemic condition stimulates the conversion of xanthine dehydrogenase enzymes to XO. When exercise ceases and the organs/tissue are reoxygenated, XO then generates superoxide and hydrogen peroxide as end products of hypoxanthine degradation into xanthine (Kang et al., 2013).

4. *Nicotinamide adenine dinucleotide phosphate oxidase structure*: The reduced form of NADPH oxidase is normally found in a dormant form in polymorphonuclear leukocytes, monocytes, and macrophages. During muscle contraction, this dormant form can get activated resulting in a generation of a large amount of superoxide which gets further converted into hydrogen peroxide by superoxide dismutase antioxidant (Babior et al., 2002; Birben et al., 2012).

The generation and accumulation of RONS can overwhelm the muscles' antioxidant buffering capacity which may easily cause inhibition of force production and lead to muscle fatigue, muscle atrophy, poor performance and some other exercise-induced illnesses (Xiao et al., 2012; Steinbacher & Eckl, 2015). Undeniably, excessive production of RONS during exercise causes damage of cellular components and poor exercise performance. However, moderate RONS production plays a crucial regulatory role in the modulation of skeletal muscle force production, controlling of cell signalling pathways and regulation of gene expression which could stimulate and/or enhance antioxidant enzyme generation and/or subsequently strengthen the endogenous antioxidant defence system (Berzosa et al., 2011; Steinbacher & Eckl, 2015).

2.4.1 Exercise, inflammation, and oxidative stress

Exercise or any physical activity involves muscle contractions which may induce muscle damage and soft tissue injury which may trigger an inflammatory response (Bowtell & Kelly, 2019). At the early stage, infiltration of inflammatory cells especially polymorphonuclear (PMN - neutrophils, granulocytes, phagocytes, and macrophages) to muscle or tissue damage sites, play an essential role of removing damaged proteins and realising growth factors and other substances to repair muscles and tissue damaged, however, in doing so, this process further generates a substantial amount of RS, particularly hypochlorous acid (Bessa et al., 2016; Hägglund et al., 2018; Bowtell & Kelly, 2019). These activated inflammatory cells release toxic molecules and hydrolytic enzymes within their granules through the respiratory burst process which also involves the generation of several RONS (Carrera-Quintanar et al., 2017). In case of excessive inflammation, inflammatory response may trigger overproduction of the pro-inflammatory cytokines (e.g., tumour necrosis factor α [TNF-α] and interleukin-1β [IL-1β]) which may activate nuclear factor (NF) κB pathway and subsequently elevation of C-reactive protein (CRP) which reflects or regarded as a biomarker of systemic inflammation (Thirupathi & Pinho, 2018; Perrone et al., 2020). Furthermore, in the case of chronic inflammation, the inflammatory response and attraction of PMN to the injury site further enhance RONS production and this subsequently forms a vicious cycle (Ji & Yeo, 2021).

2.5 Exercise and the human metabolome

Any disturbance or stimuli, internal (physiological and intracellular) or external (environment) cause changes in metabolite concentrations of any living organism (Schranner et al., 2020). Exercise is one of the “disturbances” known to cause changes in metabolites, especially in energy metabolic pathways (e.g., lipids, protein, and purine nucleotide metabolism) and other branches of metabolisms (Davison et al., 2018; Schranner et al., 2020). Different kinds of exercise (Figure 2.4) cause challenges to cellular homeostasis because of the mobilisation, utilisation, and conversion of carbohydrates, tricarboxylic acid, amino acids, lipids, and their metabolite derivatives to meet the body’s energy demands during exercise (Kim et al., 2019).

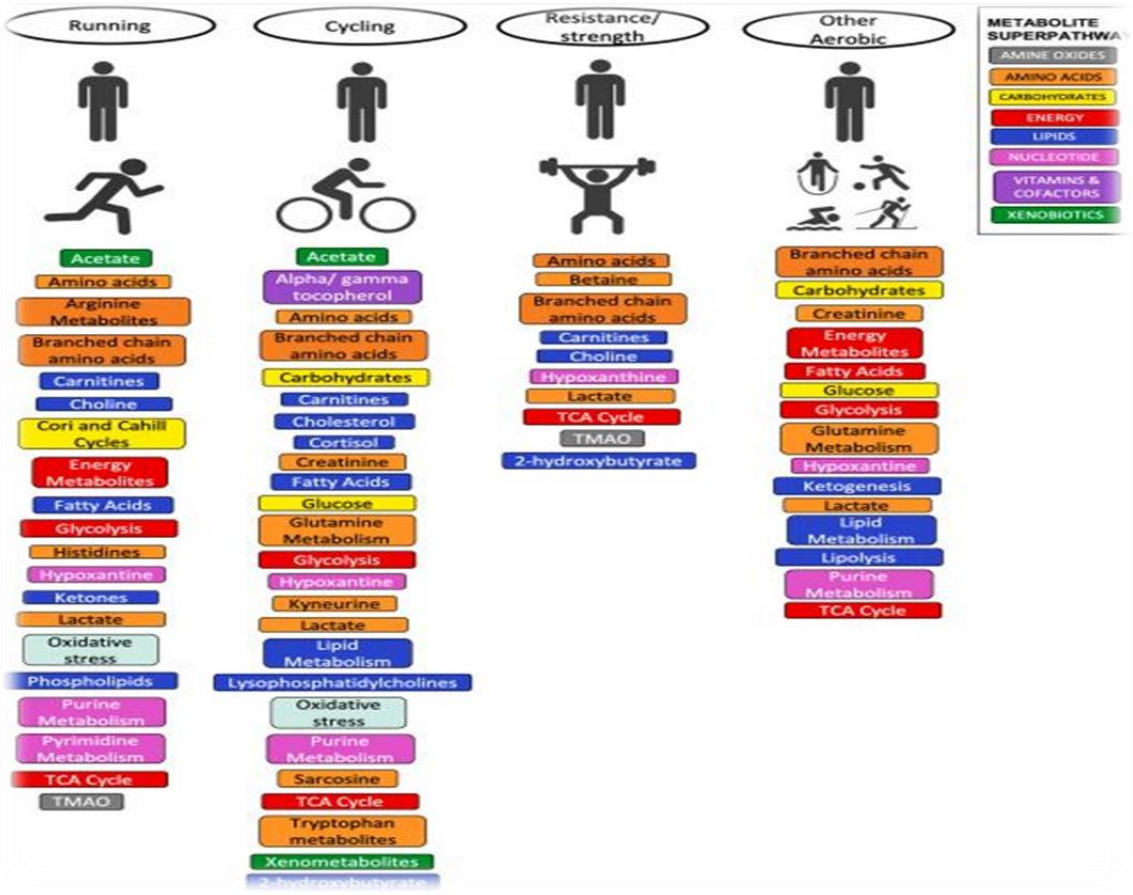


Figure 2.4: Various metabolites and/or pathways involved in different types of physical activities. Adapted from: (Kelly et al., 2020a).

Because of their sensitivity to disturbance/stimuli (internal or external), metabolites are considered to possess the ability to chemically reflect what is happening within the organism. Hence, metabolomic analysis is deemed to be the best approach to investigate human and/or animal drug testing, nutritional analysis and/ or other exploratory physiological analysis (Duft et al., 2017; Wishart, 2019). Metabolomic is defined as a comprehensive characterisation of metabolites (small molecules with molecular weight <1500 Da) and other chemical species (both exogenous and endogenous) in biological specimens in response to different perturbations or interventions (Khoramipour et al., 2022). This analysis approach has been of interest in exercise/sport studies, not only for commercial reasons such as determining the effect of sports supplements but for personalised nutrition and dietary management of various diseases (McNiven et al., 2011; Duft et al., 2017). The metabolomic analysis provides a platform to understand the mechanisms of performance-enhancing products from a metabolite point of view, which could help in the design of a potential modulator that will mitigate exercise-induced ailments (Miao et al., 2018). Also, due to its potential for new biomarker discovery that might be used for early diagnosis and/or monitoring of exercise-induced ailments/pathologies,

metabolomic gained the attention of many researchers in medical and health related fields (Kordalewska & Markuszewski, 2015; Ammar et al., 2017). In 2019, Bongiovanni et al. (2019) introduced concept of "Sportomics" into the scientific and sport and exercise literature, referring to when metabolomic effects of physical exercise on individuals are investigated. Metabolomic analysis mostly follows two analytical approaches, targeted and untargeted metabolomic analysis (Kordalewska & Markuszewski, 2015, Nieman et al., 2018). However, because of different sample types and analysis technique, other different analysis approaches have also emerged. These include **(1)** metabolic fingerprinting, **(2)** metabolic footprinting, **(3)** metabolic profiling, and **(4)** lipidomic (Khoramipour et al., 2022).

2.5.1 Targeted and untargeted metabolomics analyses

Targeted metabolomic analysis approach aimed at analysing known metabolites with similar properties (e.g., amino acids and carbohydrates) or those that belong to a certain group or metabolic pathway that modulates a biological function of interest. This approach is mainly ideal for hypothesis testing (answering a hypothesis which has already been formulated) and biomarker detection (Duft et al., 2017). Untargeted is also known as unbiased, global or discovery metabolomic analysis, and it focus on analysing samples without prior knowledge of which metabolites should be analysed (Wishart, 2019). This approach allows analyses of systemic metabolite changes and compared to a particular physiological state (e.g., pre, during or post) and provides an opportunity to better understand the prospect of metabolite or identify trends, patterns and/or discover novel markers that provide important information (Kordalewska & Markuszewski, 2015; Duft et al., 2017; Nieman et al., 2018). Metabolites detected during untargeted metabolomic analysis are (semi) quantitated and considered potential biomarkers of indicative predefined conditions (e.g., metabolites that differentiate between diseased and healthy individuals) or reflect the causative and/or associative relationships with a physiological state of interest (Heaney et al., 2019).

2.6 Exercise and genetic variations

Exercise is known to improve and promote and/or maintain good health, mostly by decreasing risks of many chronic diseases and ill-health experienced worldwide. However, less is known regarding the genetic molecular mechanisms behind these benefits (Capomaccio et al., 2015). Traditionally, it is assumed that athletes would respond similarly to a standardised exercise or stimulus (Pickering & Kiely, 2017) because this assumption often overlooks an individual's genetic variations which are known to influence exercise outcomes in many different ways (Figure 2.5) and inter-individual exercise responses. Genetic variations analyses in exercise context provide information that can be used to manipulate exercise factors (e.g., intensity, duration, resting period, frequency) to improve exercise outcomes or adopt personalised

training programmes that best fit individual athletes' genetic make-up (Bouchard et al., 2015; Pickering & Kiely, 2017).

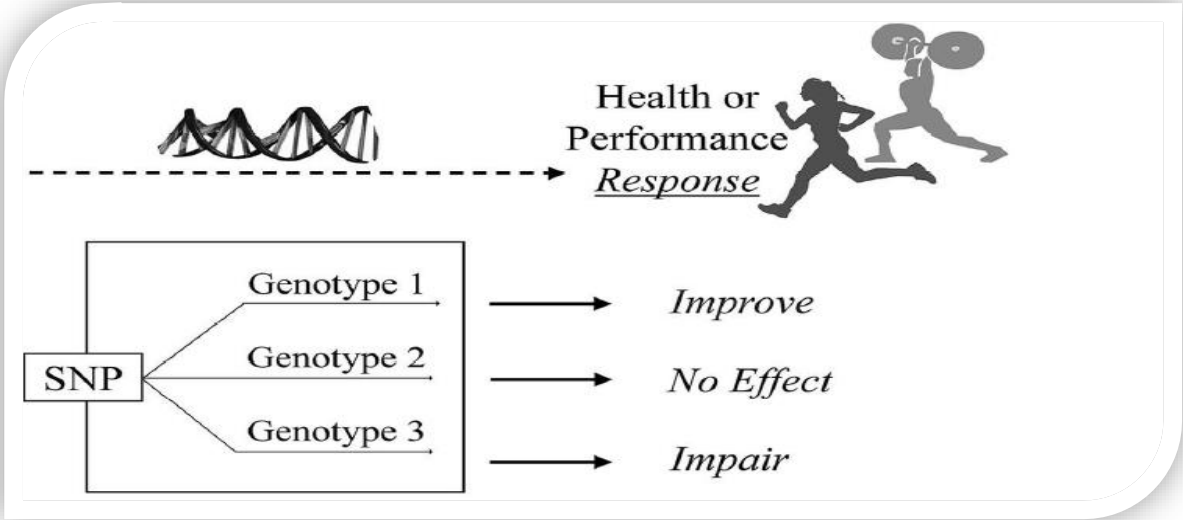


Figure 2.5: Schematic on how genetic variation may influence phenotype.
Adapted from:(Guest et al., 2019)

However, although, genetic testing played a crucial role in identifying gene abnormalities that underpin several diseases, September et al. (2012) cautioned that genetic test can should not considered diagnostic in nature but should be used in conjunction with other intrinsic and extrinsic factors to determine risk for specific injury. Hence, pathology genetic support testing (PGST) approach can be considered to have diagnostic, therapeutic and prognostic value provided that the testing is performed and interpreted by an expert in the field (Gray & Semsarian, 2020). Furthermore, Castelletti et al. (2022), cautioned that despite genetic testing benefits there are number of limitations and pitfalls associated with genetic testing that needs to be recognised.

2.6.1 Genes associated with exercise performance.

In the last two decades, about 200 performance-enhancing gene polymorphisms (PEP - collective of genetic factors that enhance or influence exercise performances) have been reported in the general population, but only about 20 have been found in athletes and only 10 were able to be replicated (Naureen et al., 2020). Angiotensin conversion enzyme (ACE) and Actinin – 3 (ACTN-3) are the most commonly researched and well documented PEP and both have been consistently associated with endurance and power performance phenotypes (Naureen et al., 2020).

2.6.1.1 ACTN-3

The human genome has four ACTN genes (ACTN1, ACTN2, ACTN3 and ACTN4) coding for α-actinin (α-actinin-1, α-actinin2, α-actinin3 and α-actinin4) which is a group of tissue specific binding protein that regulates muscle metabolism (Grealy et al., 2013; Ben-Zaken et al., 2019). Both α-actinin 2 and 3 are found in muscle and help to anchor sarcomere actin filaments in the Z disc of the skeletal muscle. This helps to maintain the myofibrillar array and regulate muscle length and tension during exercise, while α-actinin 1 and 4 are not found in the muscles, but they are responsible for cytoskeletal anchoring (Ben-Zaken et al., 2019). Also, α-actinin 2 and 3 are 80% identical, however, α-actinin 3 expression is confined to type II muscle fibre only, while expression of α-actinin 2 occurs in both type I and type II muscle fibre (Ben-Zaken et al., 2019). Due to poor collagen network regulation which subsequently affects muscle's mechanical properties (Baumert et al., 2018), the ACTN-1 gene mainly encoding α-actinin 1 of type I collagen tend to negatively influence muscle strength and recovery post-exercise training. The ACTN-3 gene coding for α-actinin 3 protein is sometimes referred to as R577X (rs1815739) genetic variant or single nucleotide polymorphism (SNP). This SNP was first discovered accidentally in the late 1990s (North et al., 1999). This SNP (R577X) is a result of the transition of the cytosine (C) amino acid to thymine (T) amino acid in the ACTN3 gene sequence and formed a premature stop codon (X) instead of the arginine (R) codon at the 577 positions of α-actinin 3 sequence (Maciejewska-Skrendo et al., 2020). This R577X SNP has three genotypes (RR, XX and RX) and two alleles (R and X). The R allele allows normal functioning and full production of α-actinin 3, whereas the X allele diminishes the expression and production of α-actinin 3 due to mRNA degradation (Maciejewska-Skrendo et al., 2020). Therefore, because of allele X effects, homozygosity of 577X (XX genotypes) causes a deficiency of α-actinin 3 protein in muscle, however, this deficiency does not make an individual prone to disease due to compensatory action of α-actinin 2 protein, but the deficiency alters individual muscles functions and metabolism (Blazek et al., 2016).

The first study to associate ACTN-3 with sport and/or exercise performance was published in the early 2000s. The study by Yang et al. (2003) found a significantly high frequency of the 577R in elite sprinters, both male and female. Since then, many other studies have associated the RR genotype with elite power performances while the XX genotype has been associated with low muscle strength and poor sprinting ability (Eynon et al., 2013; Zouhal et al., 2021). Because of α-actinin 3 deficiency, the XX genotype is associated with poor muscle contraction capacity and force production, unlike the RR genotype which is associated with greater muscle contraction capacity and great power production (Maciejewska-Skrendo et al., 2020). Studies reported that XX homozygote individuals are more prone to exercise injuries unlike their RR, RX genotypes counterparts (Deuster et al., 2013; Zouhal et al., 2021). Deficiency of α-actinin 3 in these individuals causes lower muscle strength, impaired strain tolerant capacity, reduced

muscle volume and decreased bone mineral density. These challenges negatively impact muscles and ligaments' capacity to endure exercise, thus making individuals more susceptible to muscle contraction damage (Coso et al., 2017; Del Coso et al., 2020; Zouhal et al., 2021). The RR genotype is considered to be speed-specific and tends to benefit sprinter and jumper athletes due to the nature of their sports game which often needs a high-speed level to move rapidly from one point to another, unlike the power performance athletes who hardly benefit from the RR genotype (Grealy et al., 2013).

2.6.2 Genes associated with skeletal muscle damage and soft tissue injury risk.

Similar to exercise performance, genetic variants play a crucial role in individuals' capability to recover and resist/ susceptibility to injuries (Naureen et al., 2020). Muscle damage and soft tissue injuries especially anterior cruciate ligament (ACL) rupture and achilles tendinopathy are the most common injuries often athletes suffer, and genetic variants is also a common factor in them (Appel et al., 2021). Since the first study linked genetics to exercise (North et al., 1999)**,** several genes genetic variations have been identified and associated with skeletal muscle damage and/or soft tissues injury risks. These genes include collagen genes, growth differentiation factor 5 gene, Caspase 8 genes, Adenine monophosphate deaminase and many others.

2.6.2.1 Collagen gene

Collagen refers to a superfamily of proteins found in the extracellular matrix (ECM) of almost every tissue and helps with maintaining tissue structural integrity and regulates other various biological processes (Collins & Posthumus, 2011). There are different types of collagens, namely type I, II, III, V and XI. The collagen V (COL5) gene is the most researched one and encodes type V collagen protein which forms a crucial structural component of connective tissue and helps to regulate fibrillation of type I and III collagen in the musculoskeletal system (McCabe & Collins, 2018). This gene (COL5) encodes for the alpha-1 chain type V collagen (COL5A1) gene which is found on the long arm of chromosome 9 and has many genetic variations COL5A1 >AGGG (rs71746744); COL5A1 C>T (rs12722); COL5A1 ATCT (rs16399); and COL5A1 A>T (rs1134170), which all associated with skeletal muscle damage and soft tissue injury risk, especially the mutant homozygote genotype (McCabe & Collins, 2018).

2.6.2.2 Growth differentiation factor 5 gene

The growth differentiation factor 5 (GDF5) gene encode the growth differentiation factor 5 protein which regulates the growth and maintenance of tendon, muscle, and bone (McCabe & Collins, 2018). The GDF5 gene plays a very important role in maintaining and regulating ligament homeostasis, however, its genetic variants (T>C, rs143383) within the 5 UTR affect the GDF5 expression in the connective tissues (Raleigh et al., 2012). Furthermore, this genetic

variant has three genotypes (CC, TT and CT) and two alleles (C and T). Unlike the C allele, the T allele is associated with muscle and soft tissue injury risk and osteoarthritis conditions due to poor or less production of GDF5 protein (McCabe & Collins, 2018). A study observed that GDF5 deficiency in tendons may lead to the inability to develop intra-articular ligaments and consequently weaker tendons due to a 40% lack of collagen (Harada et al., 2007).

2.6.2.3 Adenine monophosphate deaminase 1 (AMPD1)

Adenine monophosphate deaminase is a skeletal muscle enzymatic gene that encodes adenosine monophosphate deaminase enzyme isoform 1 (AMPD1), which plays a crucial role in regulating skeletal muscle energy metabolism during exercise (Varillas-Delgado et al., 2023). This gene genetic variant (rs1760, c.34, C>T) produce three genotypes (CC, CT, and TT) and two alleles (C and T) (Gronek et al., 2018). The C allele produces normal sufficient AMPD1 enzyme, but the T allele produces insufficient AMPD1 enzyme, hence, the presence of this SNP is often associated with induced muscle symptoms such as early fatigue, cramps, pain, and muscle weakness post-exercise (Varillas-Delgado et al., 2023). Therefore, individuals who possess the AMPD1 gene mutant homozygote genotype (TT) are more likely to suffer or sustain skeletal muscle and soft tissue injuries because of lack or insufficient production of AMPD1 in their skeletal muscles.

2.7 Common interventions used to modulate/mitigate exercise-induced ailments.

2.7.1 Dietary/nutritional antioxidant or nutraceuticals

For decades, it has been demonstrated and proven that nutritional supplements/nutraceuticals could be useful non-invasive tools in improving exercise performance, reducing OS and muscle damage as well as improving lifespan by decreasing risks and other possible pathological effects associated with exercise (Beaton, Louise et al., 2002; Theodorou et al., 2011; Ballmann et al., 2014; Yavari et al., 2015). Dietary antioxidants refer to foods or nutritional supplements prepared and consumed to provide polyphenols that are either missing or insufficiently consumed in the human diet (Sorrenti et al., 2020). Dietary antioxidants are known to significantly decrease the toxic effects of OS in a human by acting either as radical chain reaction inhibitors, metal ions chelators or as antioxidant enzymes cofactor (Huang et al., 2005). The potential positive health impacts of dietary antioxidants against exercise-induced OS deleterious effects on lipid, protein, and DNA has been investigated by in many studies (Sinha et al., 2009; Villaño et al., 2010; Ballmann et al., 2014). Some studies demonstrated that dietary antioxidants help the endogenous antioxidants system to detoxify and/or neutralise harmful effects of OS (Willcox et al., 2004), alleviate muscle fatigue, and/or improve exercise recovery (He et al., 2016).

Living organisms such as humans naturally possess an antioxidant defence system. This system is not always 100% effective, especially in cases of excessive RONS production, and its effectiveness also tends to decline with age, infection and/or when an individual's immune system gets compromised (Yokota et al., 2001). Thus, the intake of dietary antioxidants is advisable since it can help to alleviate and/or mitigate these challenges. Many studies demonstrated that dietary antioxidants could reduce LPO by chelating prooxidative metals, scavenging chain initiation radicals (e.g., hydroxyl, alkoxyl and peroxyl), trapping aggressive ROS (e.g., superoxide, hydrogen peroxide), and breaking chains of radical sequences or quench singlet oxygen (Sinha et al., 2009; Villaño et al., 2010; He et al., 2016). A lot of dietary-rich antioxidant foods have been identified, and they could serve crucial roles in the fight against OS related pathologies and ailments by protecting against RONS production or boosting the endogenous antioxidant defence system (Yavari et al., 2015). Dietary antioxidant -rich food such as fruits, vegetables and herbs reap positive health benefits against exercise-induced OS challenges, and most of them contain a lot of antioxidant supplements (e.g., Vitamin C, vitamin E, β-carotene) and some essential minerals (e.g., selenium and zinc) which all could enhance and boost up the body antioxidant defence system (Fang et al., 2002; Rahimi & Falahi, 2017). Nutritional antioxidant supplements such as vitamin C and vitamin E health impacts to counteract or mitigate exercise-induced OS deleterious effects have been well explored (Mastaloudis et al., 2004; de Oliveira et al., 2019).

A study investigated the effects of vitamin C supplementation in two groups (one group with low baseline vitamin C level and another group with high baseline vitamin C level) reported a decrease in exercise performance and increased oxidative damage in the low baseline vitamin C group, but after 30 days of vitamin C supplementation, investigators noted a vice versa results (Paschalis et al., 2016). In the same study, a high F2-IsoP and PC level were observed in a low vitamin C group at rest compared to a high vitamin C group, but after 30 days of vitamin C supplementation and 45 minutes of cycling exercise for 2 days, F2-IsoP and PC level markedly decreased in individuals with low baseline vitamin C, whereas only a few minimal effects observed in a high baseline vitamin C group. Moreover, (Davis et al., 2010) evaluated the use of supplements and reported an effective reduction of OS and inflammatory response in fit and physically active young males after 8 weeks of 500 and 250 mg/day quercetin and vitamin C supplementation respectively.

Vitamin E is another nutritional antioxidant supplement known to have positive health impacts on exercise. One of the first studies to investigate the relationship between exercise and OS, (Dillard et al., 1978) reported a reduction in both resting and exercise-induced pentane levels after vitamin E supplementation. While McBride et al. (1998) and Beaton, Louise et al. (2002) found a decrease in plasma and serum creatinine kinase (CK - muscle damage indicator)

levels in participants involved in different exercise regimes despite 1200 IU vitamin E supplementation for 30 and 14 days respectively. Some studies dismissed positive findings associated with vitamin E and exercise-induced damage ailments or outcomes. For instance, Avery et al., (2003) reported an increase in participants' plasma and serum CK levels after consuming 1200 IU of vitamin E supplement, 21 days before and 10 days after exercise bouts. Also, Cannon et al. (1991) showed that supplementation of vitamin E (800 IU/day) for 48 days fails to prevent the release of plasma CK, but instead, it reduced plasma inflammatory indices (IL- 6 and IL-1β) in young males after 45 minutes of downhill running exercise. A recent study by de Oliveira et al. (2019) reported that combined vitamin supplementation for 15 days (500 mg/day and 400 IU/day vitamin C and E respectively) significantly reduced OS effects in football players but failed to attenuate muscle damage or muscle soreness and did not also improves athletes' performances or recovery.

However, inappropriate and/or excessive use of dietary/nutritional antioxidant supplements may also cause antioxidative stress (negative effects of antioxidants). Some studies cautioned against the excessive use of nutritional supplements since it may become detrimental and/or blunt the needed positive response. For instance, smokers and asbestosis patients may suffer lung cancer if they consume excessive vitamin C (> 33,000 IU/day) (Wooltorton, 2003). It may also cause jaundice, arthralgia, diarrhoea as well as gastric upset at the intake of 2000 mg/day. A study by Theodorou et al. (2011) concluded that excessive use of vitamin C and E (1000 mg/day and 400 IU/day respectively) for 11 weeks, delayed the wound healing process and muscle strength restoration in athletes after exhaustive exercise training. While the use of combined supplements (2 capsules/day, containing vitamin E, vitamin C, β-carotene, lutein, selenium, zinc and magnesium) for 28 days failed to protect spring trained athletes against LPO, muscle damage and inflammation induced by a kayaking 1000 m bout (Teixeira et al., 2009). However, a balance is needed, because dietary/nutritional antioxidant supplement deficiency can negatively affect an individual overall health system. Mounting evidence indicates that supplement deficiency diets have negative health impacts and subsequent increases in OS damage. A human study by Carr et al. (2000) found that vitamin E deficiency may result in reduced liver antioxidant enzymes (GPx, CAT and GR) activities which subsequently enhanced liver LPO. While Paschalis et al. (2016) observed an increase in LPO and neurodegeneration in mice brain after consuming a vitamin E deficient diet compared with those who consumed a vitamin E rich supplement diet. Clearly, the controversy regarding antioxidant supplementation on exercise performance or adaption is still very much debatable in scientific community. Currently, there is no convincing research-based evidence supporting antioxidant supplementation in regard to training adaptations, however, there is a growing literature (Gomez-Cabrera et al., 2005; Wadley et al., 2013; Morrison et al., 2015) suggesting that the use of antioxidant supplement may actually hamper or prevent the signalling of

important adaptations such as muscle mitochondrial biogenesis, insulin sensitivity and hypertrophy. Merry and Ristow (2016) suggests that the type of antioxidant employed (general vs. targeted) and duration of treatment (pre-treatment vs. treatment only during training) and/or volume of training are factors determining the effect antioxidant supplementation on exercise training adaptations.

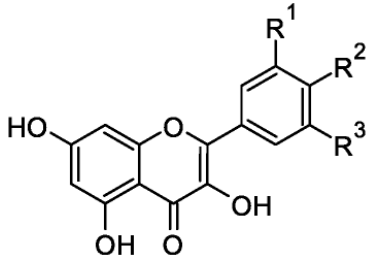
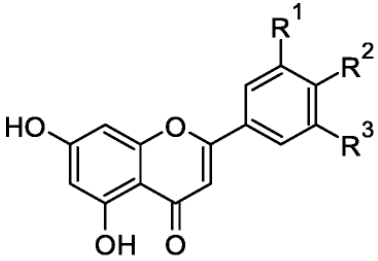
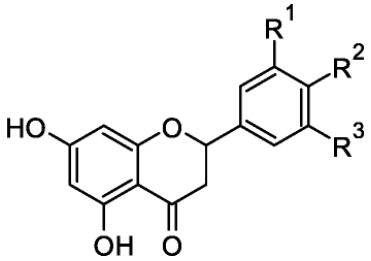
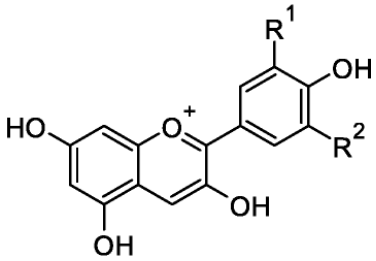
2.7.2 Phytochemical polyphenolic constituents

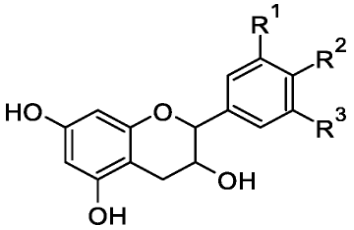
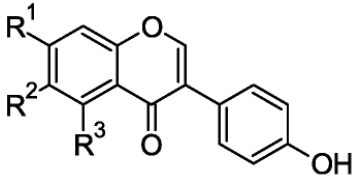
Phytochemicals are believed to be more compatible with human physiology because the chemical constituents present in them are of the physiological function of living flora (Sen et al., 2010). Phytochemicals also contain natural substances that can promote health and alleviate illness and have been proven to be safer and better patient tolerance (Sen et al., 2009; Del Rio et al., 2013). Currently, in Europe and Asia, most healthcare providers still advise their patients to make use of herbal tea due to its phytochemical polyphenolic constituents (McKay & Blumberg, 2007). According to the World Health Organisation (WHO), about 80% of the world's population still relies on the use of indigenous/traditional plants/herbal extracts for their primary health needs (Anon, 2002). And, about 75-80% of people in the world, especially in developing countries still strongly believe in the use of phytochemicals/traditional medicinal plants/herds for primary healthcare because of affordability, better cultural acceptability, fewer side effects and better compatibility with the human body (Sen et al., 2010). In recent years, non-nutritional components of plants such as tea and herbs have been researched in terms of their potential positive impacts on health particularly toward exercise (Morillas-Ruiz et al., 2006; Villaño et al., 2010; Marnewick et al., 2011; Kim et al., 2014; Chen et al., 2013; Liu et al., 2017; Watanabe et al., 2014). Most studies indicate that abundant polyphenols in herbal plants act as chain-breaking antioxidants and can mop up active RS that are normally engaged in oxidation reactions (Scalbert et al., 2005; Pandey & Ibrahim Rizvi, 2009; Villaño et al., 2010; Sissing et al., 2011; Sanguigni et al., 2017).

Polyphenols are phytochemical subgroups which are biologically active plant compounds or plant secondary metabolites characterised by the presence of one or more hydroxyl groups attached to aromatic ring structures (Bravo, 1998; Manach et al., 2004; Sadowska-Krępa et al., 2019). Polyphenols constitute one of the most widely distributed groups of natural products in the plant kingdom and can be classified based on their origin, biological function, and chemical structure. Polyphenols exhibited antioxidant potential which is determined by their functional hydroxyl groups (OH) that further determine the ROS synthesis suppression and scavenging, chelation of trace elements responsible for FRs generation, and the improvement of antioxidant defence systems (Elejalde et al., 2021). In plants, polyphenols play various roles such as attracting pollinating agents, and molecular signalling as well as protecting against parasites, herbivores, oxidative cell damage and microbial infection (Han et al., 2007; Del Rio

et al., 2013). Additionally, polyphenols are grouped into flavonoids and nonflavonoids (Table 2.7). Flavonoids are the most studied and represent the largest group of polyphenols with over 6000 derivatives (Bhagwat et al., 2013). Flavonoids are further divided into subgroups of anthocyanins, flavanols, flavonols, flavones, flavanones and isoflavone, while non-flavonoids are divided into phenolic acid, lignans and stilbenes. Like other dietary antioxidants, polyphenols could play a vital role against exercise-induced oxidative damage via their antioxidant and anti-inflammatory bioactivities (Liu et al., 2017) which have been highly attributed to their chemical structures which make them good electron or hydrogen atom donor to neutralise and stabilise RS (Zhang & Tsao, 2016).

Table 2.7: Polyphenols groups and their subgroups structures and sources

Polyphenols groups	Sub-groups	Structure	Examples compounds	Major sources
Flavonoids	Flavonols		Quercetin, myricetin, kaempferol	Onions, leeks, broccoli, celery, buckwheat, apples, apricots, variety of berries, tea, red wine
	Flavone		Apigenin, luteolin	Parsley, celery, hot peppers, rosemary
	Flavanones		Naringenin, hesperetin, eriodictyol	Citrus fruits: grapefruit, oranges, lemons, tomatoes, aromatic plants
	Anthocyanidins		Cyanidin, malvidin, delphinidin, pelargonidin	Blackberries, blueberries, black grapes, strawberries, cherries, black currant, red wine, cabbage, beans, onions

	Flavanols		Catechin, epicatechin, gallocatechin, epigallocatechin, epigallocatechin gallate	Apples, apricots, peaches, plums, raspberries, blackberries, cherries, cranberries, tea, chocolate, red wine
	Isoflavones		Genistein, daidzein	Soya
Nonflavonoids				
Phenolic acid	Hydroxylbenzoic acids		Gallic acid, protocatechuic acid	Blackberries, onions, raspberries, tea
	Hydroxycinnamic acids		Caffeic acid, ferulic acid, coumaric acid, sinapic acid	Coffee, blueberries, apples, cider, cereal brans, spinach, broccoli, citrus juices
Lignans			Secoisolariciresinol, matairesinol, lariciresinol, pinoresino	Linseed, grains, soybeans, beans, broccoli
Stilbenes			Resveratrol	Grape skin, blue and blackberries, peanuts, red wine
Phenolic alcohols			Tyrosol, hydroxytyrosol	Virgin olive oil, red and white wines, beer

Source of structure: https://www.google.com/search?q=polyphenols+subgroups&tbm=isch&chips=q:polyphenols+subgroups,online_

2.7.2.1 Dietary polyphenolic constituents

Dietary sources of polyphenols include fruits, vegetables, cereals, and beverages such as coffee, wine, and herbal teas (Aherne & O'Brien, 2002; Watson, 2018). A well-balanced diet with the recommended 5-7 daily servings of fruits and vegetables and moderate amounts of chocolate, tea, or wine, has been estimated to provide over 1 g of total phenols per day (Lotito & Frei, 2006). Apples provide about 400 mg of total phenols while a glass of red wine or a cup of tea provides about 100 mg of polyphenols, while fruits such as grapes, pears, cherries, and berries contain up to 200 to 300 mg of polyphenols per 100 grams (Pandey & Ibrahim Rizvi, 2009; Mushtaq & Wani, 2013). However, the actual total polyphenol content of food products varies greatly under the influence of several factors such as cultivar, seasonal and climate variations, growth conditions, stage of harvesting, storage conditions, processing, and cooking preparation (Amarowicz et al., 2009; D'Archivio et al., 2010; Hussain et al., 2016; Watson, 2018;). As mentioned previously, phytochemical polyphenols can protect against exercise-induced OS challenges via their antioxidant and anti-inflammatory effects (Pandey & Ibrahim Rizvi, 2009; Liu et al., 2017). Phytochemical polyphenols exhibit their antioxidant and anti-inflammatory effect either directly or indirectly to neutralise the deleterious effects of OS at the cellular level (Scalbert & Williamson, 2000). Studies support the notion that dietary phytochemical polyphenols exhibit strong antioxidants against RONS as a scavenger *in vitro*, metal chelator and inhibitor (Coimbra et al., 2006; Rahimi & Falahi, 2017). Furthermore, studies demonstrated beneficial effects of phytochemical polyphenolic sources such as green tea compound on scavenging RONS and its role in the prevention or as possible therapy of exercise-induced OS pathologies as well as other related exercise-induced ailments (Fang et al., 2002; Marnewick et al., 2005; Jówko et al., 2015; Rahimi & Falahi, 2017; Deley et al., 2017). Green tea polyphenol (GTP) is known to inhibit oxidative damage of low-density lipoprotein (LDL) and effectively scavenge superoxide and hydroxyl radical (Rahimi & Falahi, 2017), inhibit growth and stimulate the apoptosis of human cancer cell lines *in vitro* (Yang et al., 1998), raise serum high-density lipoprotein (HDL) concentration and decrease serum total cholesterol and MDA concentration in humans (Fang et al., 2002).

A study that investigated the effect of green tea reported a significant reduction of oxidative DNA damage after 2 weeks of green tea extract (GTE) consumption in obese men (Rahimi & Falahi, 2017). Also, a study conducted among postmenopausal osteopenia patients (171), showed a positive effect of green tea against OS deleterious effects. In the study, green tea intake (500 mg/daily) significantly decreased urine 8-OHdG concentration after 1, 3 and 6 months compared to the placebo control group (Qian et al., 2012). Another study by McAnulty et al. (2004) observed a significant decrease in hydroperoxide radical generation in athletes after consuming 150 g (2/3 cup) of blueberry for 7 days before treadmill running exercise, while

Jówko et al. (2015) concluded that supplementation of GTP (980 mg/d) prevented OS induced by high intensity repeated spring tests in male sprinters. In their study, Jowko and co-workers noted a markedly raised in participant plasma's total polyphenol and total antioxidant capacity at rest, and also decreased MDA and SOD plasma levels after the sprint test, however, no improvement in sprint performances nor protection against muscle damage was observed after GTP supplementation.

Many other human and animal studies also demonstrated the protective effects of polyphenols against exercise-induced OS. A study by Swamy et al. (2011) reported a reduction in exercise-induced OS damage and pro-inflammatory cytokines secretion after 7 days of high polyphenolic diet consumption. While Somerville et al. (2017) concluded that 7 days of polyphenol supplementation increased athletes' exercise performance by 1.90%, whereas (Malaguti et al., 2013) observed a 2.82% increase after the use of quercetin supplement. An animal study by Liu et al. (2017) reported a reduction in serum proinflammatory markers (IL-6, IL - 1β, TNF- α) and muscle damage markers (CK) in rats performed a single exhaustive swimming exercise after being fed with daily 300 mg/kg of tea polyphenols for four weeks. Additionally, an assessment of catechin modulatory effects on exercise-induced OS and muscle damage concluded that catechin treatment for 8 weeks attenuates loss of muscle force, muscle damage and OS biomarkers (CK, LDH, MDA) in mice, and it further significantly improved GSH:GSSH ratio after a downhill running exercise (Haramizu et al., 2011). Chang et al. (2010) noted a significant improvement in rats' swimming performance after consuming a combined polyphenol-rich diet (catechin, chlorogenic acid, ellagic acid and quercetin). In the same study, a polyphenol-rich diet raised muscle glycogen concentration and decreased the muscles, liver, and blood MDA, CK and LDH levels. Therefore, based on these, one can easily conclude that the use of phytochemical polyphenols could benefit exercise performances directly or indirectly. The direct benefit may include the reduction of muscle damage and fatigue that often occur during or after exercise, while indirect benefit can involve overall improvement of training performances and/or better training recovery (Myburgh, 2014).

It is also speculated that dietary polyphenol improves exercise performance via modulating mitochondrial biogenesis in two ways: firstly, they stimulate stress-related cell signalling pathways that raise expression of the gene encoding nuclear respiratory factor-2 ([NRF-2] - a cytoprotective protein and plays a role in mitochondrial biogenesis). Secondly, polyphenols can raise peroxisome proliferator-activated receptor -y coactivator (PGC-1) and activate sirtuins ([SIRT1] gene that stimulates several biological and physiological processes involved in mitochondria biogenesis and skeletal muscle functions) (Chung et al., 2010; Lappalainen, 2010; Malaguti et al., 2013). Additionally, polyphenols can increase the endothelial NOS (arterial vasodilator), which further improves the rate of blood flow during exercise as it

maximises cardiac output and eventually improves overall exercise performance (Bassett & Howley, 2000; Labonté et al., 2013). However, Nieman et al. (2007) noted that high dose of phytochemical polyphenolic compound supplementation (1g/d, quercetin, 3 weeks) in ultramarathon athletes before 160 km race, fails to reduce inflammatory response levels and OS indices, but it reduced the upper respiratory tract infection incidences. A recent study (de Carvalho et al., 2019) reported that 7 days intake of phytochemical polyphenolic compound (616 mg/daily, cocoa flavanols mixed with low-fat chocolate milk) consumed immediately and 2 hr post-exercise, failed to exert oxidative stress or exercise performances recovery benefits in elite rugby players.

2.7.2.2 Bioavailability of polyphenolic constituents

Bioavailability refers to the proportion of the food consumed, digested, absorbed, and metabolised through normal metabolic pathways. Polyphenols are found either attached to a sugar (glycosides) which increases their solubility or unattached to sugar (aglycones) (Martin & Appel, 2010; Del Rio et al., 2013). However, most polyphenols occur as esters or glycosides or as a polymer which cannot directly absorbed, hence, first undergo a conjugation process before being absorbed (Massimo et al., 2007; Hussain et al., 2016). Conjugation process occurs mainly in the colon, small intestines, and the oral cavity and later in the liver and this process mainly includes methylation, sulfation and glucuronidation, thus, polyphenol metabolites in circulation are found in forms such as methylated, sulfated and glucuronidated derivatives (Manach et al., 2005; Saura-Calixto et al., 2007; Habauzit & Morand, 2012). Therefore, the bioavailability of polyphenolic constituents depends on digestion, absorption, and metabolism of that particular polyphenolic compound.

The absorption of polyphenols is known to occur along the gut, however, the exact mechanism remains unknown, although, mechanisms such as passive (diffusion) and active (sodium-dependent glucose transporter 1) have been hinted to play a role. The rate and extent of polyphenol absorption vary greatly due to various factors such as polyphenols' chemical structure, molecular weight, as well as type and extent esterification (Manach et al., 2005; Habauzit & Morand, 2012). For example, fruits and cocoa contains proanthocyanidins which is hardly absorbed in the gut because of its large molecular size, whereas absorption of catechin often declines due to their increased esterification (Scalbert et al., 2002). Additionally, the human body recognises polyphenols as xenobiotics, hence, their low absorption ratio compared to other nutrients. Only about 5 – 10% of consumed polyphenols are absorbed in the small intestine, and 90 – 95% proceed to the colon and undergo intestinal microbiota conjugation before being absorbed (Sorrenti et al., 2020). Upon absorption, polyphenols bound to albumin that then facilitate their transportation to the liver where they get further metabolised to smaller phenolic compounds/metabolites (chemically distinct from the parent

compounds found in plant foods) that reach the blood and tissues before excreted in urine or bile (Manach et al., 2004; Marín et al., 2015), however, the processes differ from polyphenol to polyphenol (Krogholm et al., 2010).

2.7.2.3 Polyphenols' mechanism of action against reactive species

Polyphenol bioactivity is attributed to aglycon structures, with the antioxidative potency mainly attributed to the orthodiol structure in aglycons (Sorrenti et al., 2019). Polyphenols are considered and proposed to act as an adjuvant (substance that enhance the body's immune response to an antigen) through their antioxidant and anti-inflammatory activities/mechanisms as briefly discussed below (Nijveldt et al., 2001; Manach et al., 2004; Chuang & McIntosh, 2011; Sanguigni et al., 2017; Hussain et al., 2016).

1. Free radicals' scavenger- polyphenols B ring contains a hydroxyl group which enables them to react with radicals (e.g., superoxide) which subsequently results in a less reactive radical or inactivation of radicals.
2. Modulation of immune responses - polyphenols can act as adjuvants and can interfere with nuclear factor kappa B (NFκ B) and mitogen-activated protein kinase (MAPK) cellular process to reduce inflammatory responses.
3. Inhibition of xanthine oxidase activity - Some polyphenols particularly flavonoids (e.g., quercetin and luteolin) are known to inhibit XO activities (a key source of FRs).
4. Leukocyte immobilisation - In case of inflammatory response and/or tissue damage, polyphenols modulate receptor-directed Ca2+ channel resulting in decreased number of leukocytes as well as inhibition of neutrophil degranulation – which would have otherwise result in the release of more oxidants.
5. Interaction and inhibition of enzymes involved in the oxidation process - many studies demonstrated that polyphenolic compounds could modulate and/or inhibit activities of pro-inflammatory molecules and many enzymes (e.g., cyclooxygenase, lipoxygenase, and nitric oxidase synthase) that are involved in arachidonic acid metabolisms, which then reduced the production of arachidonic acid, leukotrienes, prostaglandins, and nitric oxide production which are known major mediators of inflammation.
6. Inhibition of LPO and prevent possible radical-mediated depletion of vitamin E and β-carotene.

Based on the studies discussed above, phytochemical polyphenols play crucial roles in attenuating and neutralising OS's deleterious effect. However, some studies cautioned against the use of phytochemical polyphenolic compounds, stressing that they have some drawbacks and can become prooxidant at high doses (Scalbert & Williamson, 2000; Coimbra et al., 2006; Martin & Appel, 2010). However, despite several drawbacks, the use of phytochemicals

polyphenols against exercise-induced OS has been proven to be clinically effective and relatively less toxic than most of the currently used synthetic drugs (Sen et al., 2010; da Silva et al., 2018). Surprisingly, to date, there is still no official protocol/procedure to mitigate exercise-induced OS and other ailments (Wan et al., 2017). Nevertheless, many athletes opted to use some nonspecific treatment and preventative approaches such as synthetic drugs to alleviate and mitigate exercise-induced OS and muscle damage and other ailments.

2.7.3 Synthetic products/drugs

Apart from the use of nutraceutical supplements and dietary antioxidants, the use of synthetic drugs such as ephedrine, amphetamine, caffeine, ibuprofen and other many non-steroidal anti-inflammatory drugs (NSAIDs) to prevent and/or mitigate exercise-induced ailments as well as improvements in exercise performances is well known (Wan et al., 2017). However, these synthetic drugs appear to have mixed benefits with some studies questioning their safety and efficacy (Nieman et al., 2006; Van Wijck et al., 2012; Ji et al., 2016).

Amphetamine - one of the most common synthetic drugs used by athletes to improve and mitigate exercise-induced effects. Amphetamine is used and/or acts as an ergogenic drug. At low to moderate dosages, amphetamine enhances physical performance by increasing muscle strength, improve reactive time and delays muscle fatigue onset (Morozova et al., 2016). However, amphetamine is a stimulant and highly addictive drug and is known to have serious side effects such as anorexia, insomnia, weight loss etc (Heal et al., 2013). Additionally, it may also cause emotional and cognitive effects such as euphoria, changes in sexual desire and elevated mood.

Caffeine - Generally, caffeine is a substance found in a variety of beverages, chocolate-based food, and in some drugs, where it acts as an adjuvant (a substance that enhances the body's immune response to an antigen) (Olcina et al., 2006). Like amphetamine, caffeine also acts as an ergogenic drug as it helps to reduce fatigue and increase concentration and alertness (Paluska, 2003). Also, a study by Olcina et al. (2006) found that ingestion of caffeine (5 mg/kg) before the cycling test, improved participants' time to exhaustion, workload, maximum oxygen consumption and decreased their respiratory exchange ratio, however, it failed to modulate and/or reduced LPO. Caffeine is also a stimulant, its ingestion or intake can decrease cerebral blood flow, and antagonise adenosine receptors in blood vessels which could lead to reducing adenosine-mediated vasodilation and consequently decreased myocardial blood flow (Namdar et al., 2009). Additionally, at high doses (>500 mg – 600 mg/day) caffeine can cause tremors, restlessness, and tachycardia (Nawrot et al., 2003).

Ibuprofen - one of the most commonly used NSAIDs to alleviate pain and inflammations sustained during exercise. A study by Nieman et al. (2006) concluded that, the use of 600 - 1200 mg of ibuprofen failed to alleviate muscle damage, soreness, and inflammation in athletes after a 160 km race. Nieman and colleagues also noted an increase in serum ALT, AST, BUN, and lower urine creatine in ibuprofen users post-race compared to non-users which somehow indicates slight kidney function disturbance. Moreover, similar to all other NSAIDs and synthetic drugs, intake of ibuprofen is known to cause side effects such as nausea, dyspepsia, dizziness etc.

2.8 Proposed intervention to modulate/ mitigate exercise-induced ailment.

Due to the toxicity, ineffectiveness and several side effects of synthetic drugs used to mitigate and/or alleviate exercise-induced OS effects and other ailments, it forced a shift in the scientific community to start searching for natural antioxidant remedies (Nagulendran et al., 2007; Sen et al., 2010). Many studies demonstrated that antioxidants of plant origin are more promising and possess great therapeutic potential (Habauzit & Morand, 2012; Cases et al., 2017; Yada et al., 2018; Bowtell & Kelly, 2019; Sorrenti et al., 2020; Elejalde. Edurne; Villarán. Mari Carmen; Alonso. Rosa María, 2021; Jantan et al., 2021; Goode et al., 2022; Bonilla et al., 2023; Moslemi et al., 2023). Additionally, phytochemical polyphenols are natural products and prove to be safe, inexpensive, and more compatible with normal human physiology (Del Rio et al., 2013; Yavari et al., 2015). In fact, most of the drugs used are stimulants, costly and are known to cause serious side effects and lack tolerance in certain individuals, limiting their usage. Therefore, new, and safer additional measures are needed to substitute and/or supplement the currently existing interventions used to mitigate exercise-induced OS damage and other related ailments. Consumption of a balanced diet rich in natural antioxidants may be the most effective and possible safe way to alleviate and mitigate the harmful effects of exercise-induced oxidative damage and improve exercise performances (Yavari et al., 2015; Liu et al., 2017; Copetti et al., 2020). Literature also supports the use of natural phytochemical polyphenolic antioxidant compounds such as Rooibos herbal tea as a potential modulatory and ergogenic aid intervention that alleviates, attenuates, neutralises and/or detoxifies deleterious effects of exercise-induced OS and enhances and improves exercise performance and recovery.

2.8.1 Rooibos herbal tea (*Aspalathus linearis*)

Rooibos (*Aspalathus linearis*) is a South Africa indigenous shrub naturally found around the Cederberg Mountain range and nearest regions in the province of the Western Cape (South Africa) and is characterised by needle-shaped leaves, reddish-brown branches, and strong two-metre-long taproot (McKay & Blumberg, 2007). Many research studies have been done on Rooibos (Joubert, E; Ferreira, 1996; Marnewick et al., 2000; Kreuz et al., 2008; Persson et

al., 2010; Dludla et al., 2017) and it increasingly gained the attention of researchers in the scientific field, due to its rich and unique polyphenolic content with bioactive that can provide a wide spectrum of health benefits and strong antioxidant properties (Marnewick et al., 2004; Ulicna et al., 2006; Villaño et al., 2010; Joubert & de Beer, 2011; Marnewick et al., 2011; Canda et al., 2014; Watanabe et al., 2014; Dludla et al., 2017). A study by the South African government, Department of Environmental Affairs (DoEA) indicated that the Khoi and San indigenous peoples appear to be the first users of Rooibos as a beverage. The same study has intimated that traditional knowledge (TK) in the use of Rooibos was likely passed down from generation to generation by the Khoi and San indigenous people before the arrival of European settlers in South Africa (Department of Environmental Affairs, 2014).

The native Khoi-Khoi people were the first to use Rooibos as a beverage some centuries ago due to its agreeable taste and aroma. And now, because of its affordability and increasing suggested health benefits, Rooibos is currently marketed globally (Joubert et al., 2008; Raynolds & Ngcwangu, 2010). Depending on the production and processing procedure chosen, two kinds of herbal tea are produced from Rooibos, namely fermented (red) or unfermented (green) Rooibos tea (Joubert & Schulz, 2006). For the fermented Rooibos tea, Rooibos shrub stems and leave are cut into small fine pieces, wetting, and piling them in heaps for 12 – 24 h. This subsequently stimulate chemical oxidation process during which fermented Rooibos attains its characteristic aroma and red colour. While green or unfermented Rooibos tea is made by immediately oven-drying the fresh harvest to prevent oxidation before cutting into pieces and sieving (McKay & Blumberg, 2007; Chen et al., 2013). The oxidation process is believed to reduced overall antioxidant capacity of the fermented Rooibos tea due to the loss of aspalathin and nothofagin which are important antioxidant-rich components (Joubert, 1996; McKay & Blumberg, 2007).

2.8.1.1 Rooibos (*Aspalathus linearis*) chemical composition

In general, Rooibos contains an abundance of phenolic compounds with various bioactive that can provide a wide spectrum of health benefits (Marnewick et al., 2009; Joubert & de Beer, 2011; Canda et al., 2014; Davies et al., 2019) including the unique dihydrochalcone, aspalathin, and cyclic dihydrochalcone, aspalanin (Shimamura et al., 2006) only found in Rooibos (Figure 2.6).

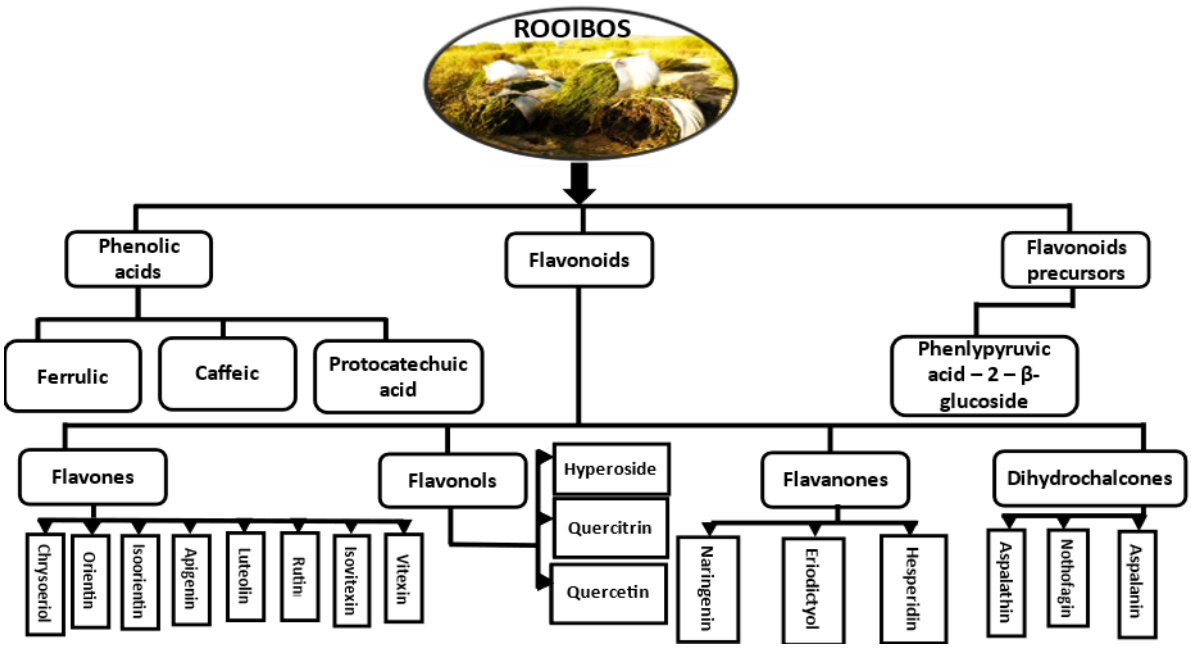


Figure 2.6: Most common polyphenolic constituents present in Rooibos (*Aspalathus linearis*)

2.8.1.2 Rooibos (*Aspalathus linearis*) bioavailability

For the last two decades, there has been an increase in the number of studies that validate and attest to the health benefits of Rooibos herbal beverage/products (Joubert et al., 2008; Villaño et al., 2010; Chen et al., 2013; Canda et al., 2014), however, only a few studies to date explored Rooibos bioavailability (Kreuz et al., 2008; Stalmach et al., 2009; Breiter et al., 2011). When consumed, most polyphenolic compounds in Rooibos such as aspalathin are absorbed in the gut and undergo bacterial actions (conjugation) in the colon and small intestines to form free and absorbable metabolites (aglycones). A study by Kreuz et al. (2008) investigated the *in vivo* aspalathin metabolites, and only methylated aspalathin metabolites was found in the urine, but not in plasma, after 7 and 11 days of oral intake of Rooibos. This finding is also supported by Stalmach et al. (2009) with O-methyl-aspalathin-O-glucuronide, O-linked methyl sulphate, glucuronide metabolites, (unfermented tea) and an eriodictyol-O-sulphate (fermented tea) isolated/found in human urine. Breiter et al. (2011) also found methylated aspalathin as the main excreted metabolite in human urine which further affirm the presence of Rooibos metabolites in human.

2.8.1.3 Rooibos (*Aspalathus linearis*) bioactivity

Rooibos is flavonoid-rich and contains an abundance of phenolic compounds with various bioactive properties, hence, it serves as a greater dietary antioxidant source (Villaño et al., 2010; Canda et al., 2014). Flavonoids are a subgroup of polyphenols and are known to exhibit antioxidant effects of potential health benefits, hence, it can be beneficial to athletes and fitness individuals because they can lessen harmful effects experienced during exercise and/or

improve exercise performance (Hong et al., 2014; Watanabe et al., 2014). Their antioxidants effects include reducing activities of arachidonic acid metabolising enzymes (phospholipase A2, cyclooxygenase, lipoxygenase), reducing LPO, inhibiting proinflammatory cytokines (IL-1b, IL-2. IL-6, TNF), modulating NOS and exhibit immunomodulatory effects (Marzocchella et al., 2011; Hussain et al., 2016). They can also chelate metals, scavenge radicals, trap aggressive RONS (e.g., superoxide, hydroxyl, hydrogen peroxide), break chains of radical sequences or quench singlet oxygen (Hudson & Lewis, 1983; Nijveldt et al., 2001; Sanguigni et al., 2017). Many human and animal studies have evaluated and demonstrated possible positive impacts, antioxidant role and protective effect of Rooibos herbal tea or products against OS damage, exercise-induced ailments, and other pathologies. Marnewick et al. (2003) reported that, even although Rooibos fails to make any impact in the male Fisher rats liver's TAC, it enhanced the GST-alpha and Uridine Diphosphate glucuronyltransferase (UDP-GT) enzymes activities and increased significantly the GSH:GSSH ratio after 10 weeks of Rooibos herbal tea consumption. Furthermore, Kucharská et al. (2004) concluded that consumption of Rooibos herbal tea diminished carbon tetrachloride (CCl4) induced hepatic steatosis and cirrhosis and further reduced the hepatic MDA as well as plasma bilirubin, ALP, and ALT in rats after 10 weeks of exposure to the CCl4 pro-oxidant. In 2005, Marnewick et al. (2005) observed that consumption of fermented and unfermented Rooibos herbal tea decreased MDA formation and further significantly improved the blood reduced to oxidized glutathione (GSH:GSSG) ratio. In another study, Marnewick et al. (2011) report that consumption of fermented Rooibos herbal tea greatly improved the lipid profile as well as redox status in adults at risk for developing heart disease. In the same study, Marnewick and colleagues observed a significant increase in plasma total polyphenols as well as a decrease in plasma LPO markers after six weeks of Rooibos herbal tea consumption (six cups/day). A study by Villaño et al. (2010) despite inconclusive findings on the lipid profile, reported that intake of both fermented and unfermented Rooibos herbal teas boosted and enhanced the human endogenous antioxidant defence system.

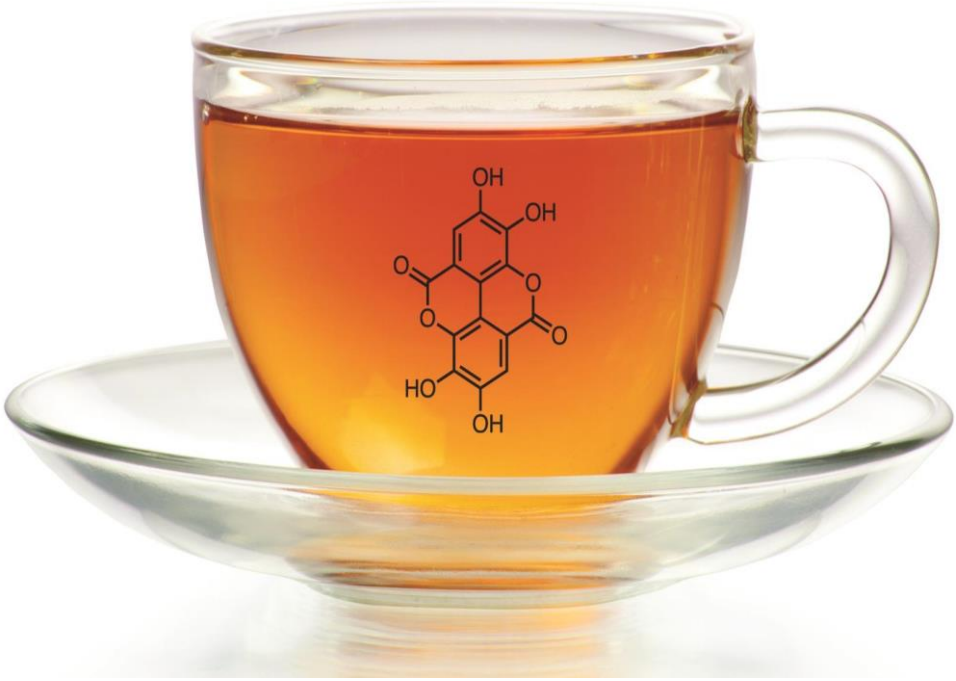
A human study by Persson et al. (2010) stated that the intake of Rooibos herbal tea may also have cardiovascular health benefits. In the study, Persson and co-workers (2010) observed that angiotensin-converting enzyme (ACE) was inhibited in healthy adults who consumed 400 mL of Rooibos herbal tea beverage. Inhibition of ACE is currently one of the treatment approaches used to treat and manage hypertension and congestive heart failure as it decreases the tension of blood flow and blood volume in the circulation. Furthermore, Dludla et al. (2017) reported that six weeks daily dose of aspalathin (130 mg/kg) maintained cellular homeostasis and protected the heart against hyperglycemia-induced OS damage in mice. Dludla and colleagues noted that the aspalathin cardioprotective effect was through the upregulation of nuclear factor erythroid-related factor-2 ([Nrf2] a gene that regulates the

expression of antioxidant protein/enzymes). Moreover, an *in vivo* study by Chen et al. (2013) found a significantly improved survival rate of the *Caenorhabditis elegant* with decreased oxidative damage caused by juglone (superoxide anion radical generator) after Rooibos herbal tea treatment. In the same study, quantitative real-time polymerase chain reaction (PCR) results indicate that aspalathin targets stress and age-related genes, decreases the endogenous intracellular RONS level and promotes longevity under stress. Canda et al. (2014) concluded that intake of Rooibos herbal tea may enhance and boost the liver's antioxidant capacity enabling it to detoxify and/or reduce damages induced by several toxicants.

Some studies also suggested that intake of Rooibos beverages and/or products may benefit exercise performances by increasing physical endurance resistance and delay fatigue onset (anti-fatigue effect). A study by Watanabe et al. (2014) noted a significant improvement and prolonged swimming time in mice fed with green Rooibos. Watanabe and colleagues also noted a high glycogen level in the liver and skeletal muscles which resulted in improved physical endurance of mice fed with green Rooibos compared to the control group. However, despite much research work done on OS to date, there's still a lack of a standardised OS human model, hence, only a few therapeutic interventions exist which were developed based on knowledge from *in vitro* and animal OS research models. However, due to several biological and lifestyle factors (Alberto De La Riva et al., 2023), knowledge from the *in vitro* and animal OS research models in the OS field does not always translate to humans. Furthermore, the current therapeutic/interventions used to mitigate and/or ameliorate OS damage and exercise-induced ailments mostly include synthetic drugs (Ji et al., 2016; Wan et al., 2017) which are known to be stimulants, costly and are known to cause serious side effects and lack tolerance in certain individuals and there's questionable safety and efficacy over them. Therefore, new, affordable, and safer additional measures are needed to substitute and/or supplement the currently existing interventions.

Interestingly, the literature supports the use of natural phytochemical polyphenolic compounds such as Rooibos herbal tea as it showed promising results due to its antioxidant and abundant phenolic compounds with various bioactive properties. Although the antioxidant effects of Rooibos herbal tea have been well investigated and documented (Marnewick et al., 2005; Persson et al., 2010; Marnewick et al., 2011; Chen et al., 2013; Watanabe et al., 2014), it's modulatory effect and metabolites influence in human in relation to exercise-induced OS have rarely been studied, thus, to date, no study as yet reporting on the modulation of exercise-induced oxidative stress and metabolites shifts after exercise and Rooibos herbal tea extracts consumption. Hence, the current study using a placebo-controlled intervention exercise model aims to fill that gap and further elucidate on potential health benefits/effects offered by Rooibos herbal tea. Lastly, current literature has a very limited understanding regarding Rooibos

influences on human metabolites/metabolic pathways and genetic variations influences on the extent of OS and muscle and soft tissue damage during exercise. Therefore, considering the personalised strategies that medical and health related fields, including sports, are heading into, the use of metabolomic and genetic approaches with the aim to holistically understand the response to certain interventions will not only lead to the development of OS intervention therapeutics but will certainly provide useful information that will allow personalised interventions or adopting a different lifestyle that would best fit individual.



CHAPTER THREE

RATIONALE AND AIMS OF THE STUDY

This study was conducted based on a widely acknowledged consensus that despite the known positive and negative impacts of oxidative stress on human health, this phenomenon (oxidative stress) is not yet well enough understood within the context of exercise and sport performance, to allow for effective clinical manipulation and possible development of preventative or therapeutic interventions, and one of the major factors frustrating these efforts is the lack of standardised human models. Even though the *in vitro* and experimental animal models are helpful, they do not generally translate well to humans in the field of oxidative stress.

3.1 Research aims and objectives

This study was performed in four phases with the ultimate aim of establishing a human oxidative stress model and using the standardised fermented Rooibos extract as a potential therapeutic intervention to modulate the deleterious effects of oxidative stress experienced during exercises. The study also aimed to evaluate Rooibos' influences on acute metabolite changes following an exercise regime and the influence of genetic variation in genes related to oxidative stress and sport injury and/or muscle damage. The study objectives for different phases were:

1. To establish/develop an exercise-induced oxidative stress human model suitable for herbal or phytochemical nutraceutical interventions, by assessing:

- Participants' plasma lipid and protein oxidation markers
- Participants' blood redox status

2. To assess the modulation of exercise-induced oxidative stress using a standardised fermented Rooibos beverage and to further elucidate if acute Rooibos ingestion could improve exercise performance outcomes and/or recovery, by assessing:

- Rooibos and placebo beverage phenolic constituents, total polyphenol content (TPC) and antioxidant capacity
- Plasma TPC and antioxidant capacity
- Plasma lipid and protein oxidation markers
- Blood glutathione redox status
- Serum biochemical analytes
- Exercise performances parameters

3. To evaluate the influence of a submaximal exercise regime in conjunction with the fermented Rooibos beverage on participants' relevant metabolite changes and/or metabolic pathways, by assessing.

- Plasma metabolites and metabolomic pathways

4. To determine the influence of genetic variation in genes related to oxidative stress and injury following an acute fermented Rooibos beverage and exercise regime, by assessing:

- Plasma oxidative damage biomarkers and biochemical
- muscle and soft tissue injury risk genetic variants

3.2 Rationale

Exercise induces a dramatic increase in oxygen uptake in skeletal muscles with RONS formed as by-products which may lead to poor exercise performances and prolonged recovery times. Additionally, accumulation and/or ineffective removal of RONS from cells may subsequently lead to damage to important macromolecules (deoxyribonucleic acid [DNA], lipid and protein) as a result of oxidative stress damage. Due to the lack of a standardised OS research human intervention model, there is little therapeutic interventions directly attributed to the research into oxidative stress. Since oxidative stress was conceptualised in the eighties (Sies & Cadenas, 1985) surprisingly enough, to date, there is still no official protocol/approach/practice to prevent or mitigate exercise-induced oxidative stress damage and related ailments, mainly due to a lack of reliable human intervention models. However, many athletes or individuals involved in exercise events/activities opt to use nonspecific treatment and preventative approaches such as the use of synthetic drugs. The safety and efficacy of these drugs have been questioned by some studies (Nieman et al., 2006; Wan et al., 2017). Moreover, synthetic drugs are stimulants, expensive, and can only be obtained through a medical doctor's prescription and some are known to cause serious side effects and lack tolerance in certain individuals**.** Therefore, novel interventions are needed to supplement or substitute the use of synthetic drugs. Hence, it is of great need to identify and develop safer, more effective, and well-tolerated non-prescription prophylaxis procedures or preventative approaches.

For the past 2 to 3 decades, scientific research into the use of natural products in particular herbal products which are often polyphenol-rich compounds has increased dramatically due to their bioactive properties (Oteiza et al., 2021). The research on bioactive as health promoters started in the 1980’s driven by the assumed relevance of generic “antioxidants” for human health, but at the beginning of the 21st century, more health benefits associated with fruit and vegetable consumption further drove great interest of researchers on plant bioactive.

Furthermore, there’s an increasing trend worldwide in consumers shifting to natural remedies as preventative strategies in the maintenance of health, hence, the increased use of natural plant products as a means of modulating exercise-induced OS pathologies and other related exercise ailments emerges to have several advantages over synthetic drugs. These natural plant products: **a)** are easily accessible, **b)** contain unique compositions of various bio-active, **c)** exhibit low or no toxicity, **d)** can react to most or all types of reactive species, and **e)** are more compatible to normal human physiology (Anon, 2002; Sen et al., 2010; Hong et al., 2014). However, there is a limited understanding and/or literature on the influences of polyphenolic-

rich products on the human metabolome and molecular mechanisms. It is important to know and understand the role or influence of natural polyphenolic-rich products on metabolites and molecular mechanisms that possibly translate into their protective and modulatory effects. However, there's a need to first establish or develop an oxidative stress human model, since the existing in *vitro* and experimental animal model research outcomes do not always translate to humans. Rooibos (*Aspalathus linearis*) a popular indigenous South African herbal tea gained many researchers' attention due to its rich and unique phytochemical and polyphenolic content with potent bio-activities (antioxidant, anti-inflammatory and anti-fatigue properties) which are of potential health use to prevent, detoxify and attenuate exercise-induced OS effects, and inflammatory responses as well as improving exercise performance and recovery (Watanabe et al., 2014; Nagasawa & Kaori, 2017; Davies et al., 2019), hence its consideration for the current study. The rationale for each of the five study phases is provided below in the form of an introduction to each phase, in an attempt to highlight the respective knowledge gaps, this study seeks to address.

3.3 Rationale Phase I – Establishment of an exercise-induced oxidative stress model for testing herbal interventions, such as Rooibos.

Oxidative stress was conceptualized several decades ago and has been defined as the imbalance of pro-oxidative molecules and antioxidant molecules within a system (Sies & Cadenas, 1985). The role of free radicals and antioxidants are still not fully clear, but it is known that radicals play several signalling and inflammatory functions within the body (Sharifi-Rad et al., 2020; Ji & Yeo, 2021). Within these contexts free radicals may, in general, be seen as beneficial mediators, however, it is well known that with ageing and diseases infection the natural antioxidant defence systems which regulate the levels and activity of these radicals are frequently overwhelmed, resulting in tissue damage (Jones, 2008; Kurutas, 2015; Powers et al., 2020). As such oxidative stress is seen to contribute to the pathophysiology of several diseases and in some cases to be involved in the aetiology thereof (Sharifi-Rad et al., 2020).

Despite this general consensus on the importance of oxidative stress to human health, it is widely acknowledged that this phenomenon is not yet well enough understood to allow for effective clinical manipulation thereof (Sharifi-Rad et al., 2020; Ji & Yeo, 2021). One of the main reasons why there is a relative lack of knowledge regarding oxidative stress and the natural role of radicals and antioxidants may be the great biological diversity which is exhibited both in and between species regarding these mediators (Alberto De La Riva et al., 2023). These same authors, further expand on how several body systems and lifestyle factors significantly impact the expression of antioxidant defences and the generation of free radicals. Environmental factors including exposure to exogenous (environmental and synthetic), and endogenous (natural) toxic agents influence the antioxidant defence system and contribute to

the formation and/or exacerbate generation of free radicals (Davis et al., 2010; Gupta et al., 2014; Phaniendra et al., 2015). The antioxidant deficiencies expressed in individuals, often develop as a result of decreased antioxidant intake, decreased efficiency of the endogenous antioxidant system (including the decreased synthesis of endogenous antioxidant enzymes and low molecular weight antioxidant, such as glutathione) due to disease, ageing or increased antioxidant utilisation (Kurutas, 2015).

Since free radicals are generated as by-products of normal cellular metabolism, the exposure of humans and other living organisms to radicals is unavoidable. However, the extent of damage suffered from the production and/or accumulation of free radicals depends on the nature of the targeted macromolecule, concentration and location of the targeted macromolecular and occurrence of secondary damaging events such as chain reactions (Davies, 2016). This lack of predictability and controllability of the free radical production and antioxidant expression may have contributed to the general lack of human models that are suitable for conducting intervention studies. It is clear in the literature that there are many *in vitro* and animal models which have been developed over the years to conduct oxidative stress-based research. We could, however, not locate literature on human models which induce oxidative stress for research purposes. This may in part be due to the fact that beyond biological variation, it is much more difficult to control lifestyle factors and stress in a human population than in an experimental animal cohort (Alberto De La Riva et al., 2023). In addition, it would be necessary to employ a method which can generate oxidative stress in a predictable and controllable fashion in a human subject without undue risk of further harm. As such, some researchers have attempted to conduct research on the phenomenon by enrolling cohorts of participants who suffer from conditions which have an oxidative stress component to its pathophysiology. One of the main challenges with this approach is that it would be impossible to exclude other factors of the causative disease and secondly, the oxidative stress produced in most disease states are in general relatively unique. In order to study such a complex phenomenon, it would be highly desirable to study a standardised reproducible type of oxidative stress which excludes other disease factors.

After many years of research study, there are few therapeutic interventions that could be directly attributed to the research conducted on oxidative stress (Pizzino et al., 2017). One of the major factors that frustrates these efforts is the lack of human models. It is clear that *in vitro* models and experimental animal models help understand the phenomenon of oxidative stress, it is, however necessary to translate this knowledge to humans if preventative strategies and therapeutics are to be developed. Previous studies attempting to measure oxidative stress in human subjects without pathology have enrolled athletes who participated in endurance events to measure the associated oxidative stress effects (Thirupathi & Pinho, 2018). Due to

the uncontrollable factors associated with enrolling athletes in this way, this cannot be seen as a standardised model for the measurement of oxidative stress and the testing of interventions such as herbal remedies. As such it is important that a human model be developed for the study of oxidative stress and potential interventions, because current *in vitro* models and experimental animal models' outcomes does not always translate to human (Oteiza et al., 2021). This study, therefore, aimed to develop an exercise induced oxidative stress model using human participants which is suitable to test herbal interventions.

3.4 Rationale Phase II – The modulatory role of a standardised fermented Rooibos beverage to serve as an ergogenic aid using a sub-maximal exercise regime in adult males.

The increased oxygen consumption during certain conditions such as exercise or physical activity may lead to the continuous formation of reactive species (RS) mostly reactive oxygen and nitrogen species (RONS) (Elejalde et al., 2021). Depending on their concentration, RONS may be harmful or beneficial to living organisms. At low and/or moderate concentrations, RONS are involved in physiological processes such as cellular signalling, defence against infectious agents, upregulation of endogenous enzymatic antioxidant activity (Steinbacher & Eckl, 2015) and conditioning of the endogenous antioxidant system in athletes (D'Angelo & Rosa, 2020). However, at high concentrations, RONS may result in the occurrence of oxidative stress (OS) and subsequent damage of important macromolecules such as lipids, proteins, and genetic material with resultant alteration of their functions (Gupta et al., 2014; Elejalde et al., 2021). During exercise, high oxygen consumption and utilisation results in the generation and accumulation of more RONS in skeletal muscles this leads to exercise-induced OS and other ailments such as fatigue, muscle damage, pain, and overall poor exercise performances and prolonged recovery (Mastaloudis et al., 2001; Finaud et al., 2006; Powers & Jackson, 2008; Powers et al., 2020). To date, there is still no official protocol/approach/practice used to prevent or mitigate exercise-induced ailments (Wan et al., 2017). Nonetheless, many athletes opt to use certain non-specific treatments (synthetic drugs) or preventative approaches such as the use of high/mega doses of single antioxidant supplements. These approaches do have drawbacks such as high costs, may act as stimulant drugs, can only be obtained through a medical prescription, and may result in serious side effects and low tolerance. Additionally, the safety and efficacy of these drugs have been questioned in some studies (Nieman et al., 2006; Wan et al., 2017). Therefore, the quest for novel or alternative interventions continues and more studies are investigating the use of dietary natural polyphenolics constituent as they contain components with differing structures and resultant differing mechanistic bioactivities as possible supplements and/or ergogenic aids.

Rooibos, made from the plant *Aspalathus linearis* is a popular indigenous South African herbal tea, harvested in the Cederberg mountainous region of South Africa, Western Cape province. In 2014, Rooibos was granted geographical indication (GI) status in the European Union (EU) and is the first African food to join Champagne, Irish Whiskey, Porto, and other iconic products in the European Commission register of protected designations of origin (PDO) and protected geographical indications (GI) (SARC, 2023). It has gained the attention of many researchers in the recent past due to its rich and unique phytochemical and polyphenolic content and related health promoting bioactivities which are of potential health use to prevent, detoxify and attenuate exercise-induced OS effects as well as improve exercise performance and recovery (Chen et al., 2013; Hong et al., 2014; Watanabe et al., 2014). Although the antioxidant effects of Rooibos herbal tea have been investigated and well documented (Marnewick et al., 2005; 2009; Persson et al., 2010; Marnewick et al., 2011; Chen et al., 2013; Canda et al., 2014; Smith & Swart, 2016; Dludla et al., 2017; 2020), it's bioactivities in humans, in relation to exercise-induced OS has been rarely reported on. Thus, the current study using a randomised placebo-controlled dietary intervention model is aimed to assess the modulation of exercise-induced oxidative stress using a standardised fermented Rooibos beverage and to further elucidate if Rooibos ingestion could improve exercise performance outcomes and/or recovery in the participants.

3.5 Rationale Phase III – The influence of exercise and Rooibos consumption on the human serum metabolome.

Metabolomics is a field of omics science, that involves a comprehensive measurement, identification, and quantification of small molecule (<1500 Da) metabolites in the metabolome. The mmetabolome itself refers to the complete set of low molecular weight metabolites found in a cell, tissue, biofluid, or organism and indicates the physiological or pathological status of any organism (Khoramipour et al., 2022). Application of metabolomics to analyse the change in metabolome offers novel opportunities for a better understanding and evaluating an organisms' response to several challenges such as drugs/therapeutics, intense stress or exercise, food nutrients or any other stimulus to the metabolome (Montoliu et al., 2013). Among other things, the human metabolome includes all small molecules such as peptides, lipids, amino acids, nucleic acids, carbohydrates, vitamins, minerals, food additives, drugs and any other chemical that humans ingest, metabolise, catabolise, or come into contact with (Wishart et al., 2018).

Metabolomics analyses are mostly achieved through the separation of metabolites via liquid or gas chromatography followed by the detection of individual metabolites using retention time from the chromatographic separation, mass-to-charge ratio, and fragmentation pattern of each

ionised metabolite to identify the detected metabolites through matching against databases of known metabolites (Schranner et al., 2020). Metabolites are the end products of cellular metabolism and play a crucial role in energy production, storage, cell signalling, and apoptosis and provide information on the physiological state of an organism. Thus, metabolite measurement, through metabolomics offers an ideal route to measure both phenotype and physiology (Tian et al., 2021; Khoramipour et al., 2022). Targeted and untargeted metabolomics analysis are the two main metabolomics analysis methods (Kelly et al., 2020b), with nuclear magnetic resonance (NMR) and mass spectrometry (MS) used as the analytical techniques/ platforms. The NMR is high reproducibility, non-destructive to samples, needs minimal sample preparation and provides structural information, which is useful in the identification of unknown metabolites, but compared to MS, the NMR has lower sensitivity (usually 1–10 umol/L) and resolution (Shi et al., 2016; Kelly et al., 2020b). However, MS have low reproducibility but have higher resolution and sensitivity than NMR and is usually coupled with separation techniques, particularly liquid chromatography (LC) and gas chromatography (GC). When compared to NMR, MS-based techniques can detect several metabolites with a broad range of concentrations (Shi et al., 2016). Even though, GC-MS is often regarded to be simpler and faster than LC-MS (Schranner et al., 2020), these techniques each has its own advantages and limitations. Technological advancements in each platform (GC-MS or LC-MS) further provided improvements in analyses carried out. Noting that, each system measures a fraction of the metabolome, under investigation. Nevertheless, because of different separation modes that can be utilised, such as normal phase LC, reversed phase LC and hydrophilic interaction chromatography, this makes the LC-MS more flexible and suitable than GC-MS. However, the final choice of analytical platform or technique often depends on the study purpose and the nature of the samples, because each analytical technique has its advantages and disadvantages (Shi et al., 2016).

The ultimate objective of metabolomics is to measure the physiological state and/or changes occurring within the organism in response to an intervention or stimulus (Heaney et al., 2019). Changes in human metabolome as a result of different stimuli have been observed for many decades. In 1975, Wahren et al. (1975) argued that after 40 minutes of upright continuous bicycle exercise, changes in the study participant's metabolic status were comparable to the metabolic status of a few days of starvation. Exercise is an external stimulus which causes disturbance to the metabolome and the whole body’s homeostasis which often subsequently alters the metabolome metabolite concentration (Khoramipour et al., 2022). During exercise, these alterations and/or changes reflect the mobilisation, utilisation, and conversion of energy metabolites such as carbohydrates and triacylglycerols (fats) to meet the ATP demand of the exercising muscles (Schranner et al., 2020). Metabolomic changes associated with exercise mostly include changes in fatty acid metabolism, mobilisation and lipolysis, the TCA cycle,

glycolysis, amino acid metabolism, carnitine metabolism, purine metabolism, cholesterol metabolism and insulin sensitivity among others (Heaney et al., 2019). Similar to exercise, dietary consumption is also an external stimulus, whether it is consumed acutely or chronically it has an effect on metabolome metabolite concentration (Nieman, 2021). When food and/or beverage is consumed, it undergoes several metabolism processes before it gets transported to the blood/tissue. This causes disturbance to the metabolome or a change in metabolite concentration (Marín et al., 2015), which may either be beneficial or detrimental to the organism's health. Thus, analyses of these metabolites' concentration via metabolomics analysis provide a picture regarding the effects of exercise, nutrition or an intervention on the metabolic pathways or metabolic state of an individual in physiology and pathological condition (Bongiovanni et al., 2019).

In the recent past, metabolomics has gained increasing attention of many researchers in sciences like biomedicine and exercise physiology (Suárez et al., 2017; Nieman et al., 2018; Takis et al., 2019; Wishart, 2019; Chen et al., 2020; Tu et al., 2021). The main reason for the growing application/usage of metabolomics in biomedicine is because metabolomics studies can be conducted to (**1**) obtain healthy reference value measurements, (**2**) compare case versus control that would explore how different stimuli affect the metabolome, or (**3**) collect data over short or long periods to explore longitudinal effects (Khoramipour et al., 2022). The usage of metabolomics provides a comprehensive view of the metabolome and its modulation by interventions. This makes the metabolomics analysis approach ideal for analysing/probing processes as they occur in real-time or over a period (e.g., seconds, minutes, hours, or days) and this could help to provide an early diagnosis or prognosis and even detect the efficacy of a specific therapeutic treatment (Bongiovanni et al., 2019; Khoramipour et al., 2022).

The health benefits of Rooibos consumptions have been demonstrated in several studies (Marnewick et al., 2005; Sissing et al., 2011; Smith & Swart, 2016; Dludla et al., 2017; S. E. H. Davies et al., 2019; Dludla et al., 2020), and the ergogenic aid potential of Rooibos consumptions is currently an active research area. However, to date, there's no clear consensus on how Rooibos consumption influences metabolites, which is often speculated to result or translate into health benefits and positive effects and subsequently improvements in exercise performance and recovery. Hence, this study phase aimed to assess the influence of Rooibos and placebo beverages consumption on study participants' plasma metabolites shift/changes that occurred during the submaximal exercise regime.

3.6 Rationale Phase IV – The influence of genetic variations in genes related to oxidative stress and injury on exercise outputs following the consumption of an acute dose of a standardised fermented Rooibos beverage.

Apart from the health benefits individuals yield from exercise, exercise also induces physiological challenges to the body including inflammatory response, muscle damage and OS burden due to increased RONS (Collins et al., 2015; Baumert et al., 2016; D'Angelo & Rosa, 2020). Although muscle damage is sometimes needed for adaptation purposes, but if the damage occurs excessively it may lead to poor recovery and increase injury risk (Baumert et al., 2016). Furthermore, some exercise-induced damages and injury risks could be mitigated or exacerbated by individuals' genetic background. Many genetic variations have been identified, and depending on where that genetic variation occurs within the gene, can directly affect gene expression of such gene (Baumert et al., 2018; Baumert et al., 2022). Some genes genetic variations have been associated with various exercise phenotypical characteristics as well as injury susceptibility, while some have the potential to influence the structure, tissue functions, and mechanical properties of musculoskeletal soft tissues (Becer & Çirakoğlu, 2015; Appel et al., 2021). This potentially explains the inter-individual variability in injury frequency and severity and also why some individuals suffer and/or experience a higher magnitude of muscle damage or injuries than others despite participating in the same exercise regime (Del Coso et al., 2020; Hall et al., 2022). The most common genetic variations are **1**) single nucleotide polymorphism (SNP) – where one nucleotide substitutes another, and **2**) insertion/deletion (indel) polymorphisms – where a specific nucleotide sequence is present (insertion) or absent (deletion) from the allele (Baumert et al., 2016).

When considering athletes and/or sports professionals, genetic variations found in genes encoding key musculoskeletal proteins have implications on their performance and recovery and predispose them to exercise injury (Tharabenjasin et al., 2019; Appel et al., 2021; Mohd Fazli et al., 2022). Furthermore, genetic variants or SNPs have been found in genes that encode antioxidant enzymes (SOD, CAT, and GPx) and these often compromise the endogenous antioxidant defence system's capacity to counter against RONS (Kotowska & Jówko, 2020). For instance, SNPs in the mitochondrial manganese superoxide dismutase (MnSOD) gene whereby cytosine (C) in the GCT codon is replaced/ substituted by thiamine (T) and results in mutated valine (GTT) from an alanine (GCT) (Ala16Val, rs-4880) (Kotowska & Jówko, 2020; Wu et al., 2021). This modification produces a β-sheet structure instead of an α-helix structure, resulting in a reduced MnSOD activity, which in turn increases oxidative stress damage effects in the mitochondria, due to decreased or insufficient transportation of the MnSOD antioxidant enzyme (Pourvali et al., 2016; Jówko et al., 2017; Synowiec et al., 2021). Another genetic variation is TNF-α -308 G>A, whereby guanine (G) is substituted by Adenine (A) at nucleotides -308 within the TNF-α promoter region (Ferguson et al., 2008). This

SNP also influences exercise oxidative stress damage effects, particularly inflammatory related conditions because it alters the functions, production and/or subsequent expression TNF-α gene (Umapathy et al., 2018; Tiongco et al., 2020).

Despite the acknowledged genetic variations implications on individuals' health, the utilisation of genetic testing is still not commonly practised in clinical diagnosis, due to several factors especially ethical dilemmas that could occur as a result of genetic testing. However, the literature demonstrates that genetic testing is slowly and increasingly being used as a pathology support genetic testing (PSGT) approach to support the traditional clinical diagnosis methods because it identifies health risk gene variants and makes a preclinical genetic diagnosis. This helps to mitigate/prevent the negative effects of phenotypic expression, disease progression and worsening of conditions (Castelletti et al., 2022). The PSGT refers to the analysis of DNA sequences to identify genetic variants that cause or predispose an individual to a certain pathology or ailments. In recent years, studies utilised the PSGT approach to explore the relationship between genetics and susceptibility, prognosis or provide scientific basis for the pathogenesis and/or possible treatment for various pathologies such as acute and chronic hepatitis liver diseases (Tang et al., 2020), kidney diseases (Umapathy et al., 2018), diabetes (Pourvali et al., 2016), diabetic nephropathy (Tiongco et al., 2020), cardiovascular diseases and stroke (Synowiec et al., 2021). However, not all genetic variants cause or predispose individuals to pathologies or ailments (Castelletti et al., 2022). The fact that exercise yields health benefits, understanding which genetic make-up contributes or compromises such benefits is equally important (Harvey et al., 2020). Additionally, knowing the likelihood of athletes' or individual response to an exercise or training regime is what every fitness coach would prefer since it would help to employ better adaptation mechanisms to maximise fitness and recovery and also reduce exercise injury risk. This study phase employed the PSGT approach to assess the study participants' genotype-phenotype relationship by analysing genetic variants in genes involved in antioxidant balance, inflammatory response, and exercise injury risk and comparing them with exercise-induced OS damage blood biochemical biomarkers.

CHAPTER FOUR

MATERIALS AND METHODS

4.1.1 Study design and ethics

The study design included a randomised, single-blinded placebo-controlled crossover dietary intervention trial (RCIT), where forty healthy adult males (18 – 50 years old) consumed an acute dose of either the standardised fermented Rooibos or placebo beverage (375 mL) together with a standardised snack, waiting 90 minutes (to ensure optimal bioavailability) before completing a modified submaximal exercise ramp test with 10 sets of sprints, on a Wattbike cycle ergometer. The study protocol was approved by the Faculty of Health and Wellness Sciences Human Research Ethics Committee, Cape Peninsula University of Technology (Project number: CPUT/HWS-REC 2018/H2) and followed guidelines as stated in the Declaration of Helsinki. Written informed consent was obtained from all the study participants before commencement of the study.

4.1.2 Participant recruitment and screening

As indicated in Figure 4.1, about 54 physically active adult males (18 – 50 years old) were initially screened for study eligibility, but only 40 were included in the study, randomly divided, and completed the crossover trial. Potential participants were recruited via posting advertisements and study leaflets at the study site, the Human Performance Laboratory (HPL) situated on the Mowbray campus of the Cape Peninsula University of Technology as well as distributing leaflets to various sports clubs in the Cape Town area. Interested individuals were screened for study eligibility with inclusion criteria including physically active males with no current or past history of using anabolic steroids, not on any chronic illness medication (hypertension, hyperglycaemia, hypercholesterolemia or metabolic disorders), not using any ergogenic nutritional/ antioxidant supplements, not consuming more than 2 alcohol beverages per day; no unusual dietary habits (e.g. vegetarian or vegan diets), and not having sustained any muscular or skeletal injury in the previous two months before the study. Additionally, screened fingerpick blood values were expected to be within the normal reference ranges for total cholesterol (3.5 - 5.0 mmol/L), fasting glucose (5. 6 – 7.0 mmol/L), haemoglobin (13.3 – 17.2 g/dL) and blood pressure (<120/80 mmHg - <140/90 mmHg) as per reference ranges published on Pathcare Laboratory (South Africa) website. Those who met the inclusion criteria were recruited into the study. Due to physiological and performance differences in exercise capacity and hormonal response to exercise between males and females (Accattato et al., 2017; Copetti et al., 2020), only adult males were recruited for this study.

4.1.3 Randomisation and dietary intake

Recruited participants were randomly assigned into two groups, either consuming the rooibos beverage or the placebo beverage, whereafter they were crossed over before the repeat of the exercise regime**.** Recruited participants were requested to keep dietary records for three non-consecutive days including two weekdays and one weekend day during the week before their exercise days. To assist them with this, each received a set of household measuring items (teaspoons, cups, and a jar), dietary record booklets and a dietary restriction list (to ensure a minimal intake of flavonoid-rich foods and beverages) was also given to the participants. The participants were also asked to not change dietary habits and physical activity during the study period. However, participants were instructed to abstain from exercise activity for 48 h and also avoid alcohol intake 24 h before their exercise days.

4.1.4 Intervention beverages

The intervention beverages (fermented Rooibos and placebo) were freshly prepared on the morning of the exercise days. The placebo beverage was prepared by dissolving a store-bought peach and apricot-flavoured sachet (13.11 g) in 375 mL of still bottled water, whereas the Rooibos beverage was prepared by dissolving the fermented Rooibos soluble extract (1.6 g) in 375 mL of the peach and apricot flavoured still water. The cold water-soluble fermented Rooibos extract is commercially available and was a gift from Rooibos Ltd (Clanwilliam, South Africa).

4.1.5 Study exercise test protocol

Study participants were asked not to do any exercise 48 h before the test day. After an overnight fast (8 – 12 h), participants reported to the Human Performance Laboratory of the university and were given a standardised light breakfast (162.8 g, two slices of white bread topped with margarine, pastrami, and cheese) and consumed 375 mL of either the Rooibos or placebo beverage. Ninety minutes thereafter, participants commenced with the modified Wattbike submaximal ramp test protocol as detailed below.

1. 5 minutes warm-up, at 50 – 60 rpm on air resistance setting 3 on the Wattbike Pro
2. Pedal in a seated position for 1 minute at the starting power of 100 watts (W), at a cadence rate of between 70 –100 rpm
3. Increase the air resistance setting and/or cadence as necessary every 1 minute to ensure a 15 W increase in power (W) output every 1 minute. This allowed the body to adapt to the increasing workload and steady rate heart rate to be achieved (at that level)

4. Keep increasing the power (W) output by 15 W every min until the rider reaches the rate of perceived exertion (RPE) of somewhat hard - level 13 on the Borg scale rate of perceived exertion
5. Or if the rider experiences any adverse symptoms, requests to stop or experiences an emergency
6. Once the participant reaches level 13 on the Borg scale, he stopped cycling for 1 minute and then asked to complete sets of 10 second sprints (10 max or until the participant reaches the RPE of maximal exertion - level 20 on the Borg scale), separated by 15 sec of passive recovery rest periods. Each participant attended two of these exercise sessions with 7 days apart, serving as the washout period as shown in Figure 4.1.

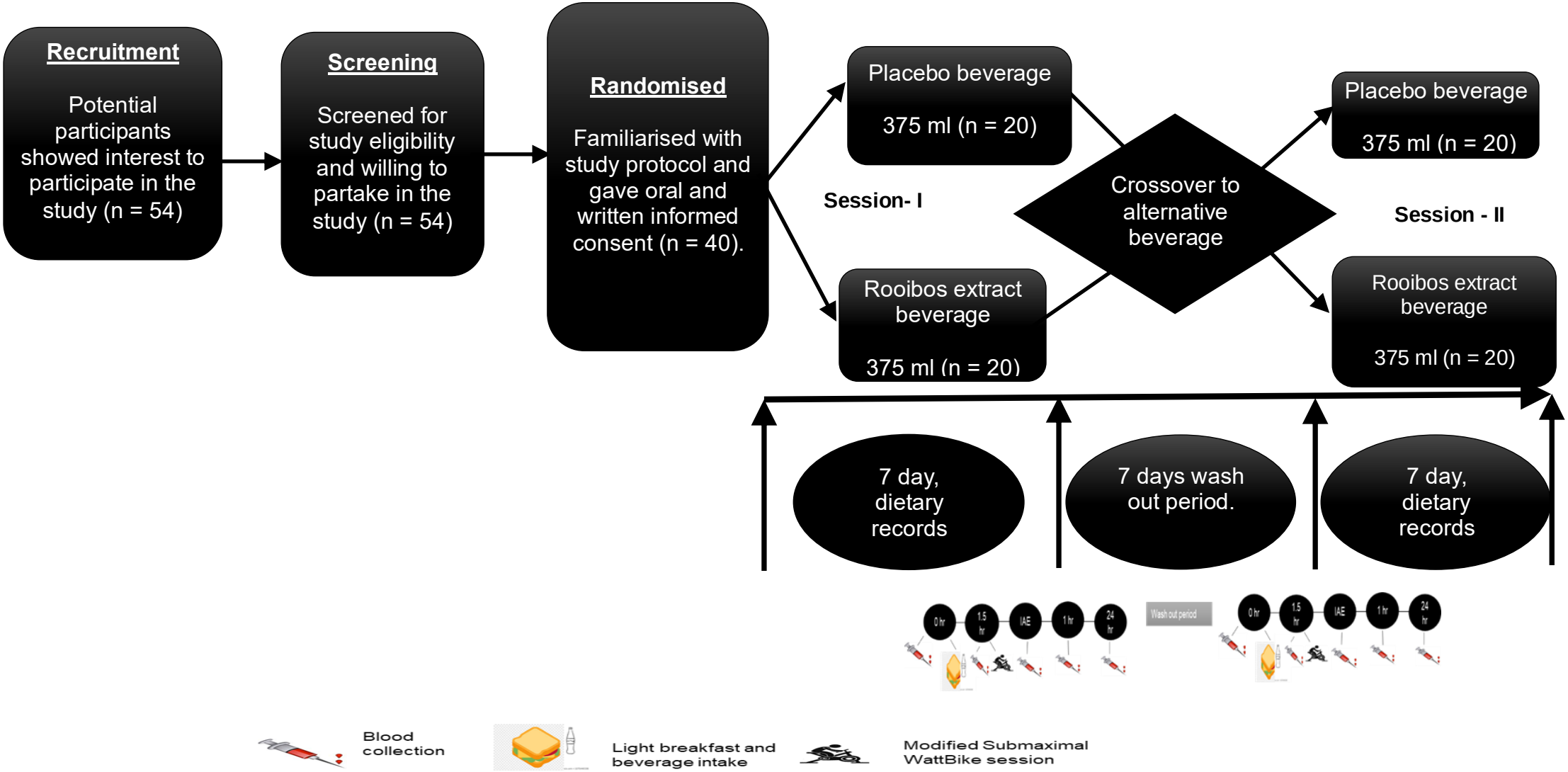


Figure 4.1: Schematic diagram showing the study trial design and participant flow

4.1.6 Anthropometric and blood pressure measurements

Anthropometrics including stature, mass, and waist circumference, were taken for all participants during their first session. A digital weighing scale (Soehnle Professional) was used to determine their mass (kg), while a free-standing stadiometer (Scales 2000, Rochdale Park, Durban, SA) was used to measure the stature (m). A two-metre non-stretchable measuring tape was used to take the waist circumference and the body mass index (BMI) was calculated manually using the standard formula (mass [kg]/stature [m^2]). Blood pressure (systolic and diastolic) was measured before the start of each exercise session (Rossmax Medical®, Kraaifontein, SA).

4.1.7 Blood sample collection and processing

Blood was drawn from the participant's antecubital vein into a serum separate tube (SST), sodium fluoride (NaF) tube and ethylenediaminetetraacetic acid (EDTA) tubes at five different time points during each session (0 h, 90 min after the ingestion of beverage and snack but before the exercise, immediately after the exercise was completed [IAE], 1 h and 24 h post-exercise). Both 0 h and 24 h bloods were fasting samples. After collection, 100 µL of the EDTA collected blood was aliquoted into a 2 mL Eppendorf tube containing 10 µL of methyl-2-vinylpyridinium (M2VP) for GSSG analysis. Another 50 µL of EDTA collected blood was aliquoted into a 2 mL Eppendorf tube for GSH analysis. The remaining blood in the EDTA tube was centrifuged at 2000 x g, 4 °C for 10 min whereafter the plasma, buffy coat and red blood cells were aliquoted into different Eppendorf tubes. The NaF blood samples were centrifuged and aliquoted for glucose analysis, while the SST blood samples were first allowed to clot before being centrifuged to obtain serum aliquots. All sample aliquots were stored at - 80 °C until the time of analysis.

4.1.8 High performance liquid chromatography of study beverages

An Agilent Technologies 1200 series system high performance liquid chromatography (HPLC) was used to determine the main polyphenolic constituents of the study intervention beverages as described by Bramati et al. (2003). Briefly, the HPLC system consisted of a quaternary pump, an autosampler, a degasser, a diode array, and a multiple wavelength detector. A 5 µm, (150 mm x 4.6 mm i.d) column was used for the separation and acquisition was set at 287 nm for aspalathin and at 360 nm for other major Rooibos components. Water (solvent A) and methanol (solvent B) with 300 µL/L of trifluoroacetic acid in each solvent served as the mobile phases. The elution gradient started with solvent A at 95%, then 75% after 5 min and to 20% after 25 min and back to 95% after 28 min. The flow rate was set at 0.8 mL/min, with 20 µL as the injection volume and column temperature set at 23 °C. The beverages' peaks were identified based on the retention time of the standard individual phenolics. The concentration

of each compound in the beverages was calculated using the standard area and sample area. Results were reported as mg/375 mL of beverage.

4.1.9 The antioxidant content of the study beverages and participant samples

4.1.9.1 Total polyphenolic content (TPC)

The method described by Singleton and Rossi (1965) was used to assess the TPC in the study beverages and the participants plasma samples pre-and-post beverage intake. Briefly, all reagents were freshly prepared, 0.040 g of gallic acid (Sigma, SA) was dissolved in 50 mL of 10% ethanol to make the gallic acid stock standard solution. This solution was used to prepare the standard series (0, 25, 50, 100, 250 and 500 mg/L) with double distilled water (ddH_2O) used as diluent. The assay working solution, Folin Ciocalteu reagent (FCR) was prepared by diluting 1 mL of FCR (Merck, SA) with 9 mL of ddH_2O in a 15 mL tube. A thawed plasma sample or study beverage aliquot was equilibrated to room temperature (RT) and diluted (10x dilution) with ddH_2O. The assay was done in triplicate, in a clear 96 well microplate (Figure 4.2) and the reaction mixture in each well contained 25 µL of standard/sample (plasma/beverage) and 125 µL of FCR. The microplate was incubated for 5 min at RT then 100 µL of 7.5% sodium carbonate **(**Na_2CO_3) was added to each well. The microplate was further incubated for 2 hr at RT before reading the absorbance in a SpectraMax i3x platform plate reader (Molecular Devices, China) set at 25 °C and 765 nm. Microsoft® Excel 2016 program was used to do data processing and calculations based on a calibration curve plotted using the standard series and absorbance readings. Results were expressed as mg gallic acid equivalents (GAE) per 375 mL (mg GAE/375 mL) or mg GAE/mL in beverage and plasma, respectively.

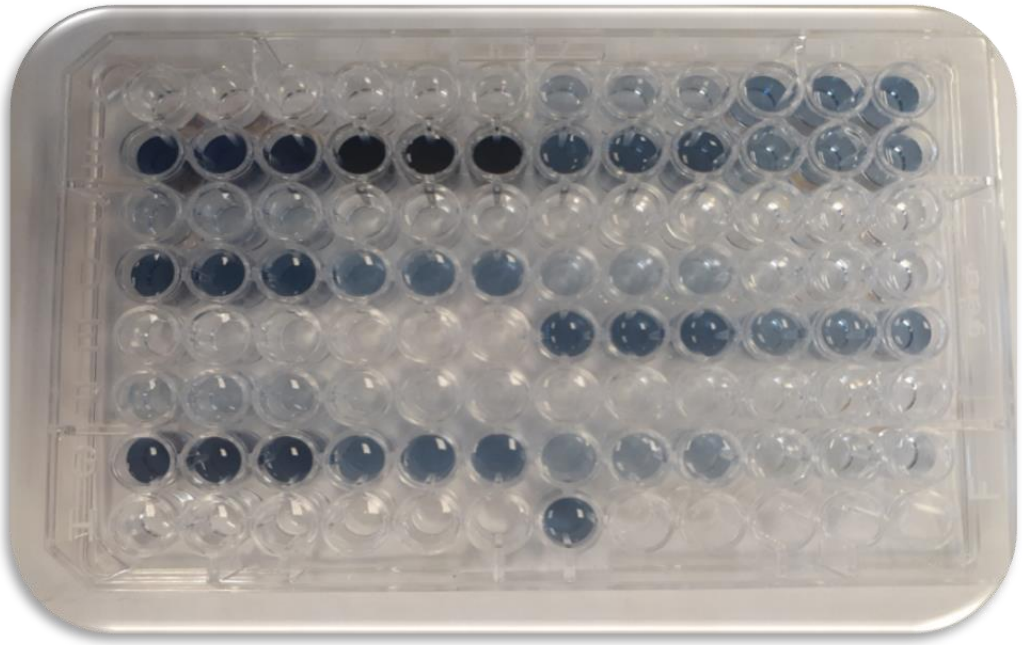


Figure 4.2: The 96-well clear microplate utilised for the total polyphenolic content assay

4.1.9.2 Flavanol and flavonol contents of the study beverages

The flavanol content of both study beverages was determined as described by Treutter (1989). All reagents were freshly prepared, briefly, 32% hydrochloric acid (HCl) and absolute methanol (MeOH, Merck, SA) were mixed (1:4) and used to prepare the assay working solution 4-dimethylaminocinnamaldehyde (DMACA) reagent by dissolving 0.0025 g of DMACA powder (Merck, SA) in a 50 mL tube. Catechin hydrate (0.0145 g) was dissolved in 500 mL of MeOH and used to prepare the standard series (0, 5, 10, 25, 50 and 100 µM) using ddH_2O as the diluent. A study beverage aliquot was diluted (10x dilution) with ddH_2O as a diluent, and, the assay was performed in triplicate, in a clear 96 well microplate. The mixture in each well consisted of 50 µL of standard/sample (beverage) and 250 µL of DMACA solution. The microplate was incubated for 30 min at RT before reading the absorbance in a SpectraMax i3x platform plate reader (Molecular Devices, China) set at 25 °C and 640 nm. Microsoft® Excel 2016 program was used to do data processing and calculations based on a calibration curve plotted using the standard series and absorbance readings**.** Results were expressed as catechin equivalents per 375 mL of study beverage. The flavonol content was determined as described by Mazza et al. (1999). All reagents were freshly prepared, briefly, 95% EtOH (Merck, SA) and 2% HCL solutions were prepared with ddH_2O as a diluent, then quercetin (4 mg) (Sigma-Aldrich, SA) was dissolved in 50 mL of 95% EtOH and used to prepare the standard series (0, 5, 10, 20, 40 and 80 µM) using ddH2O as a diluent. Study beverage aliquot was diluted (10x dilution) with ddH_2O, and then the assay was done in triplicate, in a clear 96 well microplate. The mixture in each well consisted of 12.5 µL of standard/sample (beverage) followed by 237.5 µL 2% HCl solution. The microplate was incubated for 30 min at RT before

reading the absorbance in a SpectraMax i3x platform plate reader (Molecular Devices, China) set at 25 °C and 640 nm. Microsoft® Excel 2016 program was used to do data processing and calculations based on a calibration curve plotted using the standard series and absorbance readings**.** Results were expressed as quercetin equivalents per 375 mL of study beverage.

4.1.10 Total antioxidant capacity of study beverages and plasma

Two assays were used to determine the antioxidant capacity and included the ferric reducing antioxidant power (FRAP) assay and the Trolox equivalent antioxidant capacity (TEAC) assay. The FRAP assay measures the sample's ability to reduce iron from the ferric (Fe3+) to ferrous (Fe2+) tripyridyltriazine (Fe2+ TPTZ) complex state at low pH (3.4). This study followed the method described by Benzie & Strain (1996) to assess the antioxidant capacity in the study beverages and participant plasma. All reagents were freshly prepared, briefly, 300 mM acetate buffer solution (pH 3.6) was prepared by dissolving sodium acetate (1.627 g) in 16 mL of glacial acetic acid (Merck, SA) with ddH_2O added to make it up to 1 L. A stock standard solution was prepared by dissolving 0.0088 g of ascorbic acid (Sigma, SA) in 50 mL of ddH_2O. This solution was used to prepare the standard series (0, 50, 100, 250, 500 and 1000 µM), with ddH2O used as diluent. The FRAP working reagent (straw-coloured solution) was prepared in a 50 mL tube by mixing 30 mL of 300 mM sodium acetate buffer, 3 mL of TPTZ solution (0.0093 g TPTZ dissolved in 3 mL of 0.1 M HCl), 3 mL Iron (III) chloride ($FeCl_3$) solution (0.053 g ferric chloride dissolved in 10 mL of ddH_2O) and 6 mL of ddH_2O. Thawed plasma samples and study beverage aliquots were equilibrated to RT before the analysis. The assay was done in triplicate with each well of a clear 96 well microplate (Figure 4.3) containing 10 µL of standard/sample (beverage or plasma undiluted) and 300 µL of FRAP reagent. The microplate was incubated at RT for 30 min before taking the absorbance reading in a SpectraMax i3x platform plate reader (Molecular Devices, China) set at 25 °C and 593 nm. Microsoft® Excel 2016 program was used to do data processing and calculations based on a calibration curve plotted using the standard series and absorbance readings. Results were expressed as µmole ascorbic acid equivalents per 375 mL of beverage (µmole AAE/375 mL) or µmole AAE/ mL in beverage and plasma, respectively.

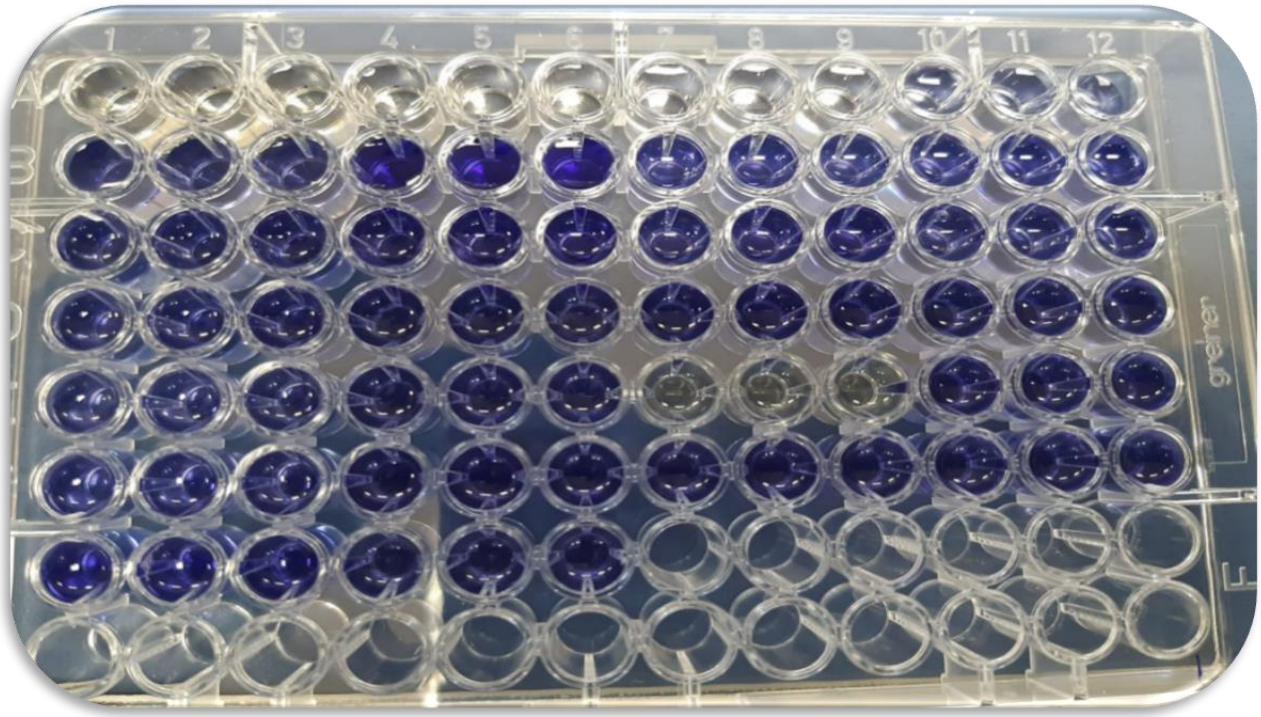


Figure 4.3: The clear, 96 well microplate utilised for the ferric reducing antioxidant power assay

The TEAC assay works on the principle of scavenging and/or inhibiting the generation of 2,2'-azino-di-3-ethylbenzthialozine sulphonate **(**ABTS•+) radicals. In this study, a method described by Re et al. (1999) was followed to assess TEAC in the study beverages and participants' plasma samples. All reagents were freshly prepared, briefly, 0.0192 g of 2,2'-azino-di-3-ethylbenzthialozine sulphonate **(**ABTS) was dissolved in 5 mL of ddH_2O, while 0.1892 g of potassium peroxodisulphate ($K_2S_2O_8$) was also dissolved in 5 mL of ddH_2O. The ABTS•+ radical was prepared by adding 88 µL of prepared $K_2S_2O_8$ solution to 5 mL of prepared ABTS solution. This mixture was heated in an oven set at 70 °C for 30 min and 1 mL of the dark green heated ABTS solution was diluted with 19 mL of ddH_2O or further diluted till it read 1.90 – 2.10 absorbance at 734 nm and designated the ABTS•+ working solution. Thawed plasma samples and study beverage aliquots were equilibrated to RT and diluted (10x dilution) with ddH_2O. The trolox standard solution was prepared by dissolving, 0.0125 g of trolox in 50 mL ethanol and then a standard series (0, 50, 100, 150, 250, 500 µM) was prepared from this stock solution with ddH_2O as diluent. The assay was done in triplicate in a clear 96 well microplate (Figure 4.4). The reaction mixtures in each well consisted of 25 µL of standard/sample (beverage or plasma) and 275 µL of ABTS working solution. The microplate was incubated at RT for 30 min before reading the absorbance in a SpectraMax i3x platform plate reader (Molecular Devices, China) set at 25 °C and 734 nm. Microsoft® Excel 2016 program was used to do data processing and calculations based on a calibration curve plotted using the standard series and absorbance readings. Results were expressed as µmol of trolox

equivalent per 375 mL (µmol TE/375 mL) or µmol TE /mL in beverage and plasma, respectively.

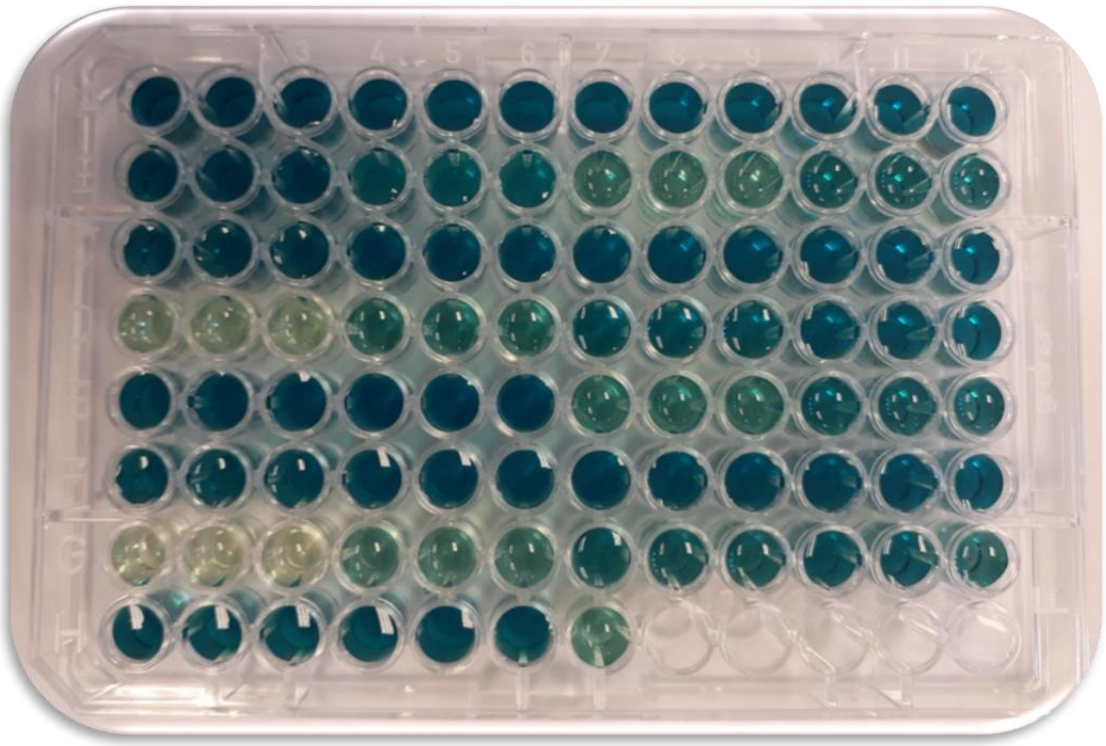


Figure 4.4: The clear 96 well microplate utilised for the Trolox equivalent antioxidant capacity assay

4.1.11 Lipid and protein oxidative damage biomarkers

4.1.11.1 Conjugated dienes (CDs)

When RONS removes an atom from a fatty acyl methylene group of lipids it results in the rearrangement of the carbon double bond and the formation of conjugated dienes (CDs) as the primary product of lipid peroxidation. This study followed a method by Glender and Recknagel (1984) to determine the plasma CDs concentration pre and post beverage intake and exercise. Briefly, plasma sample aliquots were thawed on ice then 100 µL was pipetted in a 2 mL Eppendorf tube followed by 400 µL of chloroform and methanol (2:1 ratio) mixture (Merck, S.A), vortexed for 10 s and centrifuged (Eppendorf 5810R, Germany) at 10 000 g, 4 °C for 10 min. After centrifuging, 200 µL of the bottom layer (chloroform) of the tube was transferred into a new 2 mL Eppendorf tube and left in the fridge overnight with the lid open to allow the chloroform to evaporate. The next morning, the dried sample was reconstituted in 1000 µL of cyclohexane (Merck, SA), vortexed for 10 s and used as an assay sample. The assay was performed in triplicate in a clear 96 well microplate, each well filled with 300 µL of sample. The absorbance was read against clean cyclohexane as a blank in a SpectraMax i3x

platform plate reader (Molecular Devices, China) set at 25 °C and 232 nm. The sample plasma CD concentration was calculated (equation 1) as the difference between plasma sample absorbance and blank sample absorbance and the final results were reported as nmol/ mL plasma.

Equation 1

$$\frac{A232s - A232b}{\xi}$$

Where A232s = absorbance of the sample at 234 nm

A232b = absorbance of blank at 234 nm

Ξ = coefficient of extinction 2.95 x 10*4,

Normally, ξ is based on a 1 cm cuvette, therefore, since a microplate well has a length of 0.9 cm, appropriate factoring was done in the calculations.

4.1.11.2 Thiobarbituric acid reactive substances (TBARS)

TBARS are indicators of late lipid peroxidation decomposition products with malondialdehyde (MDA) reacting with thiobarbituric acid (TBA). This study followed a method described by Draper et al (1993) with slight modifications. Briefly, all reagents were freshly prepared, 4mM butylated hydroxytoluene (BHT, Merck, SA) solution was prepared by dissolving 0.0088 g of BHT in 10 mL of ethanol (Sigma Aldrich, SA), while 0.1M sodium hydroxide (NaOH) solution was prepared by dissolving 0.2 g of NaOH in 50 mL of ddH_2O and this solution was used to dissolve 0.159 g of TBA (Sigma-Aldrich, SA) in 10 mL tube. A 684 µL of concentrated ortho-phosphoric acid (H_3PO4) was added to 50 mL of ddH_2O to make a 0.2M orthophosphoric acid reagent. Plasma sample aliquots were thawed and equilibrated to RT. Using a 2 mL tube, the reaction mixture consisted of 100 µL plasma, followed by 12.5 µL of BHT**,** 100 µL of H_3PO4 and 12.5 µL of TBA. The tube was vortexed for 10 s and a small hole was punched through the tube's lid before it was placed in a heating block set at 90 °C for 45 min. After heating, the sample tube was cooled down immediately by placing it on ice (to stop the MDA -TBA reaction) for 2 min before 1000 µL of butanol (Merck, SA) and 100 µL of saturated salt (sodium chloride) were added. The tube was vortexed again for 10 s, centrifuged (Eppendorf 5810R, Germany) at 10000 g, 4 °C for 2 min and the supernatant was used as an assay sample. The assay was done in triplicate in a clear 96 well microplate, each well filled with 300 µL of sample. The absorbance was read against a blank (butanol) in a SpectraMax i3x platform plate reader (Molecular Devices, China) set at 25 °C and 532 nm. The plasma sample's TBARS concentration was calculated as the difference between plasma sample absorbance and blank sample absorbance (equation 2). Results were reported as µmol/ mL plasma.

Equation 2

$$\frac{A532s - A532b}{\xi}$$

Where A532s = absorbance of the sample at 532 nm

A532b = absorbance of blank at 532 nm

Ξ = coefficient of extinction 2.95 x 10*4,

Normally, ξ is based on a 1 cm cuvette, therefore, since a microplate well has a length of 0.9 cm, appropriate factoring was done in the calculations.

4.1.11.3 Protein carbonyls (PC)

Protein oxidation implies an irreversible modification/damage of proteins caused directly or indirectly by RONS or oxidative stress by-products. This study makes use of the procedure described by Colombo et al (2016) to assess participant plasma protein oxidation. Briefly, all reagents were freshly prepared except the 10 mM DNPH solution which was prepared by dissolving 198 g DNPH (Sigma-Aldrich, S.A) in 100 mL of 2.5 M HCl and left overnight in the dark at RT. The 20% (w/v) and 10% (w/v) Trichloroacetic acid (TCA, Sigma-Aldrich, SA) solutions were prepared by 100 mL of ddH_2O, while 6 M guanidine hydrochloride solution was prepared by dissolving 27.32 g of 6 M guanidine hydrochloride (Whitehead Scientific, S.A) in 100 mL of ddH_2O, whereas 2.5 M HCl was prepared by adding 24.635 mL of 36.46 MW HCl (Merck, S.A) to 75. 365 mL of ddH_2O. For the assay, the plasma protein concentration was determined first using the Pierce bicinchoninic acid (BCA) assay method, to then normalise the samples' protein concentration to 1 mg/mL. Hereafter 200 µL of the normalised sample was pipetted into a 15 mL Sterilin tube followed by 800 µL of 10 mM DNPH solution. A blank sample was also prepared in a different 15 mL Sterilin tube and was treated similarly to the normalised sample, except that 2.5 M HCl was used instead of 10 mM DNPH solution. The 15 mL Sterilin sample tube was then incubated at RT for 60 min with mixing using a vortex every 10 to 15 min. Thereafter, 1 mL of 20% TCA was added to the sterilin tubes (sample and blank tubes), vortexed and incubated on ice for 10 min. After incubation, tubes were vortexed and centrifuged (Eppendorf 5810R, Germany) at 2 000 x g, 4 °C for 10 min. The supernatant was discarded and 1 mL of 10% TCA was added (to wash the pellets) vortexed and centrifuged again. The supernatant was discarded, and 1 mL of 1:1 (v/v) ethanol: ethyl acetate was added to further wash protein pellets. This step was repeated twice more to remove any free 10 mM DNPH debris and lipid contaminants (clear supernatant). After the last wash**,** pellets were dissolved in 500 µL of 6 M guanidine hydrochloride solution and incubated at 37 °C for 10 min. After incubation, the dissolved sample protein pellets were vortexed and centrifuged (Eppendorf 5810R, Germany) at 2 000 x g, 4 °C for 10 min. The supernatant was pipetted into a clear 96 microplate, 100 µL per well (in triplicate) and the absorbance was read in a SpectraMax i3x platform plate reader (Molecular Devices, China) set at 25 °C and 370 nm. The plasma samples' PC concentrations were calculated as described by Augustyniak et al. (2015) and the results were reported as nmol/ mL plasma.

4.1.12 Oxidative DNA damage

The comet assay is a method for visualising DNA damage in single cells. In this study, the comet assay protocol was followed as described by Draxler et al. (2021). Briefly, 10µl of whole blood samples were mixed with 200 µL 1%-agarose solution, and then 5 µL of the mixture was spotted onto 12 spot microscopy slides. For every blood sample, 4 slides were used because of 4 different treatments performed. To purify the whole blood sample DNA from cell debris, all microscopy slides were treated with a lysis buffer for one hour. Two of the microscopy slides were washed 3 times with enzyme buffer (40 mM HEPES, 0.1 M KCL, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8). On each spot of one of the two slides 30µl of the lesion-specific enzyme formamidopyrimidine DNA glycosylase (FPG) (diluted 1:3500 in enzyme buffer) was applied, using a slide unit (12-Gel Comet Assay Unit™, Severn Biotech Limited, Kidderminster, UK). On the second slide 30 µL of the enzyme buffer alone was applied. Both slides were incubated at 37 °C for 30 minutes. Treatment with enzyme buffer alone acted as a blank for the FPG treatment. The fourth slide was incubated for 15 min with the oxidising agent, hydrogen peroxide (H_2O_2) (100µM), which allowed indirect measurement of antioxidant status and represented resistance to H_2O_2. After, the treatment steps the DNA on the microscopy slides had to unwind in an alkaline electrophoresis buffer for 20 min (0.3 M NaOH, 1 mM EDTA, pH 13), followed by a 30 min electrophoresis run (25 V, 300 mA, 4 °C). The extent of DNA damage was visualised and evaluated by colouring the DNA with GelRed (GelRed TM Nucleic Acid Gel Stain, VWR, product no 41003) and using a fluorescence microscope (Nikon), The software used to accurately determine DNA damage was Comet Assay IV.

4.1.13 Redox status glutathione

4.1.13.1 Reduced glutathione (GSH)

Reduced glutathione (GSH), a low molecular weight antioxidant that forms part of the endogenous antioxidant defence system within living cells. This method referred to as a recycling assay (based on the enzymatic recycling of GSH using glutathione reductase [GR]) described by Asensi et al. (1999) was used to determine participant blood total glutathione level (tGSH, i.e., GSH + GSSG). Briefly, 75 mM phosphate buffer (pH 7.4) was freshly prepared by dissolving 10.36 g of sodium dihydrogen monophosphate (NaH_2PO4, Sigma–Aldrich, SA) in 1 L of ddH_2O, while 3 µM GSH stock solution used to prepare the standard series (0.5, 1.0, 1.5, 2.0, 2.5 µM) by dissolving 0.046 of 3 mM GSH standard (Merck, SA) in 50 mL of phosphate buffer. The assay enzyme was prepared by diluting 80 µL of GR (168U/mg, Merck, SA) in 4920 µL of phosphate buffer, while 6 mg of DTNB was dissolved in 50 mL of phosphate buffer. A 5% meta-phosphoric acid (MPA) solution was prepared by dissolving 2.5 g of MPA in 50 mL of ddH_2O, and 12 mL of phosphate buffer was added into the NADPH reagent bottle to dissolve 10 mg of NADPH (0.83 mg/mL). A whole blood sample aliquot was thawed on ice, 50 µL of sample pipetted into a new 2 mL Eppendorf tube and 350 µL of 5% MPA solution was added,

mixed for 15 – 20 s on a vortex and centrifuged (Eppendorf 5810R, Germany) at 14 000 g, 4 °C for 4 min. The supernatant (10 µL) was pipetted into a new 1.5 mL Eppendorf tube, mixed with 600 µL of buffer, vortexed thoroughly and used as an assay sample. The assay was done in triplicate in a clear 96 well microplate with each well containing 50 µL of standard/ sample, 50 µL of DTNB and 50 µL of GR. To initiate the reaction, 50 µL of NADPH was added to each well and the microplate was read immediately for five min in a SpectraMax i3x platform plate reader (Molecular Devices, China) set at 25 °C and 412 nm. Microsoft® Excel 2016 program was used to do data analyses and calculation blood sample tGSH concentration level base on the calibration curve of the GSH standard series, while GSH was calculated as indicated below (equation 3). Results were expressed as GSH concentration µmole/ mL blood.

Equation 3 $GSH = (tGSH - (2 \times GSSG))$

4.1.13.2 Oxidised glutathione (GSSG)

The same procedure described in the GSH quantification above was used to measure the participant's blood GSSG level. All reagents were freshly prepared exactly in the same way as for the GSH analysis. However, the GSH standard (Merck, SA) was used to make a 1.5 µM stock solution from which the standard series (0,5, 1.0, 1.5, 2.0, 2.5 µM) were prepared. The M2VP treated whole blood sample aliquots (100 µL) were thawed on ice and 290 µL of 5% MPA solution was added, vortexed for 15 – 20 s and centrifuged (Eppendorf 5810R, Germany) at 14 000 g, 4 °C for 4 min. The supernatant (25 µL) was pipetted into a new 1.5 mL Eppendorf tube, mixed with 350 µL of phosphate buffer, vortexed thoroughly; and used as an assay sample. The assay was done in triplicate, each well of the clear 96 well microplate contained 50 µL of standard/sample, 50 µL of DTNB and 50 µL of GR. The microplate was incubated at RT for five min then 50 µL of NADPH was added to each well to initiate the reaction. The microplate was read immediately for five min in a SpectraMax i3x platform plate reader (Molecular Devices, China) set at 25 °C and 412 nm. Microsoft® Excel 2016 program was used to do data analyses and calculation blood sample GSSG concentration level base on the standard calibration curve. Results were expressed as a GSSG concentration µmole/ mL blood.

Using the obtained GSH and GSSG concentrations, the GSH:GGSG ratio was determined for each of the participant samples, which reflects the redox status of glutathione.

4.1.14 Serum biochemical analyses

An automated chemistry analyser, Medica Easy Random Access (EasyRA, Massachusetts, USA) was used for the analysis of the following serum analytes: cholesterol profile, liver function enzymes, blood urea nitrogen, uric acid, glucose, creatinine, creatine kinase, lactate,

and lactate dehydrogenase) using the EasyRA commercial kits. Serum samples were thawed at RT and analyses were done in duplicate. Internal quality controls were run daily, and reagents were calibrated before participant sample analyses commenced. All assays were automatically analysed at their respective absorbances according to the manufacturer`s instructions. All reagent kits were purchased from Medical Electronic Distributors (S.A) and stored at 2 to 8 °C.

4.1.15 Exercise performance indicators

Exercise performance data which included time cycled, distance covered, along with the amount of power and work performed by participants were captured by the Wattbike computer monitor and downloaded via USB connection. Cardiorespiratory data (VO_2 relative oxygen uptake) was collected through a breath-by-breath analysis done on the CosMed Metabolic System. Participant heart rates were also monitored throughout the exercise regime by placing a heart rate signal (heart rate monitor polar, Finland) around the participant's chest with a beat per minute (bpm) recorded. This data (bpm) was transferred continually (via blue tooth) to the Wattbike computer monitor for integration with other exercise data.

Metabolomic analysis

4.1.16 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance spectroscopy (NMR) is an analytical technique commonly utilised in organic chemistry to confirm the structure of a synthesised compound. In the recent past, NMR analysis has been also employed in biological sample analysis and allows for the structural identification of molecules present in biological samples. NMR uses a high-powered magnet to induce a magnetic field that causes some protons to spin and align in an orientation corresponding to low or high energy states, known as α and β states (Heaney et al., 2019). When a sample is subjected to NMR analysis, radio waves are applied, causing the sample's protons in the α state to shift to the β state, however, once the applied energy is removed, the nuclei/protons return to their original energy state, resulting in resonance (an alteration in magnetic field) which is measured and/or interpreted as peaks on NMR spectra (Shi et al., 2016; Kelly et al., 2020b). The change in resonance is then compared to a standard, defined as zero, and the difference observed is known as the chemical shift. This chemical shift is characteristic of certain molecular structures; hence, it can be used to identify a molecule or part of the molecular structure present in the sample which further helps in the identification of the measured metabolite (Khoramipour et al 2022).

4.1.16.1 NMR reagents preparations

The serum/plasma buffer solution was prepared in a 500 mL glass bottle by dissolving 5.32 g of sodium phosphate dibasic (HNa_2PO_4) in 380 mL of HPLC-purity H_2O. About 400 mg of NMR

internal standard Trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP) that align the ^{1}H-NMR spectra and 5 mL of 3% sodium azide (NaN_3) that prevent bacterial growth within the sample were added to the buffer solution. The buffer solution was then mixed using sonication, then 100 mL of deuterium oxide (D_2O) was also added. The final buffer solution volume was adjusted to 500 mL with H_2O and drops of NaOH was added to adjust the pH to 7.4.

4.1.16.2 Samples preparation and analysis

Biological samples contain lipids and proteins that can hinder or cause NMR poor baseline if samples are analysed unfiltered. Therefore, plasma samples were filtered before NMR analyses to remove protein or any macromolecules present. The 10 kDa centrifugal filters used to filter plasma samples, are known to contain excess glycerol which results in unwanted peaks in the NMR spectrum. Hence, filters first were pre-rinsed (5 times with HPLC-purity H_2O, centrifuged at 3 000 g for 5 min, each rinse cycle) to remove excess glycerol before plasma sample filtration. After the last rinse, 250 µL plasma sample in 1.5 mL Eppendorf tube was mixed with 50 µL internal standard (mixture of 90 ppm with 15 µg/mL dimethylphenylalanine [DMPA], 15 µg/mL norleucine and 15 µg/mL of 3-phenoxybenzonic acid [3-PBA]), vortexed and pipetted into pre-rinsed centrifugal filters and centrifuged at 3000 g for 40 min. After centrifuge, the filtrate was aliquoted into different 1.5 mL Eppendorf tubes for different analyses, 100 µL for liquid chromatography mass spectrometry (LC–MS) analysis, 60 µL for nuclear magnetic resonance (NMR) analysis, and 40 µL of each sample into one 5 mL tube - this aliquot was used as pool quality control sample for both NMR and LC–MS analyses.

Due to the limited sample volume, the NMR microanalysis approach (miniaturised method) was used for this work as described by Mason et al. (2018). Briefly, instead of using the normal pipette, an eVol automated analytical syringe equipped with a 100 µL syringe and a 180 mm long bevel-tipped needle was used to aspirate 6 µL of buffer solution followed by 54 µL of prepared sample filtrate (1:10, buffer: sample ratio). The mixture (buffer-sample filtrate) was then mixed by purging the mixture total volume (60 µL) into a 2 mm NMR sample micro-tube, suck it up and pipette/purge it back again into the micro-NMR tube. The eVol syringe was then washed by aspirating 100 µL of HPLC-purity H_2O and purging it into a waste container, this cleansing was repeated three times before the next sample was prepared. After all samples have been prepared, a depth gauge was used to check if each sample volume in the micro-NMR tube was enough for analysis before samples were randomly loaded onto the 500 MHz NMR instrument (Bruker advance III HD) for analysis. Samples were analysed in batches with approximately 50 samples per batch. Each batch included three quality control (QC) samples with the first QC at the beginning, the second QC in the middle and the last QC at the end of the batch.

4.1.17 Mass spectrometry

Mass spectrometry (MS) is another analytical technique used in metabolomics to measure and identify multiple metabolites. The MS analysis technique offers a sensitive, specific, accurate and robust analytical approach to measure/ identify all detectable small molecule metabolites present within a sample (Dunn et al., 2010). The identification of metabolites by MS is based on mass-to-charge ratios (m/z) and has been improved by coupling the MS with some chromatographic techniques such as liquid or gas chromatography (LC, GC) (Creek et al., 2011). Chromatography techniques use the affinity of molecules to a stationary phase for the deconvolution of complicated matrices (e.g. plasma/serum), that contain hundreds of metabolites whereby as the metabolites pass through the chromatographic column their affinity to the stationary phase enables them to exhibit different times between entry and exit of the analytical column (Heaney et al., 2019). This allows metabolites to be separated and introduced into the MS at intervals, and assist to decrease the MS analytical complexity and subsequently improves metabolite identification through the reproducibility of metabolite retention times. The LC technique is mostly suitable for non-volatile metabolites and often used for blood and urine samples, while GC is most suitable for volatile metabolites as it requires the metabolite to enter the analytical system in the gas phase (Heaney et al., 2019). The combination of chromatography with MS allows for the analysis of known standard reference compounds and comparison of retention time and mass spectra for definitive identification of metabolites. In this study, only the LC-MS analytical technique was employed.

4.1.17.1 LC-MS samples preparation and analysis

Plasma sample filtrate aliquots (100 µL) in 1.5 mL tubes were first thawed at room temperature then 40 µL of an internal standard isotope (mixture containing phenylalanine, methionine valine C4-, C8- and C16-carnitine, with final concentration 10 mg/L) was added to each tube and centrifuged. After the centrifuge, sample mixture was transferred to glass vials and dried by subjected under a stream of nitrogen gas at 37 °C (evaporation) and dried samples were stored at -80 °C. On the day of analysis, dried samples were again subjected to a stream of nitrogen gas for 10 minutes, then butylated (derivatised) by adding 200 – 300 µL N-butanol: acetyl chloride (butylation procedure - active hydrogens on COOH functional groups are replaced with a butyl [-C4H8] group, resulting in the formation of butyl esters of amino acids and acyl-carnitines) to each vial. Thereafter, butylated samples were vortexed for 30 seconds and incubated in a heating block at 60 °C for 30 minutes. After incubation, samples were vortexed again for 1 min and then dried again under a stream of nitrogen gas at 37 °C for 2 h. After drying, the dried sample residue was reconstituted by adding 100 µL water: acetonitrile mixture (50:50, v/v), vortexed for 1 min and sample vials were incubated at RT for 30 min to rehydrate - this allows for compounds to dissolve better. After incubation at room temperature, sample vials were vortexed again and the sample was transferred to a glass insert in 1.5 mL tubes

and centrifuged at 12 000 g for 10 min, at 4 °C. After centrifuge, 70 µL of sample supernatant was then pipetted into another glass insert in a glass vial, then finally, each vial was loaded onto the analyser.

4.1.17.2 Sample analysis

The Agilent© 1200 series autosampler LC coupled to an Agilent© 6470 triple quadrupole LC-MS instrument was used to perform LC-MS analysis as described by Mels et al. (2011) with slight modifications. Briefly, the column temperature was kept at 30 °C throughout the entire run, while water (solvent A) and acetonitrile (solvent B) both contained 0.1% formic acid and were used as mobile phases. One µL of sample was injected and the mobile phase gradient was used to enhance metabolite separation. For separation, chromatography was performed on a C18 Zorbax SB-Aq reverse phase column (2.1mm x 150 mm x 3.5 µm, Agilent©) fitted with a Phenomenex guard column**.** The chromatographic gradient started at 95 % solvent A with a flow of 0.3 mL/min for 1 min then the gradient was increased to 20 % solvent B for 2 minutes. The gradient was then maintained for 3 minutes, then it was linearly increased to 100 % solvent B at 13 minutes. During this period, the flow was linearly increased to 0.35 mL/min at 13.1 min for 5 min, then the gradient was decreased to 5 % solvent B at 18.5 min and maintained for 1.5 min. A post-run of 10 minutes was allowed to ensure equilibration of the column to give a total run time of 30 min (20 min separation and 10 min post-run) per sample. Since this was targeted metabolomic analysis approach, the resulting eluent was transferred to the MS via positive electrospray ionisation (ESI) – this allows ionisation of targeted metabolites – with capillary voltage kept at 3 500 V; nitrogen source gas at 300 °C with a 7.5 L/minute flow rate; and nebuliser pressure at 30 psi. Lastly, spectra were acquired using the multiple reaction monitoring (MRM) mode at an electron multiplier voltage of 300 V and a dwell time of 45 milliseconds for all compounds.

Genetic variation analyses

4.1.18 Blood samples DNA extraction

A QIAamp DNA blood Midi kit (Qiagen, South Africa) spin column procedure was used to extract DNA from collected whole blood samples. Except for the AL buffer and AE solutions that were ready to use, all other reagents (AW1 buffer, AW2 buffer and protease enzyme) were freshly prepared according to the kit manufacturer's instructions. Briefly, 0.3 – 1 mL whole blood was added into a 2 mL Eppendorf tube (if the volume is less than 1 mL, phosphate buffer saline (PBS) was added to bring sample volume to 1 mL), mixed gently and transferred into 15 mL Sterilin tubes then 100 µL of Qiagen protease enzyme was added and vortexed for 15 s. After vortexing, 1.2 mL of AL buffer was added, and the sample mixture was mixed by inverting the sample tubes 15 times, vortex again for 1 min and then incubated in the water bath at 70 °C for 10 min. After incubation, 1 mL of ethanol (96 – 100%) was added to the

sample tubes and mixed by inverting the tubes 10 times, followed by 30 s vortexing. The whole sample solution in a 15 mL tube was then transferred onto the QIAamp Midi column (avoid moistening the rim) inside a 15 mL tube (provided by the kit manufacturer) and centrifuged at RT, 3000 rpm for 3 min. After centrifuge, the tube containing the filtrate was discarded, while the QIAamp Midi column was placed into a new 15 mL tube then 2 mL of AW1 buffer was pipetted onto the QIAamp Midi column membrane then the tube was centrifuged at RT, 5000 rpm for 1 min. After centrifuge, without discarding the filtrate, 2 mL of AW2 buffer was also pipetted onto the QIAamp Midi column membrane and the tube was centrifuged again at RT, 5000 rpm, for 15 minutes. After centrifuge, the collecting tube with filtrate was discarded while the QIAamp Midi column was placed into another new 15 mL tube then 300 µL of AE buffer solution was pipetted onto the membrane of the QIAamp Midi column and incubated at RT for 5 min then it was centrifuged at 5000 rpm for 2 min. After centrifuging, the filtrate was reloaded onto the QIAamp Midi column, incubated at RT for 5 min and centrifuged again at 5000 rpm for 2 min. After centrifuged, the filtrate (containing DNA) was pipetted into a new 2 mL eppendorf tube. The quality, concentration and purity of extracted sample DNA was determined using a Nanodrop One (Thermo Fisher Scientific) at 260 and 280 nm and then standardised/normalised to 10 ng/ µL with PRC water used as a diluent and was stored at 4 – 8 °C until genotyped.

4.1.18.1 Genotyping analysis

The genotyping of SNPs associated with chronic inflammation (TNF-α -308, rs1800629, G>A), oxidative stress (MnSOD 47, rs4880, T > C), and exercise injury risk genes COL5A1, rs12722, C>T; ATCT, rs16399, and rs1134170, A>T; GDF5, rs143383, T>C; CASP8, rs3834129, CTTACT, and lastly MIR608, rs4919510, C>G) was done with real-time polymerase chain reaction (PCR) using TaqMan endpoint genotyping method/assay. Briefly, the method/assay master mix was freshly prepared by mixing 0.25 µL 40x SNIP mix, 5.0 µL RealQ plus TaqMan master mix probe and 2.75 µL of PCR water in a 0.5 mL PCR reaction tube. All reagents were purchased from Thermo Fisher (S.A). After master mix preparation, 2 µL of normalised DNA sample (10 ng/ µL) was pipetted into a well of 96 well microplate then 8 µL of master mix was also added. Each well contained a reaction mixture of 10 µL (2 µL DNA and 8 µL master mix). The 96 well microplate was sealed immediately with sealing foil and loaded onto the PCR analyser (Light Cycler RT-PCR 480 II, Roche Diagnostic GmbH, Switzerland) for analysis using the endpoint genotyping analysis method. Quality control procedures were identical for all SNPs and were performed for each assay and included negative and positive controls in the runs. For negative control, 2 µL PCR water replaced the DNA template, while positive controls were only used in the MnSOD, TNF-α and GDF5 assays, to provide further confidence in our results (the rest of other assays there was no positive control samples, hence, samples

were further analysed with the conventional RT-PCR). The analysis took about 1h30 min, the analysis includes 1 cycle of denaturation at 95 °C for 15 minutes, 40 cycles of cycling/amplification at 95 ℃ for 15 seconds, followed by 1 cycle of melting at 60 °C for 1 second, and lastly 1 cycle of cooling at 40 °C for 5 seconds. This genotyping analysis method (endpoint genotyping) uses two hydrolysis probes labelled with different reporter dyes (FAM and VIC/HEX), designed to detect allele x and allele y. FAM dye detects samples that are homozygous for allele X, while VIC/HEX dye detects samples that are homozygous for allele Y. Data generated during analysis were analysed with the Light Cycler 480 Software for genetic variations, whereby samples with similar intensity distributions are grouped together and identified as a genotype.

4.1.18.2 Conventional PCR analysis

After genotyping analysis, due to lack of positive control samples, some assays COL5A1-rs12722, rs16399, rs1134170, GDF5 rs143383, CASP8 rs3834129, and MIR608 rs4919510) were further analysed with conventional RT- PCR and agarose gel electrophoresis to get an amplicon for Sanger sequencing. Conventional PCR analysis, primers sequence were as follow, COL5A1 (rs16399) forward primer 5'–TTCTCTCTTGTGGCTCTCTTGTG–3' and reverse primer 5'–CCCAGTGCGCCTTCAAG–3'; COL5A1 (rs1134170) forward primer 5' – C TTGTGGTGCTATCTATCTGTTTTAAGGT–3' and reverse primer 5'–G GATAAAGAAAGCAGGGAGAACGA-3'; CASP8 (rs3834129) forward primer 5'–TTG ATT CTT TCA GAC TTT TTC CTA GGC TT-3' and reverse primer 5'– GGA AGG CAC TGA GAC GTT AAG TAA C-3' each assay primers (100 µM) was diluted to 10 µM (10 µL of primer mixed with 90 µL of PRC water), while the assay master mix was prepared by mixing 12.5 µL TaqMan reagent, 1 µL of each primer and 9.5 µL of PCR water in a 1.5 mL eppendorf tube. The PCR reaction mix sample was prepared by pipetting 24 µL of master mix and 1 µL of DNA sample in a new 0.5 mL PCR reaction tube. A negative control sample was also prepared same as the PCR reaction mix sample, but with 1 µL PRC water instead of DNA. After the PCR reaction mixture (DNA sample and negative sample) preparation, PCR analysis was run immediately for about 2h30 minutes using a T100, Thermal Cycler (BIO-RAD) PCR. The analysis included an initial 5 min of denaturation at 95 °C followed by 35 cycles of amplification in a thermocycler and 1 min denaturation at 94 °C, then annealing for 1 min at 55 – 63 °C, 1 min extension at 72 °C and final extension for 10 min at 72 °C. After analysis, the PCR reaction mixture tube was taken out from the PCR analyser and prepared for agarose gel electrophoresis.

4.1.18.3 Agarose gel electrophoresis analysis

The agarose gel (1%) was prepared by dissolving 1 g agarose powder in 100 mL of tris-acetate EDTA (TAE) buffer. This mixture was heated in a microwave till the powder mixture was completely dissolved and then 8 µL of ethidium bromide was added, then mixture solution was

allowed to cool down and poured into an agarose gel casting tray and allowed to solidify. After the PCR analysis, 12 µL from the PCR reaction tube was mixed with 2 µL of tracking dye (bromophenol blue) in a new 0.5 mL PCR tube, mixed properly and loaded onto the prepared agarose gel in the electrophoresis chamber that was run for 1hr at 100 volts, with 10 µL fast DNA ladder (0.050 – 10kb, New England Biolabs) loaded and used as reference. The gel electrophoresis product was visualised using the imaging system (Molecular imager, Gel Doc XR+, BIO-RAD) and yielded a PCR product/ amplicon 446 bp (GDF5), 551 bp (MIR608), 579 bp (COL5A1), 450 bp (CASP8) and 432 bp (COL5A1_ rs12722). The remaining sample volume in the PRC reaction tube was cleaned according to PCR clean-up kit instructions (Invetek, molecular, MS Spin PCRapace) and was sent to the central analysis facilities (CAF, Stellenbosch) for sanger sequencing.

4.2 Statistical data analysis

The statistical analysis for the study phases I, II and IV was done using IBM SPSS 29. To determine the effect of Rooibos vs placebo on the several blood biomarkers measured in the study participants, a generalised estimating equations (GEE) analysis was performed since there were repeated time points where blood data was measured. The GEE models repeated data where the dependent variable is not normally distributed or is categorical. In the case of this study, the dependent variables were modelled using the gamma logistic distribution. In the analysis of repeated data, there is a dependence on repeated measurements. Various working correlation matrices underpin these dependencies. In this study, a system of equations, based on quasi-likelihood distributional assumptions, is used to estimate model parameters in GEE, giving consistent estimators of the regression parameters and their variances under weak assumptions. The comparison of group averages is tested using pairwise comparisons using the Bonferroni method. For the metabolomic, data generated by the two metabolomic analytical platforms (LC-MS and NMR) were analysed with an online metabolomics suite MetaboAnalyst 5.0 (available online). The data were first normalised to the internal standards in Excel before an interquartile range (IQR) filter was applied in MetaboAnalyst. Thereafter, data was log transformed (base 10) and scaled by mean centring. The student's t-test was performed to identify significantly altered metabolites, and a p value < 0.05 was considered statistically significant and a false discovery rate (FDR) corrected q-value of < 0.05 was considered statistically significant. Principal component analyses (PCA) score plots and hierarchical heat maps were used to indicate the clustering and covariances of metabolites identified from each analytical platform. Data was analysed in two stages, first by comparing the placebo vs Rooibos intervention (inter group differences) at different time points (0 h, IAE and 24 h post-exercise), and secondly by comparing the differences within each group (intra group differences).

CHAPTER FIVE
RESULTS AND DISCUSSION

5.1 Phase I – Results

5.1.1 Study participant cohort

A total of 54 participants were initially screened for study eligibility. Only 40 physically active adult males (18 – 50 years old) adhered to all the inclusion and exclusion criteria and were included in the study, randomly divided, and participated in the crossover trial, with 20 starting with the placebo intervention beverage and the remaining 20 starting with the Rooibos intervention beverage (Figure 5.1). Upon analysing blood samples data and exercise data that were captured on the Wattbike monitor as well as dietary records, some participants' data were further excluded from the final study data analysis based on reasons as indicated in Figure 5.1.

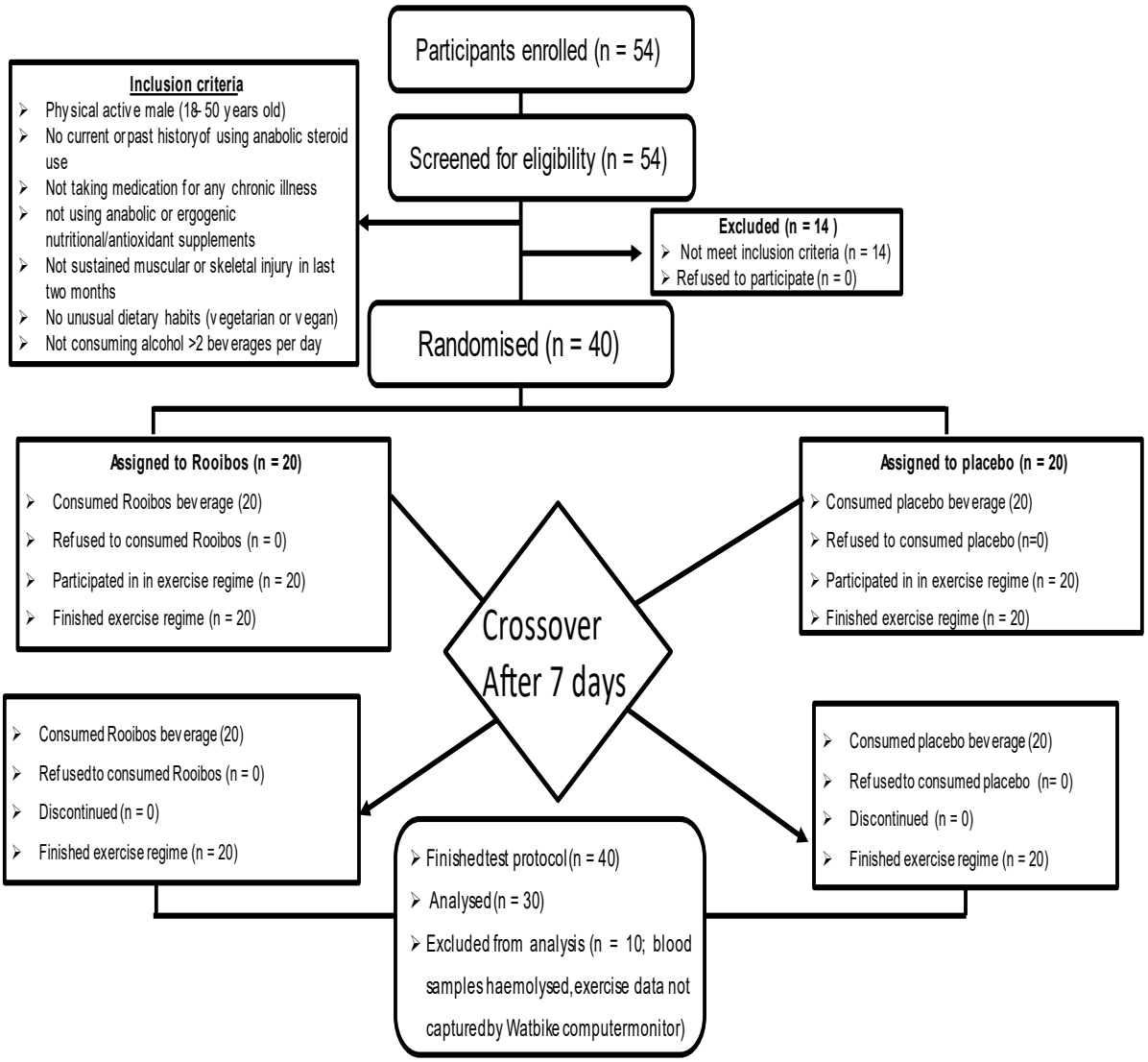


Figure 5.1: Consolidated Standards of Reporting Trials (CONSORT) flow diagram for this study

5.1.1 Characterisation of study participants

This study recruited in majority young and apparently healthy men with a mean age of 25.95 ± 6.25 years and a BMI of 25.18 ± 6.25 kg/m^2. There were no statistically significant ($p>0.05$) differences in participant's anthropometrics and cardiorespiratory data (Table 5. 1).

Table 5.1: Anthropometric characteristics of study participants (n = 30) who completed the study

Variable	Mean (SD)
Age (years)	25.95± 6.25
Mass (kg)	76.37 ± 21.64
Stature (cm)	1.74 ± 0.08
Circumference	102 ± 11.48
BMI (kg/m^2)	25.18 ± 6.15
Resting SBP (mm Hg)	125 ± 14
Resting DBP (mm Hg)	73.23 ± 8.44
Hb (g/dl)	15.30 ± 1.62
Cholesterol (mmol/L)	4.77 ± 0.62
Glucose (mml/L)	4.25 ± 0.73

Values in columns are expressed as mean ± standard deviation (SD). Abbreviations: BMI = Body mass index; DBP = Diastolic blood pressure; SBP = Systolic blood pressure; Hb = Haemoglobin.

5.1.2 Participation in the exercise regimen

The effect of intervention study beverages on participants' exercise parameters during the sub-maximal (75 – 80%) ramp test on Wattbike is displayed in Table 5.2. As described in the materials and methods section, after reaching exhaustion during the submaximal exercise, participants rested for 1 min and continued completing 10 sets of sprints, 10 sec each with 15 sec of passive recovery. As such participants completed a different number of sprints as described in Table 5.3.

Table 5.2: Statistical description of study beverage effects on participants' (n = 30) exercise parameters

	Placebo		Difference		Rooibos	
Variable (unit)	Mean ± SD	Sum	Mean %	P-value	Mean ± SD	Sum
Time (seconds)	254 ± 150	7605	14.96	0.36	292 ± 172	8755
Distance (metre)	2435 ± 1604	73048	13.60	0.42	2790 ± 1827	83709
Work (kilojoules)	30.26 ±14.97	16947.4	2.35	0.42	30.98 ± 15.65	19737.3
Power (Watts)	141 ± 43	78929	3.65	0.05	146 ± 48	93128

Heart rate (bpm)	131 ± 21.17	73511	2.47	**0.01**	135 ± 24.34	85714
Oxygen uptake ($mL.min^{-1}.kg^{-1}$)	25.84 ± 9.37	39151.30	5.53	1.70	27.31 ± 9.96	46871.35

Values in columns are expressed as mean, standard deviation (SD) and sum of scores; along with percentage difference between mean scores and P-value for one-way ANOVA. Abbreviation: BPM = Beat per minute; ANOVA = Analysis of variance.

Table 5.3: Breakdown of sprints completed by participants (n = 30) during both trial sessions

Trial session	Number of sprints
	Mean ± ***SD***
Placebo session	4.77 ±1.89
Rooibos session	5.17 ± 2.31

Values in column are expressed as mean ± standard deviation (SD).

5.1.3 Oxidative stress markers

5.1.3.1 Redox status of glutathione

The mean values of redox status markers indices measured in participants blood during this study is presented in Table 5.4 while Figure 5.2 (a, b) visualised measured blood redox status markers with significant ($p < 0.05$) changes. When considering the circulating total glutathione (tGSH) and reduced glutathione (GSH) levels, results show a significant ($p = 0.021$ and $p = 0.003$) increase (14.43% and 17.49%) in the total GSH levels and also a significant ($p = 0.017$ and $p = 0.003$) increase (15.39% and 17.88%) in blood reduced GSH levels, respectively at 1.5 h and 24 h post-exercise time points, when Rooibos was consumed compared to the levels of the placebo intervention. No significant differences were noticed for the levels of oxidised glutathione (GSGG) when either Rooibos or placebo beverages were consumed. Participants' blood GSH/GSSG ratio also increased at IAE and 24 h post-exercise and decreased at 1.5 h and 1 h post-exercise when Rooibos was consumed compared to when participants consumed the placebo. When the placebo beverage was consumed, both tGSH and GSH increased IAE and then started decreasing at 1 h to almost baseline level 24 h post-exercise. This could be an indication of possible oxidation of blood GSH molecules.

Table 5.4: Effects of intervention beverages on participants' (n = 30) blood glutathione redox status

	Different blood collection time points									
Marker	**0h PL**	**0h Rb**	**1.5 h PL**	**1.5 h Rb**	**IAE PL**	**IAE Rb**	**1h PL**	**1h Rb**	**24hr PL**	**24hr Rb**
tGSH (µmol/mL)	1392± 123	1348 ± 101	**1405 ± 129[a] (0.93)**	**1642 ± 150 (21.81)**	1738 ± 171 (24.86)	1726 ± 156[d] (28.04)	1718 ± 150[b] (23.42)	1641 ± 143[e] (21.74)	**1481 ±.122 (6.39)**	**1795 ± 139[e] (33.16)**
GSH (µmol/mL)	1370 ± 126	1348 ± 107	**1374 ± 130 (0.30)**	**1624 ± 151[h] (20.49)**	1714 ± 173[f] (25.10)	1716 ± 162[i] (27.31)	1715 ± 157[g] (25.17)	1645 ± 151[j] (22.04)	**1488 ±.127 (8.60)**	**1812 ± 145[e] (34.43)**
GSSG (µmol/mL)	5.62 ± 0.38	4.49 ± 0.50	5.26 ± 0.57 (-6.41)	6.26 ± 0.89 (39.42)	6.30 ± 0.65 (12.10)	5.55 ± 0.57 (23.61)	5.24 ± 0.47 (-6.76)	4.67 ± 0.41 (4.00)	3.72 ± 0.37[kLm] (-33.81)	3.51 (0.37)[nop] (-21.83)
GSH:GSSG Ratio	267 ± 33	295 ± 42	307 ± 57 (15.19)	293 ± 48 (-0.66)	306 ± 45 (14.78)	331 ± 48 (12.21)	374 ± 55 (40.28)	307 ± 60 (4.27)	486 ± 66[kfL] (82.27)	567 ± 85[jnq] (92.20)

Values in columns are expressed as mean ± standard error of the mean (SEM) at different time points during the study and values in brackets indicates % difference when compared to 0 h (baseline). Abbreviations: GSH = reduced glutathione; GSSG = oxidised glutathione; IAE = Immediately after exercise; tGSH = total glutathione; PL = placebo; Rb = Rooibos. Inter-group = comparing time point differences between the two study interventions, intra-group = comparing time points differences within each intervention. **Inter-group comparison significance (p < 0.05) is indicated in red.** For intra-group comparison significant differences are as follow **a** (p = **0.002**) and **k** (p<**0.0001**) compared to 0h placebo; **c** (p = **0.020**), **d** (p = **0.002**), **e** (p<**0.0001**) and **h** (p = **0.027**), **i** (p = **0.004**), **j** (p = **0.014**) compared to 0h Rooibos; **b** (p = **0.011**), **f** (p = **0.003**) and **g** (p = **0.007**) compared to 1.5h placebo; **n** (p = **0.047**) and **u** (**p = 0.009**) compared to 1.5h Rooibos; **L** (p<**0.0001**) and **x (p>0.05)** compared to IAE placebo, **o** (p = **0.015**) compared to IAE Rooibos, **m** (p = **0.014**) and **q** (p = **0.041**) compared to 1 h placebo; **p** (p = **0.033**) compared to 1 h Rooibos.

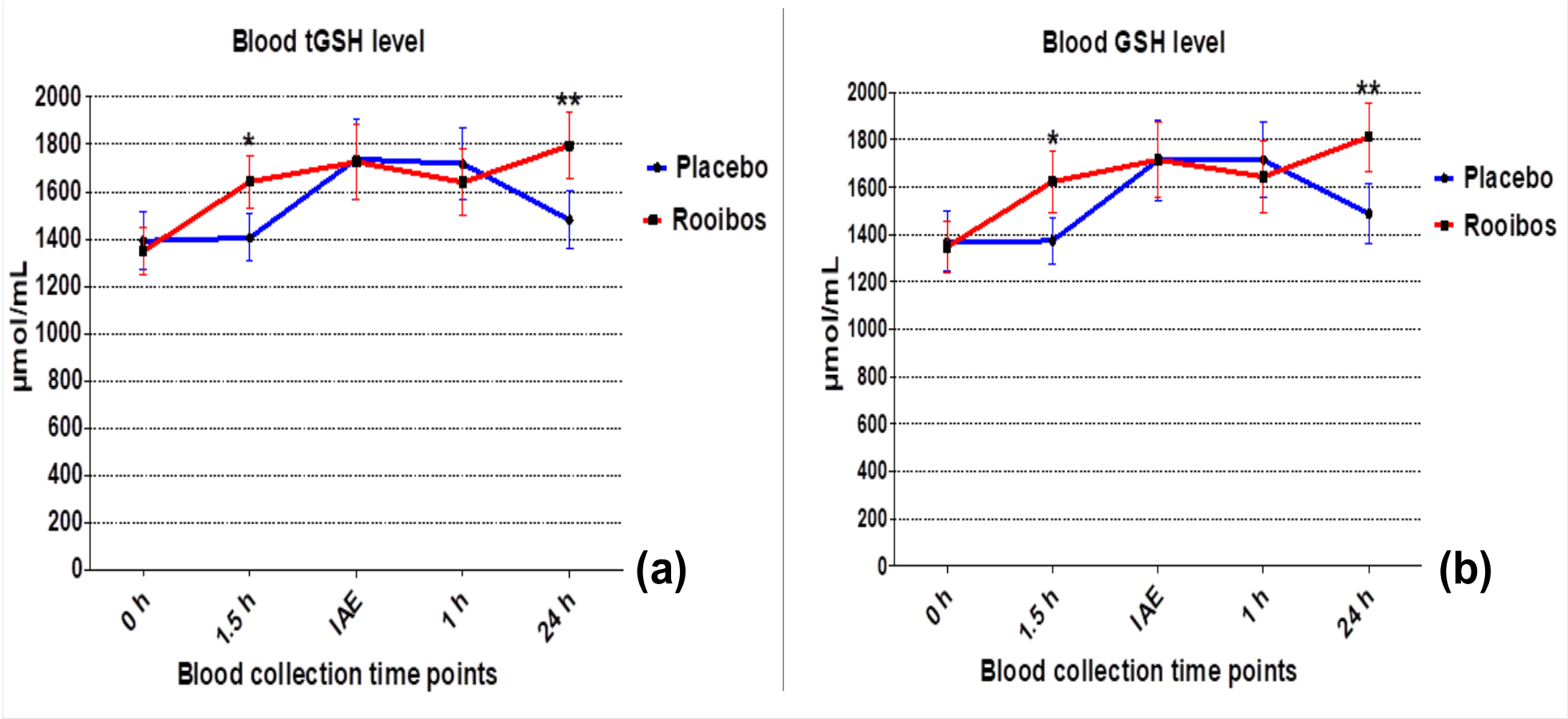


Figure 5.2: Effects of intervention beverages on participants' (n = 30) blood glutathione redox status a) total glutathione level and b) reduced glutathione level. Values are expressed as mean ± standard error of the mean (SEM). Abbreviations: IAE = Immediately after exercise; GSH = Reduced glutathione; tGSH = Total glutathione

5.1.3.2 Oxidative lipid peroxidation and protein oxidation

The mean values of lipids and protein damage biomarkers measured in participant plasma during this study is presented in Table 5.5 while Figure 5.3 show the visualised plasma level/concentration of measured biomarkers with significant ($p < 0.05$) change. For the oxidative damage lipid biomarkers (CDs and TBARS), results show lipid damage, as indicated by an increase, but not significant ($p > 0.05$) above baseline level in plasma CDs level IAE till 24 h post-exercise, irrespectively of the beverage consumed, though it was more apparent when placebo was consumed. Participants' serum TBARS level decreased at 1.5 h and remained almost similar to baseline level, throughout the study irrespective of the beverage consumed and then started increasing 24 h post-exercise. Oxidative damage protein biomarker (PC) plasma levels did not show any indication of protein damage, except at 1.5 h when Rooibos was consumed, whereby the PC plasma levels significantly ($p = 0.007$) increased (15.75%) to above baseline levels, but it later decreased to below baseline level at IAE and remained below baseline level throughout the study period. A similar pattern, although not significant ($p > 0.05$), also occurred during the placebo session, except at 1.5 h when the plasma PC levels decreased.

Table 5.5: Effects of the two intervention beverages, Rooibos and placebo on participants (n = 30) lipid and protein oxidative damage biomarkers

	Different blood collection time points									
Biomarker	***0h PL***	***0h Rb***	***1.5h PL***	***1.5h Rb***	***IAE PL***	***IAE Rb***	***1h PL***	***1h Rb***	***24h PL***	***24h Rb***
CDs (nmol/ mL)	13.98 ± 0.41	13.92 ± 0.47	13.65 ± 0.44 (-2.36)	13.92 ± 0.40 (0)	14.68 ± 0.40[a] (5.01)	14.41 ± 0.44 (3.52)	14.77 ± 0.55[a] (5.65)	14.42 ± 0.51 (3.59)	14.71 ± 0.37[b] (5.22)	15.03 ± 0.33[cd] (7.97)
TBARS (nmol/ mL)	10.37 ± 0.15	10.40 ± 0.16	9.95 ± 0.19 (-4.05)	9.61 ± 0.18 (-7.60)	10.49 ± 0.14[f] (1.16)	10.22 ± 0.17[e] (-1.73)	10.36 ± 0.20 (-0.10)	10.06 ± 0.16[g] (-3.27)	10.64 ± 0.19 (2.60)	10.41 ± 0.20 (0.10)
PC (*nmol/ mL*)	2.48 ± 0.12	2.52 ± 0.12	**2.30 ± 0.12 (-7.26)**	**2.73 ± 0.15 (8.33)**	2.37 ± 0.11 (-4.44)	2.20 ± 0.10 (-12.70 ±17 %)	2.40 ± 0.12 (-3.23)	2.29 ± 0.09[h] (-9.13)	2.14 ± 0.11 (-13.71)	2.12 ± 0.12[i] (-15.87)

Values in columns are expressed as mean ± standard error of the mean (SEM) at different time points during the study and values in brackets indicates % difference when compared to 0 h (baseline). Abbreviations: TBARS = Thiobarbituric acid reactive substance; IAE = Immediately after exercise; CDs = Conjugated denies; IAE = Immediately after exercise, PL = placebo; Rb = Rooibos, Inter-group = comparing time point differences between the two study interventions, intra-group = comparing time points differences within each intervention. **Inter-group comparison significance ($p < 0.05$) is indicated in red.** For intra-group comparison significant differences are as follow **c** ($p = 0.004$) and **f** ($p = 0.041$) compared to 0h Rooibos; **a** ($p < 0.0001$), **b** ($p = 0.016$) and e ($p = 0.042$) compared to 1.5h placebo; **d** ($p < 0.0001$), **g** ($p = 0.025$), **h** ($p = 0.040$) and **i** ($p = 0.001$) compared to 1.5h Rooibos.

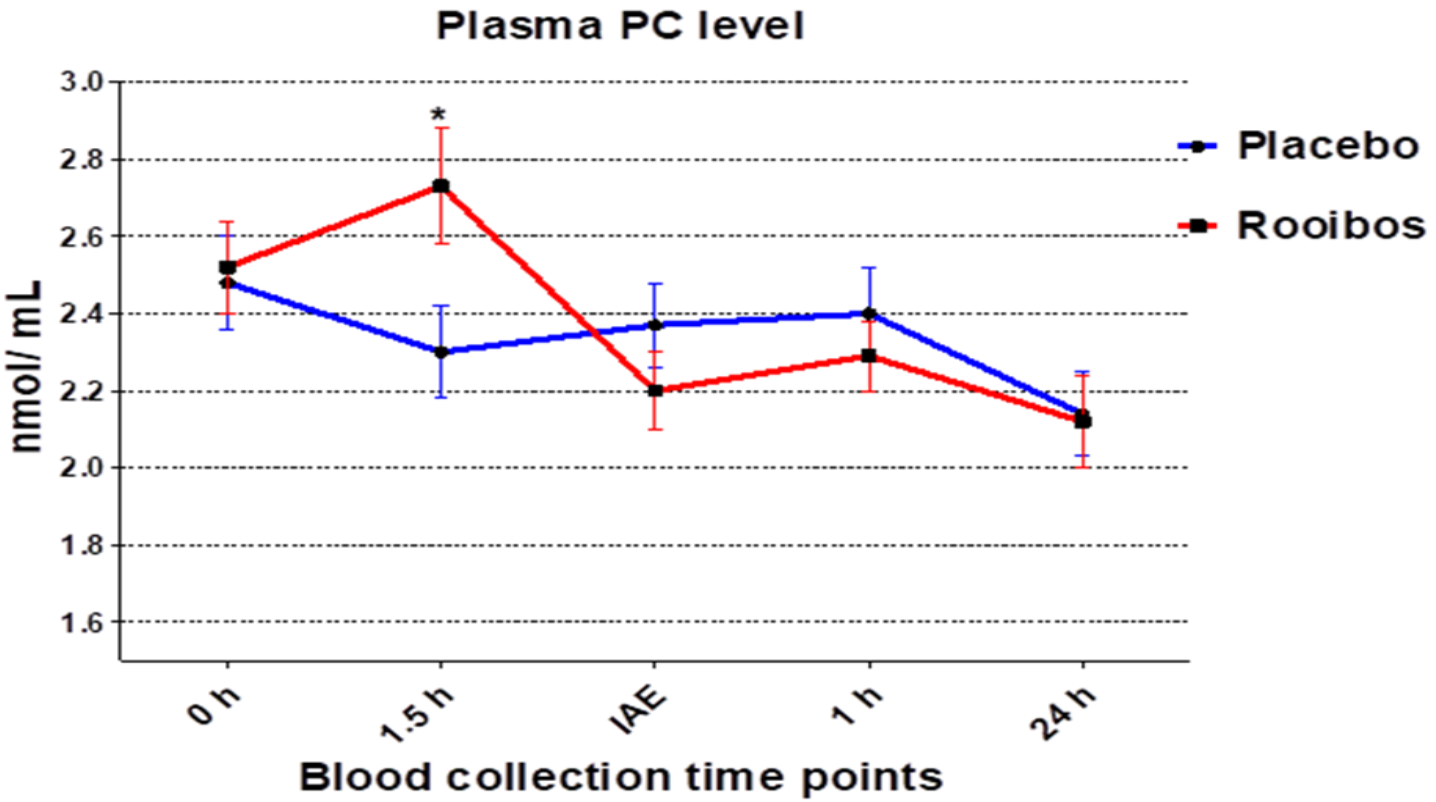


Figure 5.3: Effects of intervention beverages on participants (n = 30) plasma protein carbonyl (PC) levels at the different study time points

Values are expressed as mean ± standard error of the mean (SEM). Abbreviations: IAE = Immediately after exercise

5.1.3.3 DNA Damage

The mean values of DNA damage measured by comet assay in participants' whole blood samples during this study is presented in Table 5.6 while Figure 5.4 show the visualised DNA damage treatment with a) lysis, b) H_2O_2, and c) FPG treatments measured at all study time points. Lysis is the default treatment, while H_2O_2 treatment gives additional information about the antioxidant protection mechanisms of the cell and treatment with restriction enzyme FPG gives a more specific insight into the type of DNA damage as it detects single strand breaks. When DNA damage measured by the standard treatment, results for both lysis and H_2O_2 treatment show a small gradual increase in DNA tail damage as from 1.5 h which keep increasing till 24 h post-exercise irrespective of the beverage consumed. Results for the DNA lesion-specific enzyme [formamidopyrimidine glycosylase (FPG)] treatment show a sharp increase in DNA damage at 1.5 h which then decreased IAE, but then drastically increased again 1 h till 24 h post-exercise. These results for all three treatments provide an indication of DNA damage that occurred during the exercise regime irrespective of the interventional beverage consumed, with possibly more single strand breaks as indicated by the FPG treatment, however, none of these changes were statistically significant ($p > 0.05$) when comparing the Rooibos to placebo beverage.

Table 5.6: Effects of intervention beverages on participants (n = 30) DNA damage

	Different blood collection time points									
Biomarker	***0h PL***	***0h Rb***	***1.5h PL***	***1.5h Rb***	***IAE PL***	***IAE Rb***	***1h PL***	***1h Rb***	***24h PL***	***24h Rb***
Lysis (% tail DNA)	23.92 ± 8.28	26.63 ± 8.42	26.60 ± 7.75	27.79 ± 7.46	27.15 ± 7.41	28.34 ± 7.53	26.27 ± 7.38	29.71 ± 7.27	26.39 ± 7.23	26.45 ± 7.17
H_2O_2 (% tail DNA)	26.79 ± 8.29	31.00 ± 11.53	30.46 ± 8.62	32.26 ± 8.39	31.80 ± 7.88	33.52 ± 8.63	32.00 ± 7.06	35.55 ± 9.76	31.65 ± 8.10	33.78 ± 9.04
FPG (% tail DNA)	5.42 ± 4.51	5.50 ± 4.37)	7.30 ± 3.79	8.09 ± 4.83	6.93 ± 3.52	6.90 ± 5.24	8.29 ± 4.67	8.80 ± 4.61	9.07 ± 4.90	9.46 ± 4.45

Values in columns are expressed as mean ± standard error of the mean (SEM). Abbreviations: FPG= formamidopyrimidine glycosylase; DNA = Deoxyribonucleic acid; IAE = Immediately after exercise.

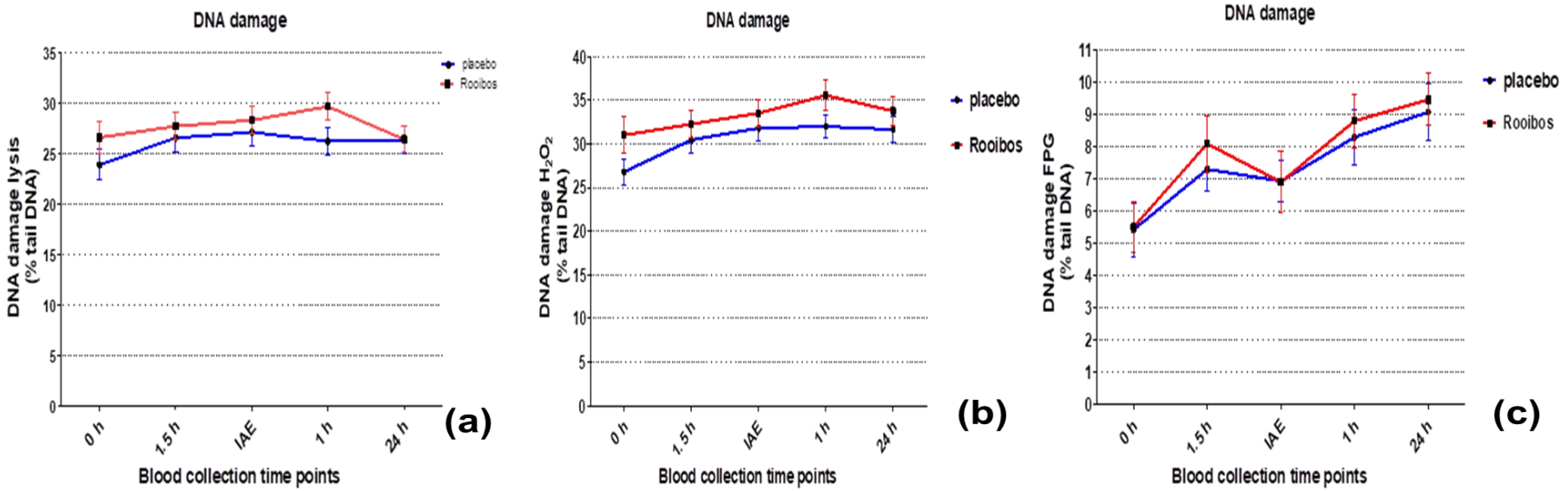


Figure 5.4: Effects of the two intervention beverages on participants (n = 30) DNA damage using the COMET assay with 3 different treatments: (a) lysis - no additional treatment, (b) treatment with H_2O_2, and (c) treatment with FPG. Values are expressed as mean ± standard error of the mean (SEM). Abbreviations: IAE = Immediately after exercise; FPG = formamidopyrimidine glycosylase; DNA = Deoxyribonucleic acid.

5.1.3.4 Clinical chemistry markers

Throughout the study, no adverse effects were reported by any of the study participants. Additionally, serum analysis, particularly liver and kidney function analytes are shown in Table 5.7 while Figure 5.5 (a, b), visualise plasma levels/concentration of measured biomarkers with significant ($p < 0.05$) change. Serum biochemical results showed changes in certain analyte levels when Rooibos was consumed compared to placebo, included the decreased AST serum levels at time points 1.5 h, IAE and 1 h post-exercise ($p < 0.0001$, $p = 0.002$ and $p = 0.001$, respectively) and at 1.5 h and IAE ($p = 0.007$ and $p = 0.051$, respectively) for ALT levels. These decreased values were still within the analytes' normal reference ranges. For the rest of the analytes that were elevated, but still within normal diagnostic reference ranges, this may be proposed to indicate that the exercise regime was rigorous enough to cause these markers to be elevated by inflammatory processes in the muscle tissues, together with the associated metabolic and excretion burdens (Georgakouli et al., 2015).

Table 5.7: Effects of intervention beverages on participants' (n = 30) serum biochemical analytes

	Different blood collection time points									
Marker	***0h PL***	***0h Rb***	***1.5h PL***	***1.5h Rb***	***IAE PL***	***IAE Rb***	***1h PL***	***1h Rb***	***24h PL***	***24h Rb***
AST (U/L)	28.87 ± 1.54	26.39 ± 1.54	**30.36 ± 1.42 (5.16)**	**25.89 ± 0.87^{q} (-1.89)**	**32.15 ± 1.52^{a} (11.36)**	**28.48 ± 0.88^{db} (7.92)**	**29.84 ± 1.29^{b} (3.36)**	**26.33 ± 0.83^{e} (-0.22)**	28.37 ± 1.11^{c} (-1.73)	26.75 ± 1.01 (1.36)
ALT (U/L)	**23.02 ± 1.96**	**18.89 ± 1.74**	**23.80 ± 1.90 (3.39)**	**20.00 ± 1.54 (5.88)**	**22.99 ± 2.08 (-0.13)**	**19.83 ± 1.63 (4.98)**	22.29 ± 1.74 (-3.17)	19.73 ± 1.57 (4.45)	23.56 ± 2.04 (2.35)	21..29 ± 2.11 (12.71)
ALP (U/L)	68.52 ± 2.12	66.62 ± 3.00	69.96 ± 1.89 (2.10)	68.22 ± 1.68 (2.40)	74.15 ± 1.96^{fg} (8.22)	74.96 ± 2.04^{id} (12.52)	68.63 ± 1.77^{c} (0.16)	69.38 ± 1.78^{e} (4.14)	67.75 ± 2.02^{c} (-1.12)	67.90 ± 1.97^{e} (1.92)
GGT (U/L)	26.78 ± 1.25	25.73 ± 1.36	26.00 ± 1.24 (-2.91)	25.70 ± 1.19 (-0.12)	26.99 ± 1.25 (0.78)	26.95 ± 1.27^{j} (4.74)	26.28 ± 1.27 (-1.87)	25.93 ± 1.14 (0.78)	25.51 ± 1.38 (-4.74)	24.62 ± 1.25^{k} (-4.31)
TBIL (µmol/L)	10.54 ± 0.98	10.05 ± 0.74	10.10 ± 0.80 (-4.17)	11.24 ± 1.05 (11.84)	10.84 ± 0.78 (2.85)	11.29 ± 0.77^{m} (12.34)	10.07 ± 0.78^{b} (-4.46)	10.08 ± 0.70^{e} (0.30)	11.77 ± 1.08^{L} (11.67)	11.54 ± 1.13 (14.83)
DBIL (µmol/L)	1.91 ± 0.12	1.93 ± 0.11	1.79 ± 0.11 (-6.28)	2.02 ± 0.10 (4.66)	1.79 ± 0.13 (-6.28)	1.97 ± 0.12 (2.07)	1.86 ± 0.13 (-2.62)	2.00 ± 0.10 (3.63)	2.02 ± 0.15^{n} (5.76)	2.25 ± 0.11^{o} (16.58)
TP (g/L)	79.41 ± 0.88	81.21 ± 1.06	80.46 ± 0.87^{p} (1.32)	81.71 ± 0.95 (0.62)	85.84 ± 1.38^{q} (8.10)	87.24 ± 1.19nd (7.42)	80.69 ± 1.16^{c} (1.61)	82.23 ± 1.07^{e} (1.26)	81.16 ± 1.13^{c} (2.20)	80.87 ± 1.05^{e} (-0.42)
ALB (g/L)	49.65 ± 0.64	48.74 ± 1.90	51.50 ± 0.52 (3.73)	51.15 ± 0.48 (4.94)	53.87 ± 1.10 (8.50)	53.86 ± 0.98^{j} (10.50)	51.28 ± 0.50 (3.28)	52.06 ± 0.66 (6.81)	49.45 ± 1.38^{c} (-0.40)	50.81 ± 0.66^{o} (4.25)
BUN (mmol/L)	4.65 ± 0.16	4.44 ± 0.18	4.70 ± 0.15 (1.08)	4.58 ± 0.13 (3.15)	4.78 ± 0.14 (2.80)	4.73 ± 0.13^{d} (6.53)	5.07 ± 0.17^{pqT} (9.03)	5.07 ± 0.16^{nde} (14.19)	4.67 ± 0.16^{u} (0.43)	4.72 ± 0.14^{R} (6.31)
CREAT (µmol/L)	85.54 ± 3.30	85.55 ± 3.72	87.27 ± 3.22 (2.02)	87.26 ± 3.59 (2.00)	103 ± 80 (20.41)	93.44 ± 5.20 (9.22)	89.19 ± 3.64 (4.27)	93.20 (3.53)^{n} (8.94)	82.55 ± 3.48^{v} (-3.50)	87.83 ± 3.24 (2.67)
UA (µmol/L)	361 ± 10.77	387 ± 13.20	378 ± 90^{a} (4.71)	396 ± 15 (2.33)	380 ± 13 (5.26)	401 ± 16 (3.62)	519 ± 15^{pqc} (43.77)	572.69 ± 20.57^{nde} (47.80)	379 ± 13^{v} (4.99)	427 ± 16^{ndR} (10.34)
GLUC (mmol/L)	5.14 ± 0.07	5.13 ± 0.10	4.53 ± 0.10^{p} (-11.70)	4.32 ± 0.12^{n} (-15.79)	4.59 ± 0.11^{p} (-10.53)	4.69 ± 0.11^{m} (-8.58)	4.95 ± 0.27 (-3.51)	4.74 ± 0.19 (-7.60)	5.14 ± 0.10^{qb} (0.19)	5.30 ± 0.17^{dk} (3.31)

Values in columns are expressed as mean and standard error of the mean (SEM) at different time points during the study and values in brackets indicates % difference when compared to 0 h (baseline). Abbreviations: TP= Total protein, ALB = Albumin; ALP = Alkaline phosphatase; AST = Aspartate aminotransferase; ALT = Alanine aminotransferase; BUN = Blood urea nitrogen; CREA = Creatinine; TBIL = Total bilirubin; DBIL = Direct bilirubin; GGT = Gamma glutamyl transferase; UA = Uric acid; GLUC = Glucose, PL = placebo; Rb = Rooibos. **Inter-group comparison significance (p<0.05) is indicated in red.** For intra-group comparison significant differences are as follow **a** (p = **0.002**), **f** (p

= **0.010**) and **p** (p<**0.0001**) compared to 0h placebo; i (p = **0.004**), **m** (p = **0.034**), **n** (**p = 0.001**) **e** (p<**0.0001**) and **h** (p = **0.027**)**, i** (p = **0.004**), **j** (p = **0.014**) compared to 0h Rooibos, **g** (p = **0.011**), **q** (p<**0.0001**), **g** (p = **0.007**), **v** (**p = 0.021**) and **T** (**p = 0.017**) compared to 1.5h placebo; **d** (p< **0.0001**), **j** (**p = 0.036**) and **u** (**p = 0.009**) compared to 1.5h Rooibos; **b** (p = **0.002**), **c** (p<**0.0001**), **T** (p = **0.010**) compared to IAE placebo, **e** (p<**0.0001**), **k** (**p = 0.012**), **o** (**p = 0.020**) compared to IAE Rooibos, **L** (p = **0.031**), **u** (p = **0.051**) and **v** (p<**0.0001**) compared to 1 h placebo; **R** (p = **0.033**) compared to 1 h Rooibos..

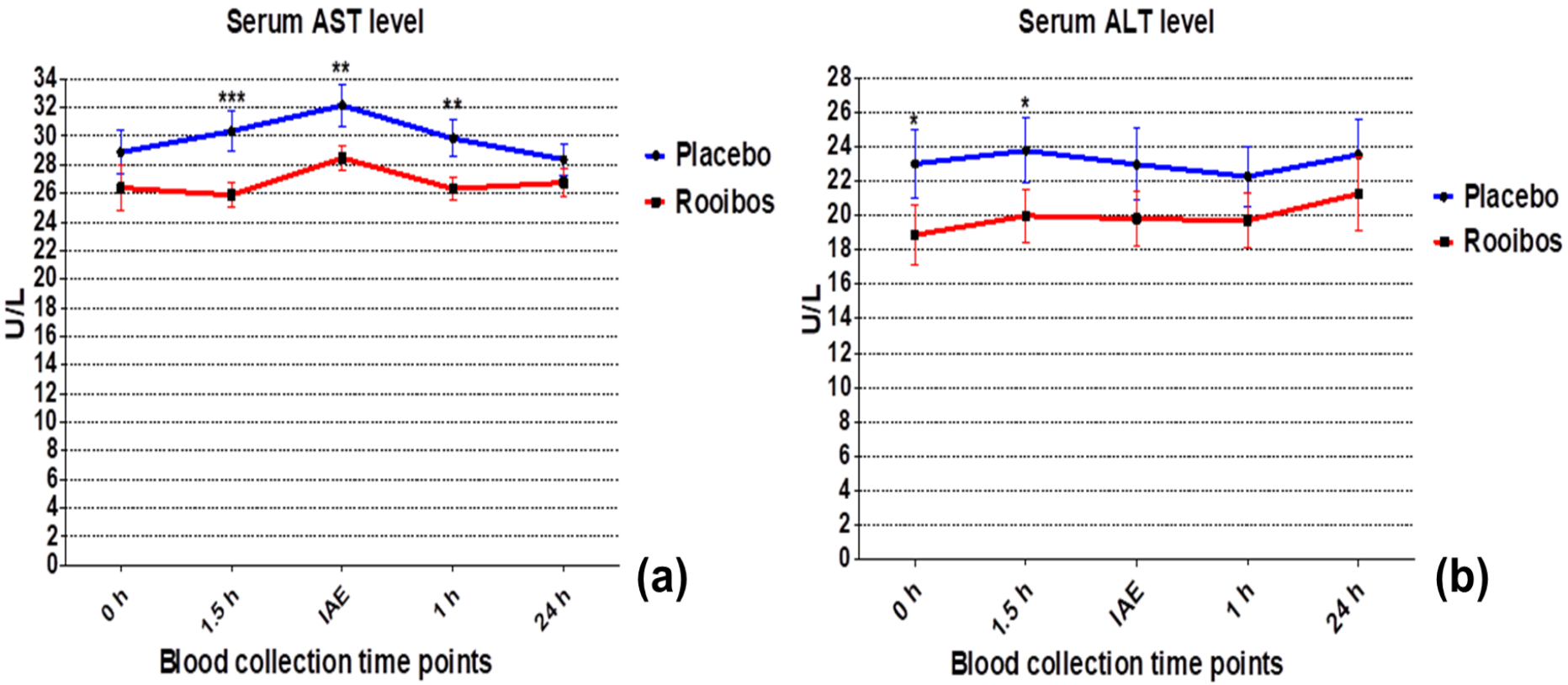


Figure 5.5: Effect of the two study beverages on participants (n = 30) serum chemistry analytes: a) Aspartate aminotransferase (AST); and b) Alanine aminotransferase (ALT), with *, **, * indicating significant differences when comparing the placebo to Rooibos intervention**

Values are expressed as mean ± standard error of the mean (SEM). Abbreviations: IAE = Immediately after exercise; ALT = Alanine aminotransferase; AST = Aspartate aminotransferase.

5.2 Phase I – Discussion

As per the aim of this study, we have designed and conducted a single-blinded cross-over intervention trial, in order to develop an exercise-induced oxidative stress model for human participants that can be used for testing various phytochemical/herbal interventions. In order to do this, the first step was to design an exercise regime to induce oxidative stress that is repeatable and applicable to individuals of different levels of fitness. One advantage to use exercise-induced oxidative stress is that it is associated with less risk and variables than oxidative stress induced by other processes that could be considered pathological (Sharifi-Rad et al., 2020). Using exercise to a point of 'failure' or exhaustion, one could have some control as to the endpoint of exercise in a manner that would lead to similar levels of oxidative stress. To ensure similarity and repeatability between the two oral interventions, the study was designed as a cross-over trial. Such that the same participants would partake in both interventions at random (blinded) and then cross over to the other intervention following a washout period. Other important factors where it was necessary to insert control in order to attempt to allow for the induction of similar levels of oxidative stress across all participants, included: general physical activity, dietary intake, and medicinal or recreational drug use of participants before or during the trial and any pathological or physiological process which may contribute to oxidative stress(Sharifi-Rad et al., 2020). All of the aforementioned factors may affect both the induction of oxidative stress by exercise, but also the effects of oral interventions there-on.

As evidenced by the oxidative stress marker results of our study, it can be deduced that the participants in this study had oxidative stress induced by the exercise regime, which was measurable in their blood, irrespective of the intervention beverage. The changes in oxidative stress were most clearly demonstrated immediately after exercise (IAE) by the increase in lipid-, protein- and DNA oxidation markers, accompanied by evidence of glutathione oxidation. It is additionally clear that for most oxidative stress markers the exercise-induced oxidative stress level was then modulated by the Rooibos beverage intervention, to return to baseline levels more rapidly than a placebo beverage intervention. As this was a cross-over trial, these results cannot be attributed to biological variation between study participants, as the same individuals were subjected to the exercise regime after both interventions. The dietary intake (via the completed dietary records) and physical activity (via the questionnaire) of participants were also controlled during the trial. Therefore, changes observed in the participants' blood oxidative stress markers could be reliably attributed to the exercise regime and study intervention beverages. These results affirm that the exercise test protocol (modified submaximal ramp test) used in this study has indeed induced oxidative stress damage in participants and also indicates a potential to successfully test oral interventions such as Rooibos. Some precedent

for this type of model is apparent in the literature, as Jowko and co-workers also observed a similar phenomenon in a crossover trial study in male sprinters after a repeated cycling sprint exercise which induced an increased MDA and other oxidative stress markers during placebo intervention, but the increase was reversed during the green tea extract intervention (Jówko et al., 2015). Based on our findings, it is however clear that the number of sprints completed by participants varies dramatically and this may have impacted how oxidative stress was induced by the exercise regime. If the reason for the difference in the number of sprints completed by participants can be attributed to physical fitness in the main, this may also have impacted metabolism and therefore radical production in these participants. This may be a variable that has not been considered by the researchers before conducting this study and may serve to further improve the reliability of this proposed model in future. However, apart from participants' physical fitness, many human and animal studies indicated that the relationship between exercise and radical production or extent of oxidative stress is complex and often dictated by duration, intensity, mode, and type of exercise (Kanda et al., 2013; Accattato et al., 2017; Moslemi et al., 2023). Generally, most radicals are generated by mitochondria as a by-product of incomplete oxygen metabolism during cellular respiration (Wang & Hai, 2016). During exercise, oxygen uptake increases due to the higher energy demand with about 10 to 20-fold oxygen flux increases in the active skeletal muscles (Yavari et al., 2015). It has also been shown that during exercise, not all of the oxygen consumed by cellular mitochondria is converted to water. Although the exact amount is not clear, a small percentage of consumed oxygen is instead reduced to radicals such as superoxide anion ($O_2 \cdot -$) which subsequently reduced to the more potent hydroxyl radical (OH·) (Neubauer, Reichhold, et al., 2008; Tryfidou et al., 2020). A study by Richardson et al. (1995) concluded that prolonged or intense muscle contractions alter the physiological environment in the muscle fibre and predispose them to a higher rate of radical generation. In 2004, Arbogast and Reid, reported that muscle contraction is associated with an increased muscle temperature, increased carbon dioxide tension, and decreased cellular pH which may stimulate and accelerate radical production within the muscles. Similarly, Ferreira and Reid (2008), also reported that increased oxygen consumption in muscle fibre during exercise, lowered oxygen intracellular tension and augmented the generation of radicals.

Even though the proposed exercise model was able to be implemented in the planned cross-over fashion, there was a need to exclude some of the study participants. This was in part due to participants who deviated from the nutritional and medical requirements as outlined upon enrolment in the study. Other reasons may be attributed to equipment and sample collection errors. Given the participant number of 40, the need to exclude 10 participants can be seen as quite a high post-enrolment exclusion rate. This points to the fact that it is quite difficult to control all aspects of human participants that may impact a complex systemic physiological

process, such as oxidative stress. It is thus that, despite our best efforts to provide guidance and monitoring tools for the participants to comply with study requirements, a few participants deviated from the allowable intake of medication and foods that have been asked to be excluded (Rehman et al., 2020). Even though this have reduced our participant number it was deemed necessary upon studying results given that there was a clear impact on findings from all identified exclusion criteria. Current models studying oxidative stress, or related diseases are mostly *in vitro* or experimental animal models. These models generally seek to study single aspects of oxidative stress or to link oxidative stress to the pathologies being studied. It would be beneficial to conduct research making use of a human model for oxidative stress research, as research conducted in animal models has not always been found to translate well to humans due to differences in lifestyle and physiological factors (Pizzino et al., 2017; Oteiza et al., 2021).

5.3 Phase II – Results

5.3.1 Characterisation of final study participants cohort

A total of 30 participants whose data was completed for the study were included in the final data analyses for this study. The anthropometric characteristics of the study participants are described in Table 5.8. Anthropometric measurements including stature, mass and waist circumference were taken during the screening phase (baseline data) and the trial's intervention phases. Blood pressure (systolic and diastolic) and other health indicators were also measured on these three occasions.

Table 5.8: Anthropometric characteristics and health indicators of the study participants (n = 30) taken at the screening session.

Variable	Screening session
Age	26.23 ± 6.53)
Mass (kg)	73.45 ±14.31
Stature (m)	1.73 ± 0.08
Waist Circumference	102 ± 8.38
BMI (kg/m^2)	24.45 ± 4.22
SBP (mm Hg)	125 ± 11.57
DBP (mm Hg)	73.33 ± 7.76
Hb (g/dl)	15.28 ± 1.23
Glucose (mmol/L)	4.91 ± 0.23
Total cholesterol (mmol/L)	4.51 ± 0.10

Values in columns are expressed as mean ± standard deviation (SD). Abbreviations: BMI = Body mass index; DBP = Diastolic blood pressure; SBP = Systolic blood pressure; Hb = Haemoglobin.

5.3.2 Characterisation of the fermented Rooibos and placebo study beverages

The antioxidant content of study beverages was quantified in terms of the total phenolic, flavanol and flavonol content using spectrophotometric analyses and main Rooibos phenolic compounds, using HPLC analysis (Table 5.9). Only the Rooibos study beverage contained phenolic constituents when compared to the placebo beverage (333.47 ± 1.72 mg GAE/375 mL vs 0.0 ± 0.89 mg GAE/375 mL), respectively. When considering the antioxidant capacity, the Rooibos beverage showed a far superior antioxidant capacity (FRAP and TEAC) when comparing with the placebo beverage (1701 ± 4.55 µmol AAE/375 mL, 2118 ± 19.3 µmol TE/375 mL vs 53 ± 2.9 µmol AAE/375 mL, 0.00 ± 1.49 µmol TE/375 mL, respectively). The HPLC analysis authenticated the Rooibos beverage by showing the presence of the unique Rooibos dihydrochalcone, aspalathin and confirmed that it was fermented rooibos, with the orientin and isoorientin quantities exceeding that of aspalathin (Table 5.9).

Table 5.9: Quantitative description of Rooibos and a placebo beverage

	Rooibos mg/ 375 mL	**Placebo mg/ 375 mL**
Flavonols (mg QE)	93.7 ± 1.67	11.3 ± 5.03
Flavanols (mg CE)	37.6 ± 3.67	0 ± 1.60
TPC (mg GAE)	333 ± 20	0 ± 0.89
FRAP (µmol AAE)	1701 ± 50	52.6 ± 2.86
TEAC (µmol TE)	2118 ± 20	ND ± 1.49
Aspalathin	8.37 ± 0.04	ND
Orientin	6.69 ± 0.03	ND
Isoorientin	15.50 ± 0.17	ND
Isovitexin	2.52 ± 0.05	ND
Vitexin	1.95 ± 0.03	ND
Hyperroside	7.26 ± 0.05	ND
Quercetin	2.37 ± 0.02	ND
Luteolin	0.57 ± 0.02	ND
Chrysoeriol	0.11 ± 0.00	ND

Values in columns are expressed as mean ± standard deviation (SD). Abbreviations: GAE = Gallic acid equivalents; AAE = Ascorbic acid equivalents; TE = Trolox equivalents; TPC = Total polyphenolic content; FRAP = Ferric reducing antioxidant power; ND = not detected; TEAC = Trolox equivalent antioxidant capacity; CE = Catechin equivalent; QE = Quercetin equivalent.

5.3.3 Plasma total phenolic content and antioxidant capacity

Note: Since, the 0 h serve as baseline – status of study participants before exercise regime and ingestion of study beverages – while IAE give an indication what has possibly changed in participants due to beverages consumption and exercise regime whereas the 24 h aimed to assess possible recovery by comparing it to the baseline level.Therefore focus when comparing intra-group differences in this section will be on time points 0 h (baseline), IAE (immediately after exercise was completed) and 24 h post-exercise, while for inter-group differences all time points will be considered.

Acute consumption of fermented Rooibos and placebo beverages before the commencement of the exercise regime did not significantly alter ($p > 0.05$) the participants' plasma TPC and antioxidant capacity (FRAP and TEAC) when measured at the different study time points (Table 5.10). Of interest, though not significant, is an apparent increase in the TPC in plasma immediately after the exercise was completed and then decreased 1 h and 24 h post-exercise, and this occured when both Rooibos and placebo were consumed. Similarly, when Rooibos was consumed, an increase in the plasma antioxidant capacity (FRAP) at 1.5 h was noticed and remained high above the baseline (0 h) level thoughout, but this increase was not significant ($p > 0.05$) when compared with the placebo. On the other hand, when Rooibos was consumed, plasma TEAC levels only increased immediately after completing the exercise regime and then decreased at 1 h and then increased again 24 h post-exercise, but none of this was significant ($p > 0.05$) when compared with the placebo.

When considering the intra-group analysis, when the Rooibos was consumed, significant ($p < 0.05$) increases and decreases in the participant plasma TPC and antioxidant capacity (FRAP) was noted at different time points, however, a similar trend was observed with the placebo beverage. However, when the placebo beverage was consumed, plasma antioxidant capacity (TEAC) level intra-group results show no significant ($p > 0.05$) change, but a significant ($p = 0.005$) decrease was observed 1 h post-exercise compared to IAE, and also significant ($p = 0.021$) increases 24 h post-exercise compare to 1 h post-exercise.

Table 5.10: Effect of study beverages on participant (n = 30) plasma polyphenol content and antioxidant capacity

	Different blood collection time points									
Marker	***0h PL***	***0h Rb***	***1.5h PL***	***1.5h Rb***	***IAE PL***	***IAE Rb***	***1h PL***	***1h Rb***	***24h PL***	***24h Rb***
TPC (*mg GAE)*	1523 ± 17	1512 ± 19	1550 ± 19.46 (1.77)	1542 ± 17^{d} (1.98)	1622 ± 19^{ab} (6.50)	1633 ± 22^{eg} (8.00)	1561 ± 20^{c} (2.50)	1562 ±.19^{fh} (3.31)	1566 ± 22^{c} (2.83)	1545 ±.22^{h} (2.18)
TEAC (µmol TE)	7723 ± 651	8072 ± 508	7909 ±.708 (2.41)	7790 ± 576 (-3.49)	8060 ± 511 (4.36)	8312 ± 502 (2.97%)	7992 ± 518 (3.48%)	7635 ± 474^{i} (-5.42)	8215 ± 512 (6.37)	8107 ± 502^{j} (0.43)
FRAP *(µmol AAE)*	594 ± 17	587 ± 20	629 ± 20 (5.90)	635 ± 20^{L} (8.17)	650 ± 18^{a} (9.43)	659 ±.19^{e} (12.26)	826 ± 23^{abc} (39.06)	839 ± 33^{egh} (42.94)	639 ± 22^{ak} (7.58)	658 ±. 2^{e} (12.09)

Values in columns are expressed as mean ± standard error of the mean (SEM) at different time points during the study and values in brackets indicates % difference when compred to 0 h (baseline). Abbreviations: GAE = Gallic acid equivalents; AAE = Ascorbic acid equivalents; TE = Trolox equivalents; TPC = Total polyphenolic content; FRAP = Ferric reducing antioxidant power; TEAC = Trolox equivalent antioxidant capacity; IAE = Immediately after exercise, PL = placebo; Rb = Rooibos. Inter-group = comparing time point differences between the two study interventions, intra-group = comparing time points differences within each intervention. **No inter-group significant differences were noted**. For intra-group comparison significant differences are as follow **a** (p < **0.0001)** compared to 0h placebo; **d** (p = **0.03)**, **e** (p < **0.0001**) and **f** (p = **0.007**) and **L** (p < **0.015**) compared to 0h Rooibos, **b** (p < **0.0001**) compared to 1.5h placebo, **g** (p < **0.0001)** compared to 1.5h Rooibos, **c** (p < **0.0001**) compared to IAE placebo, **h** (p < **0.0001**) and **i** (p = **0.005**) compared to IAE Rooibos, **k** (p < **0.0001**) compared to 1 h placebo; **j** (p = **0.0021**) compared to 1 h Rooibos, **m** (p < **0.0001**) compare to 24 h placebo.

5.3.4 Oxidative stress damage biomarkers

5.3.4.1 Oxidative lipid and protein damage biomarkers

The effects of acute consumption of the fermented Rooibos and placebo beverages on plasma lipid and protein oxidation biomarkers, measured as CDs, TBARS and PC at the different study time points is shown in Table 5.11 while Figure 5.6 visualised the biomarkers with significant ($p < 0.05$) inter-group comparison.

When considering the inter-group results, no significant ($p > 0.05$) differences were observed in the plasma CDs and TBARS at any time point when comparing the two study beverages. However, similarly, to the TPC and antioxidant capacity, certain tendencies were noted and included a decreased plasma CD and TBARS level IAE and 1 h post the exercise when Rooibos was consumed compared to placebo. Similarly, plasma PC levels was also decreased, not significantly ($p > 0.05$) at 1 h and 24 h post-exercise. A significant ($p = 0.007$) increase (15.75%) occured at 1.5 h when Rooibos was consumed compared to placebo.

The intra-group results show significant ($p < 0.05$) increases and decreases in participants' plasma CDs and TBARS levels at different time points when Rooibos was consumed, however, a similar pattern could also be observed when placebo was consumed. No significant intra-group plasma PC levels was observed when the placebo beverage was consumed, while when Rooibos was consumed a significant ($p = 0.040$ and $p < 0.0001$) decrease was noted 1 h and 24 h post-exercise, respectively when compared to plasma PC level at 1.5 h. Lastly, unlike when Rooibos was consumed, participants plasma CDs and PC levels increased much higher IAE when the placebo beverage was consumed and remained above the pre-exercise level for the CDs, confirming that the exercise regime has indeed induced oxidative stress damage in the participants. However, these changes were not statistically significant ($p > 0.05$).

Table 5.11: Effect of study beverages on participant plasma (n = 30) lipids and protein oxidative stress biomarkers

	Different blood collection time points									
Biomarker	***0h PL***	***0h Rb***	***1.5h PL***	***1.5h Rb***	***IAE PL***	***IAE Rb***	***1h PL***	***1h Rb***	***24h PL***	***24h Rb***
CDs (nmol/ mL)	13.98 ± 0.41	13.92 ± 0.47	13.65 ± 0.44 (-2.36)	13.92 ± 0.40 (0)	14.68 ± 0.40[a] (5.01)	14.41 ± 0.44 (3.52)	14.77 ± 0.55[a] (5.65)	14.42 ± 0.51 (3.59)	14.71 ± 0.37[b] (5.22)	15.03 ± 0.33[cd] (7.97)
TBARS (nmol/ mL)	10.37 ± 0.15	10.40 ± 0.16	9.95 ± 0.19 (-4.05)	9.61 ± 0.18 (-7.60)	10.49 ± 0.14[f] (1.16)	10.22 ± 0.17[e] (-1.73)	10.36 ± 0.20 (-0.10)	10.06 ± 0.16[g] (-3.27)	10.64 ± 0.19 (2.60)	10.41 ± 0.20 (0.10)
PC (*nmol/ mL*)	2.48 ± 0.12	2.52 ± 0.12	**2.30 ± 0.12 (-7.26)**	**2.73 ± 0.15 (8.33)**	2.37 ± 0.11 (-4.44)	2.20 ± 0.10 (-12.70 ±17 %)	2.40 ± 0.12 (-3.23)	2.29 ± 0.09[h] (-9.13)	2.14 ± 0.11 (-13.71)	2.12 ± 0.12[i] (-15.87)

Values in columns are expressed as mean ± standard error of the mean (SEM) at different time points during the study and values in brackets indicates % difference when compred to 0 h (baseline). Abbreviations: TBARS = Thiobarbituric acid reactive substance; IAE = Immediately after exercise; CDs = Conjugated denies; IAE = Immediately after exercise, PL = placebo; Rb = Rooibos, Inter-group = comparing time point differences between the two study interventions, intra-group = comparing time points differences within each intervention. **For inter-group comparison significance (p < 0.05) is indicated in red.** For intra-group comparison significant differences are as follow **c** (p = **0.004)** and **f** (p = **0.041**) compared to 0h Rooibos; **a** (p < **0.0001**), **b** (p = **0.016**) and e (p = **0.042**) compared to 1.5h placebo; **d** (p < **0.0001**), **g** (p = **0.025**), **h** (p = **0.040**) and **i** (p = **0.001**) compared to 1.5h Rooibos.

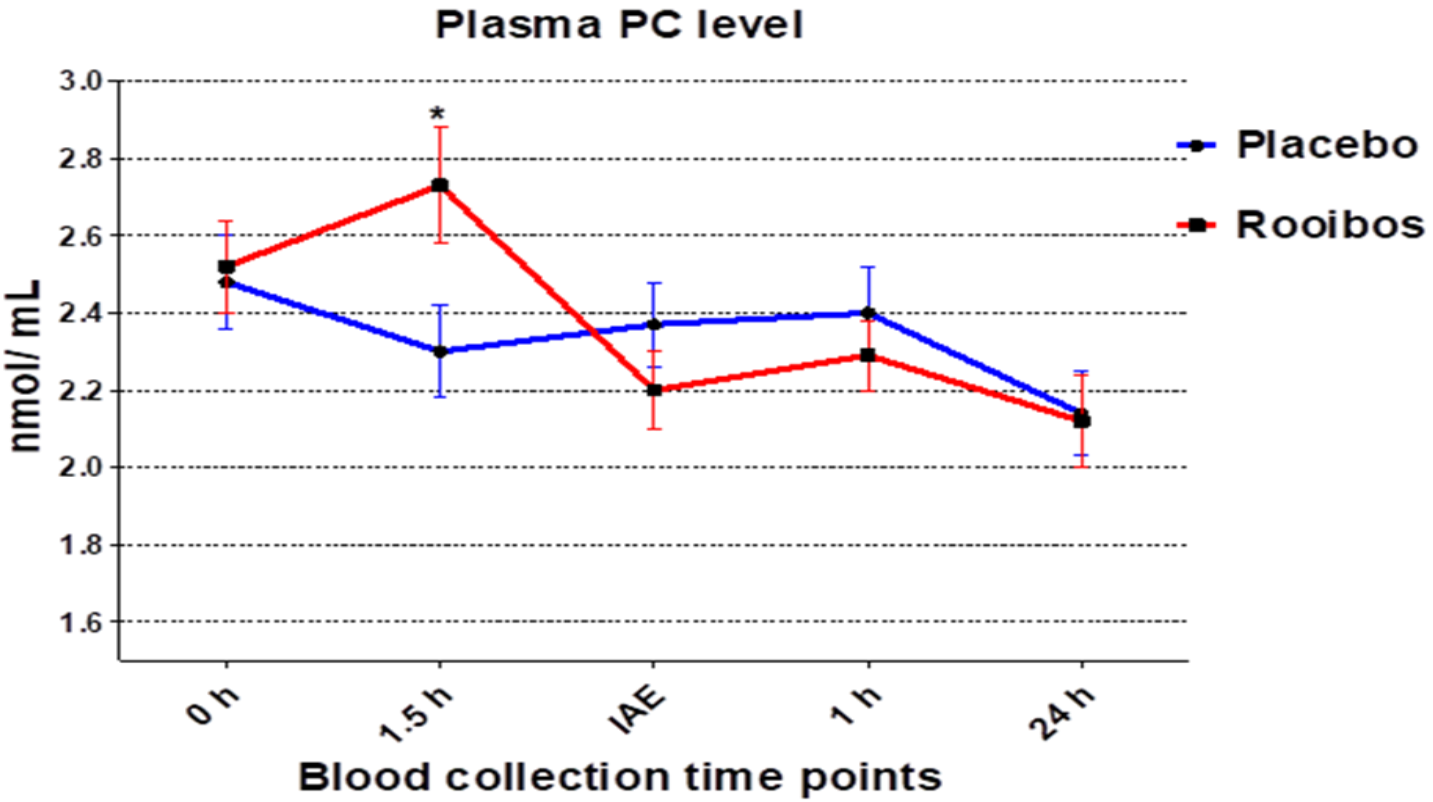


Figure 5.6: Effect of the two study beverages on the participant (n = 30) plasma protein carbonyl (PC) levels at the various study time points

Values are expressed as mean ± standard error of the mean (SEM). Abbreviations: IAE = Immediately after exercise; with * indicating significant difference ($p < 0.05$) when comparing Rooibos with placebo

5.3.5 Blood glutathione redox status

The effect of acute consumption of the intervention study beverages on participants' blood redox status at different time points is shown in Table 5.12 whereas, Figure 5.7 (a, b) dipicts biomarkers that differed significantly ($p < 0.05$) with inter-group comparisons. Results show a significant ($p = 0.021$ and $p = 0.003$) increase (14.43% and 17.49%, respectively) in the total GSH levels and also a significant ($p = 0.017$ and $p = 0.003$) increase (15.39% and 17.88%, respectively) in blood reduced GSH levels at 1.5 h and 24 h post-exercise time points, when Rooibos was consumed compared to the levels of the placebo intervention. No significant differences were noticed for the levels of oxidised glutathione (GSGG) when either rooibos or placebo beverages were consumed. Participants' blood GSH/GSSG ratio also increased at IAE and 24 h post-exercise and decreased at 1.5 h and 1 h post-exercise when Rooibos was consumed compared to when participants consumed the placebo.

The intra-group results, show a similar pattern of significant ($p < 0.05$) increases and decreases at different time points in participants' redox status (tGSH, GSH, GSSG and GSH/GSS ratio) when both Rooibos and placebo beverages were consumed.

Table 5.12: Effect of study beverages on participant (n = 30) blood redox status biomarkers

	Different blood collection time points									
Marker	***0h PL***	***0h Rb***	***1.5 h PL***	***1.5 h Rb***	***IAE PL***	***IAE Rb***	***1h PL***	***1h Rb***	***24h PL***	***24h Rb***
tGSH (µmol/mL)	1392± 123	1348 ± 101	**1405 ± 129[a] (0.93)**	**1642 ± 150 (21.81)**	1738 ± 171 (24.86)	1726 ± 156[d] (28.04)	1718 ± 150[b] (23.42)	1641 ± 143[e] (21.74)	**1481 ±.122 (6.39)**	**1795 ± 139[e] (33.16)**
GSH (µmol/mL)	1370 ± 126	1348 ± 107	**1374 ± 130 (0.30)**	**1624 ± 151[h] (20.49)**	1714 ± 173[f] (25.10)	1716 ± 162[i] (27.31)	1715 ± 157[g] (25.17)	1645 ± 151[j] (22.04)	**1488 ±.127 (8.60)**	**1812 ± 145[e] (34.43)**
GSSG (µmol/mL)	5.62 ± 0.38	4.49 ± 0.50	5.26 ± 0.57 (-6.41)	6.26 ± 0.89 (39.42)	6.30 ± 0.65 (12.10)	5.55 ± 0.57 (23.61)	5.24 ± 0.47 (-6.76)	4.67 ± 0.41 (4.00)	3.72 ± 0.37[kLm] (-33.81)	3.51 (0.37)[nop] (-21.83)
GSH:GSSG Ratio	267 ± 33	295 ± 42	307 ± 57 (15.19)	293 ± 48 (-0.66)	306 ± 45 (14.78)	331 ± 48 (12.21)	374 ± 55 (40.28)	307 ± 60 (4.27)	486 ± 66[kfL] (82.27)	567 ± 85[jnq] (92.20)

Values in columns are expressed as mean ± standard error of the mean (SEM) at different time points during the study and values in brackets indicates % difference when compred to 0 h (baseline). Abbreviations: GSH = reduced glutathione; GSSG = oxidised glutathione; IAE = Immediately after exercise; tGSH = total glutathione; PL = placebo; Rb = Rooibos. Inter-group = comparing time point differences between the two study interventions, intra-group = comparing time points differences within each intervention. **The inter-group comparison significance (p < 0.05) is indicated in red.** For intra-group comparison significant differences are as follow **a** (p = **0.002**) and **k** (p < **0.0001**) compared to 0h placebo; **c** (p = **0.020**), **d** (p = **0.002**), **e** (p < **0.0001**) and **h** (p = **0.027**), **i** (p = **0.004**), **j** (p = **0.014**) compared to 0h Rooibos; **b** (p = **0.011**), **f** (p = **0.003**) and **g** (p = **0.007**) compared to 1.5h placebo; **n** (p = **0.047**) and **u** (**p = 0.009**) compared to 1.5h Rooibos; **L** (p < **0.0001**) and **x** **(p > 0.05)** compared to IAE placebo, **o** (p = **0.015**) compared to IAE Rooibos, **m** (p = **0.014**) and **q** (p = **0.041**) compared to 1 h placebo; **p** (p = **0.033**) compared to 1 h Rooibos.

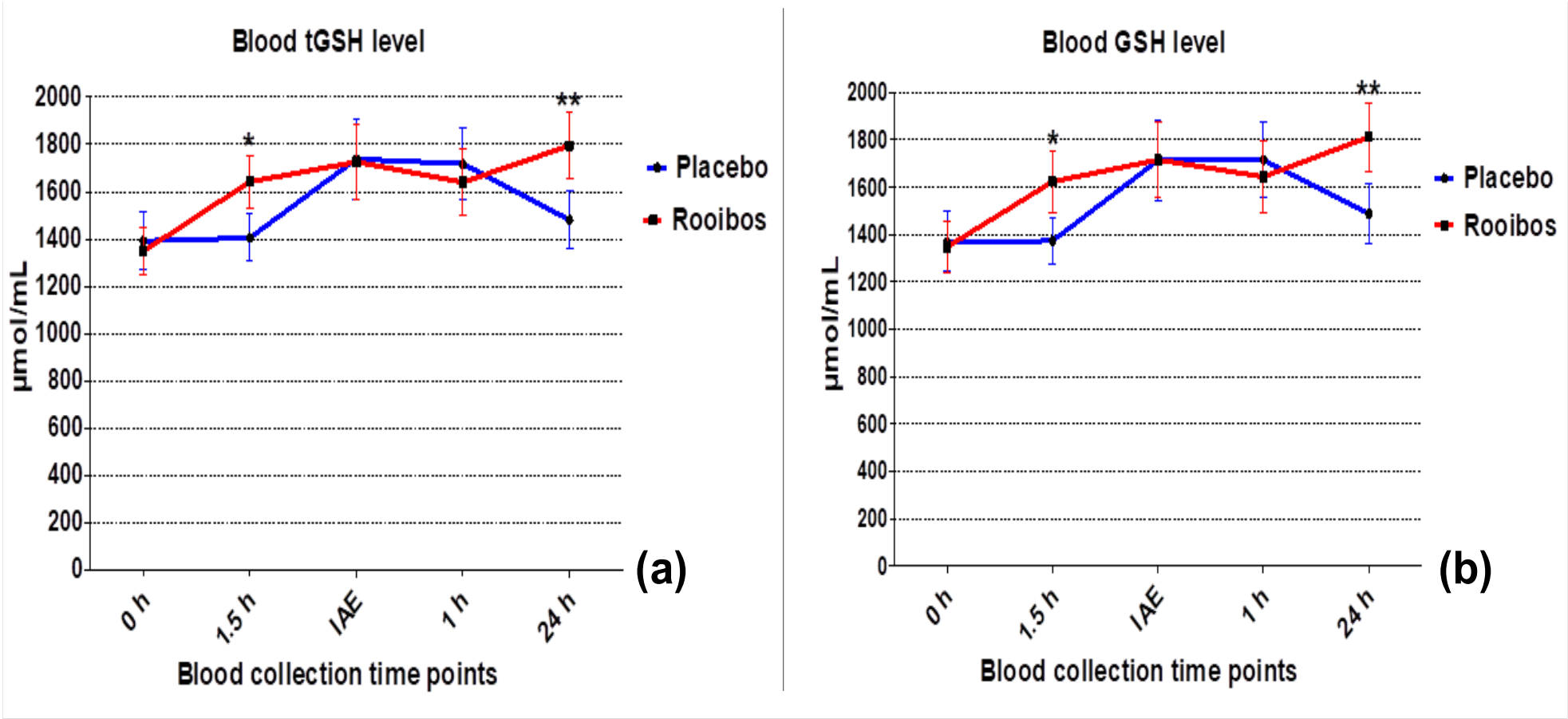


Figure 5.7: Effect of the two study beverages on participant (n = 30) blood glutathione redox status with a) total glutathione and b) reduced glutathione levels. Values are expressed as mean ± standard error of the mean (SEM). Abbreviations: IAE = Immediately after exercise; GSH = Reduced glutathione and tGSH = Total glutathione, with * and ** = $p<0.05$ when compring the rooibos with the placebo intervention at the specific time points

5.3.6 Clinical chemistry analytes

5.3.6.1 Serum biochemical analytes

The effects of both intervention beverages on participants' serum biochemical analytes, representing mostly liver and kidney function are shown in Table 5.13, whereas Figure 5.8 (a, b) visualised biomarkers with significant ($p < 0.05$) inter-group differences. Results show no adverse effects resulting from the intervention beverages as all analysed values remained within the known normal reference ranges of each analyte (Table 5.14). Significant changes in certain analyte levels when Rooibos was consumed compared to placebo, included the decreased AST serum levels at time points1.5 h, IAE and 1 post-exercise ($p < 0.0001$, $p = 0.002$ and $p = 0.001$, respectively) and at 1.5 h and IAE ($p = 0.007$ and $p = 0.051$, respectively) for ALT levels. These decreased levels were still within the analytes' normal reference ranges. The rest of the analytes intergroup results show no significant ($p > 0.05$) changes (decreases or increases) when Rooibos was consumed compared to when placebo was consumed.

When considering the intra-group comparisons, more significant changes occurred in participant serum biochemical analytes when either placebo or Rooibos was consumed. A significant ($p = 002$) increase in AST level IAE compared to baseline and then significantly ($p = 0.002$ and $p < 0.0001$) decreased 1 h and 24 h post-exercise when compared to IAE serum, when the placebo beverage was consumed. However, similar patterns of significant change were also noted when Rooibos was consumed. Rooibos consumption also caused a significantly ($p = 0.004$ and $p < 0.0001$) increased in serum ALP level IAE when compared to the baseline levels and 1.5 h and then significantly ($p < 0.0001$) decreased 1 h and 24 h post-exercise when compared to IAE. This similar pattern was also observed when placebo was consumed. The GGT serum level had no significant changes during the placebo session, but with Rooibos consumption, it significantly ($p = 0.036$) increased IAE when compared to baseline, but it later decreased significantly ($p = 0.012$) at 24 h post-exercise when compared to IAE. Furthermore, when both placebo and Rooibos beverages were consumed, serum TP levels significantly ($p < 0.001$) increased IAE when compared to baseline (0 h) and 1.5 h and then significantly ($p < 0.001$) decreased 1 h and 24 h post-exercise. A similar trend also occurred in serum UA levels, with a significant ($p = 0.023$ and $p < 0.0001$) increase 1 h post-exercise compared to baseline, 1.5 and IAE and then significantly ($p < 0.0001$) decreased 24 h post-exercise when compared to 1 h post-exercise serum level when both beverages were consumed. Serum CREAT levels were only significantly ($p < 0.0001$) decreased when the placebo beverage was consumed, 24 h post-exercise when compared to the 1 h serum level. The serum glucose levels significantly ($p < 0.0001$) decreased at 1.5 h and increased IAE respectively when compared to baseline (0 h), and then also significantly ($p = 0.038$ and $p <$

0.0001) 24 h post-exercise when compared to 1.5 h and IAE glucose serum level when Rooibos was consumed. However, this was also noted when placebo was consumed.

Table 5.13: Effect of the two study beverages consumed by the study participants (n = 30) on various serum biochemical analytes indicative of general health

	Different blood collection time points									
Marker	***0h PL***	***0h Rb***	***1.5h PL***	***1.5h Rb***	***IAE PL***	***IAE Rb***	***1h PL***	***1h Rb***	***24h PL***	***24h Rb***
AST (U/L)	28.87 ± 1.54	26.39 ± 1.54	**30.36 ± 1.42 (5.16)**	**25.89 ± 0.87^{q} (-1.89)**	**32.15 ± 1.52^{a} (11.36)**	**28.48 ± 0.88^{db} (7.92)**	**29.84 ± 1.29^{b} (3.36)**	**26.33 ± 0.83^{e} (-0.22)**	28.37 ± 1.11^{c} (-1.73)	26.75 ± 1.01 (1.36)
ALT (U/L)	**23.02 ± 1.96**	**18.89 ± 1.74**	**23.80 ± 1.90 (3.39)**	**20.00 ± 1.54 (5.88)**	**22.99 ± 2.08 (-0.13)**	**19.83 ± 1.63 (4.98)**	22.29 ± 1.74 (-3.17)	19.73 ± 1.57 (4.45)	23.56 ± 2.04 (2.35)	21..29 ± 2.11 (12.71)
ALP (U/L)	68.52 ± 2.12	66.62 ± 3.00	69.96 ± 1.89 (2.10)	68.22 ± 1.68 (2.40)	74.15 ± 1.96^{fg} (8.22)	74.96 ± 2.04^{id} (12.52)	68.63 ± 1.77^{c} (0.16)	69.38 ± 1.78^{e} (4.14)	67.75 ± 2.02^{c} (-1.12)	67.90 ± 1.97^{e} (1.92)
GGT (U/L)	26.78 ± 1.25	25.73 ± 1.36	26.00 ± 1.24 (-2.91)	25.70 ± 1.19 (-0.12)	26.99 ± 1.25 (0.78)	26.95 ± 1.27^{j} (4.74)	26.28 ± 1.27 (-1.87)	25.93 ± 1.14 (0.78)	25.51 ± 1.38 (-4.74)	24.62 ± 1.25^{k} (-4.31)
TBIL (µmol/L)	10.54 ± 0.98	10.05 ± 0.74	10.10 ± 0.80 (-4.17)	11.24 ± 1.05 (11.84)	10.84 ± 0.78 (2.85)	11.29 ± 0.77^{m} (12.34)	10.07 ± 0.78^{b} (-4.46)	10.08 ± 0.70^{e} (0.30)	11.77 ± 1.08^{L} (11.67)	11.54 ± 1.13 (14.83)
DBIL (µmol/L)	1.91 ± 0.12	1.93 ± 0.11	1.79 ± 0.11 (-6.28)	2.02 ± 0.10 (4.66)	1.79 ± 0.13 (-6.28)	1.97 ± 0.12 (2.07)	1.86 ± 0.13 (-2.62)	2.00 ± 0.10 (3.63)	2.02 ± 0.15^{n} (5.76)	2.25 ± 0.11^{o} (16.58)
TP (g/L)	79.41 ± 0.88	81.21 ± 1.06	80.46 ± 0.87^{p} (1.32)	81.71 ± 0.95 (0.62)	85.84 ± 1.38^{q} (8.10)	87.24 ± 1.19nd (7.42)	80.69 ± 1.16^{c} (1.61)	82.23 ± 1.07^{e} (1.26)	81.16 ± 1.13^{c} (2.20)	80.87 ± 1.05^{e} (-0.42)
ALB (g/L)	49.65 ± 0.64	48.74 ± 1.90	51.50 ± 0.52 (3.73)	51.15 ± 0.48 (4.94)	53.87 ± 1.10 (8.50)	53.86 ± 0.98^{j} (10.50)	51.28 ± 0.50 (3.28)	52.06 ± 0.66 (6.81)	49.45 ± 1.38^{c} (-0.40)	50.81 ± 0.66^{o} (4.25)
BUN (mmol/L)	4.65 ± 0.16	4.44 ± 0.18	4.70 ± 0.15 (1.08)	4.58 ± 0.13 (3.15)	4.78 ± 0.14 (2.80)	4.73 ± 0.13^{d} (6.53)	5.07 ± 0.17^{pqT} (9.03)	5.07 ± 0.16^{nde} (14.19)	4.67 ± 0.16^{u} (0.43)	4.72 ± 0.14^{R} (6.31)
CREAT (µmol/L)	85.54 ± 3.30	85.55 ± 3.72	87.27 ± 3.22 (2.02)	87.26 ± 3.59 (2.00)	103 ± 80 (20.41)	93.44 ± 5.20 (9.22)	89.19 ± 3.64 (4.27)	93.20 (3.53)^{n} (8.94)	82.55 ± 3.48^{v} (-3.50)	87.83 ± 3.24 (2.67)
UA (µmol/L)	361 ± 10.77	387 ± 13.20	378 ± 90^{a} (4.71)	396 ± 15 (2.33)	380 ± 13 (5.26)	401 ± 16 (3.62)	519 ± 15^{pqc} (43.77)	572.69 ± 20.57^{nde} (47.80)	379 ± 13^{v} (4.99)	427 ± 16^{ndR} (10.34)
GLUC (mmol/L)	5.14 ± 0.07	5.13 ± 0.10	4.53 ± 0.10^{p} (-11.70)	4.32 ± 0.12^{n} (-15.79)	4.59 ± 0.11^{p} (-10.53)	4.69 ± 0.11^{m} (-8.58)	4.95 ± 0.27 (-3.51)	4.74 ± 0.19 (-7.60)	5.14 ± 0.10^{qb} (0.19)	5.30 ± 0.17^{dk} (3.31)

Values in columns are expressed as mean and standard error of the mean (SEM) at different time points during the study. The inter-group comparison significance ($p < 0.05$) is indicated in red. Abbreviations: TP= Total protein, ALB = Albumin; ALP = Alkaline phosphatase; AST = Aspartate aminotransferase; ALT = Alanine aminotransferase; BUN = Blood urea nitrogen; CREA = Creatinine; TBIL = Total bilirubin; DBIL = Direct bilirubin; GGT = Gamma glutamyl transferase; UA = Uric acid;

GLUC = Glucose, PL = placebo; Rb = Rooibos. For intra-group comparison significant differences are as follow, **a** (p = **0.002**), **f** (p = **0.010**) and **p** (p < **0.0001**) compared to 0h placebo; i (p = **0.004**), **m** (p = **0.034**), **n** (**p = 0.001**) **e** (p < **0.0001**) and **h** (p = **0.027**)**, i** (p = **0.004**), **j** (p = **0.014**) compared to 0h Rooibos, **g** (p = **0.011**), **q** (p < **0.0001**), **g** (p = **0.007**), **v** (**p = 0.021**) and **T** (**p = 0.017**) compared to 1.5h placebo; **d** (p < **0.0001**), **j** (**p = 0.036**) and **u** (**p = 0.009**) compared to 1.5h Rooibos; **b** (p = **0.002**), **c** (p < **0.0001**), **T** (p = **0.010**) compared to IAE placebo, **e** (p < **0.0001**), **k** (**p = 0.012**), **o** (**p = 0.020**) compared to IAE Rooibos, **L** (p = **0.031**), **u** (p = **0.051**) and **v** (p < **0.0001**) compared to 1 h placebo; **R** (p = **0.033**) compared to 1 h Rooibos..

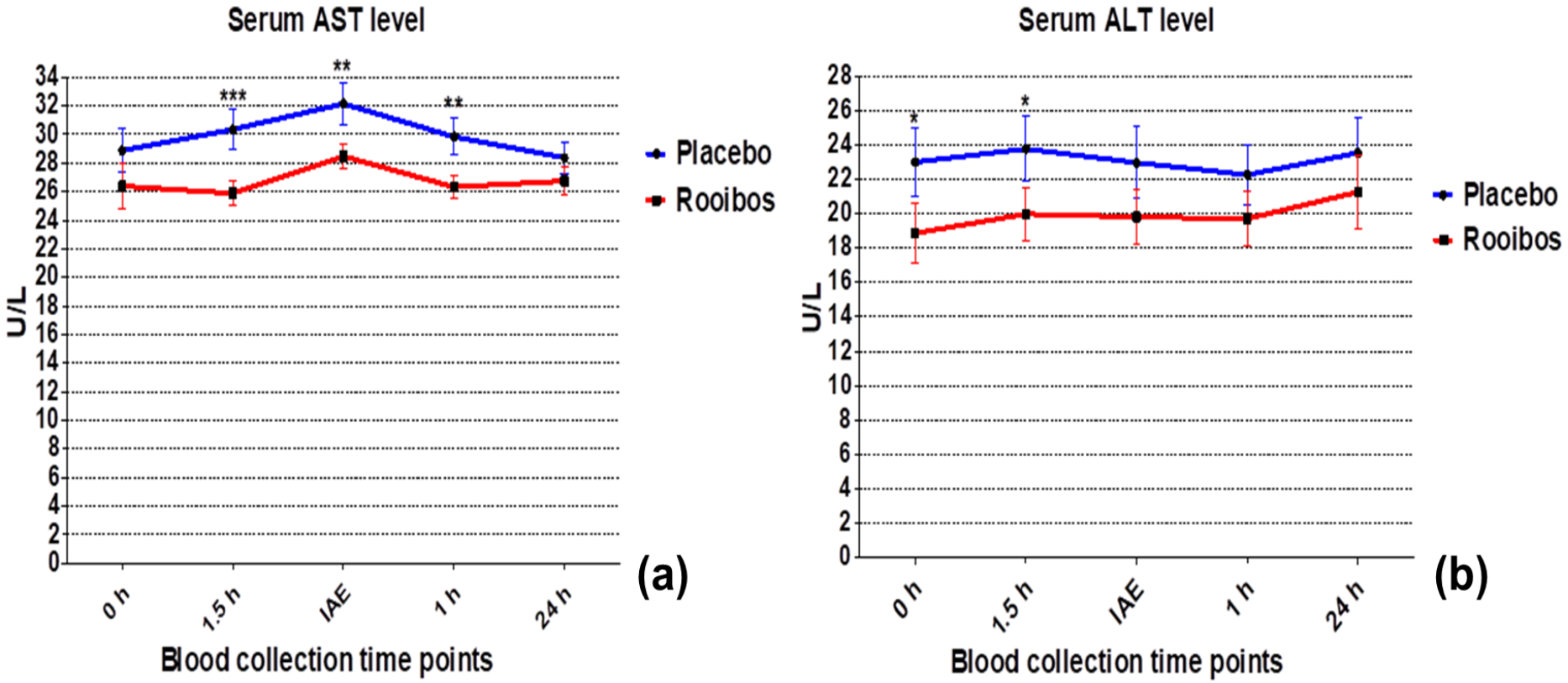


Figure 5.8: Effect of study beverages on participants (n = 30) serum chemistry analytes indicative of liver function health a) Aspartate aminotransferase (AST); and b) Alanine aminotransferase (ALT). Values are expressed as mean ± standard error of the mean (SEM). Abbreviations: IAE = Immediately after exercise; ALT = Alanine aminotransferase ; AST = Aspartate aminotransferase, with *, **, *** p <0.05 when comparing Rooibos with the placebo intervention at the various time points

Table 5.14: Blood serum diagnostic normal reference ranges for a South African population.

Serum/plasma indicators (units)	Normal values (male adults)
AST (U/L)	25.7 – 183.0
ALP (U/L)	55.0 – 316.0
ALT (U/L)	19.0 – 121.6
GGT (U/L)	30.0 – 95.0
Total protein (g/L)	42.0 – 74.0
Albumin (g/L)	26.0 – 47.0
Blood urea nitrogen (mmole/L)	3.2 – 18.7
Glucose (mmole/L)	5.0 – 12.9
Uric Acid (µmole/L)	167.0 – 605
Total Bilirubin (µmole/L)	13.5 – 134.2
Direct Bilirubin (µmole/L)	2.2 – 52.3
Creatinine (µmole/L)	81.0 – 536.0

Abbreviations: ALB = Albumin; ALP = Alkaline phosphatase; AST = Aspartate aminotransferase; ALT = Alanine aminotransferase; BUN = Blood urea nitrogen; CREA = Creatinine; TBIL = Total bilirubin; DBIL = Direct bilirubin; GGT = Gamma glutamyl transferase; TP= Total protein, UA = Uric acid; GLUC = Glucose,

5.3.7 Serum lipid profile

The effects of the two study beverages on participants' serum lipid profile analytes and at different time points during the study is presented in Table 5.15. When Rooibos was consumed, inter-group results show a significant ($p = 0.006$; $p = 0.003$ and $p = 0.009$) increase (3.04%; 5.62% and 4.69%) in serum cholesterol at 1.5 h, IAE and 1 h post-exercise, respectively. Serum triglyceride levels were also significantly ($p = 0.057$; $p = 0.045$) increased (14.29%; 14.29%) IAE and 1 hr post-exercise when Rooibos was consumed compared to placebo. For the lipoproteins, no significant ($p > 0.05$) changes were observed in the serum HDL levels at any of the study time points when Rooibos was consumed compared to placebo. However, serum LDL level inter-group results show significant ($p = 0.025$; $p = 0.001$; $p = 0.011$; $p = 0.004$) increases (5.46%; 8.16%; 7.11%; 7.39%) at 1.5 h, IAE 1 h and 24 h post-exercise when Rooibos was consumed compared to placebo.

When considering intra-group comparisons, no significant ($p > 0.05$) changes in cholesterol levels were shown when the placebo beverage was consumed. When Rooibos was consumed, a significant ($p = 0.003$) increase in cholesterol occurred IAE when compared to 1.5 h and then significantly ($p < 0.0001$) decreased 24 h post-exercise when compared to IAE serum levels. Lastly, serum triglyceride and the lipoproteins (LDL and HDL) level intra-group results show a similar trend of significant ($p < 0.05$) increases and decreases at different time points when both Rooibos and placebo beverages were consumed.

Table 5.15: Effect of study beverages on the participant (n = 30) serum lipids profile

	Different blood collection time points									
Marker	***0h PL***	***0h Rb***	***1.5h PL***	***1.5h Rb***	***IAE PL***	***IAE Rb***	***1h PL***	***1h Rb***	***24h PL***	***24h Rb***
CHOL (mmol/L)	4.33 ±0.16	4.40 ± 0.16	**4.27 ± 0.13 (-1.39)**	**4.40 ± 0.14 (0)**	**4.45 ± 0.17 (2.77)**	**4.70 ±0.18[a] (6.82)**	**4.26 ± 0.14 (-1.62)**	**4.46 ± 0.16[b] (1.36)**	4.35 ± 0.14 (0.46)	4.42 ± 0.15[c] (0.45)
TRIG (mmol/L)	0.80 ± 0.07	0.85 ± 0.07	0.92 ± 0.08 (15.00)	0.98 ± 1.00 (15.29)	**0.98 ± 0.07[d] (22.50)**	**1.12 ±0.09[gi] (31.76)**	**0.84 ± 0.06e (5.00)**	**0.96 ± 0.06[c] (12.94)**	0.79 ± 0.04[f] (-1.25)	0.87 ±0.06[hc] (2.35)
LDL (mmol/L)	2.37 ± 0.10	2.51 ± 0.11	2.38 ± 0.09 (0.42)	2.51 ± 0.12 (0)	**2.45 ± 0.13 (3.38)**	**2.65 ± 0.15 (5.58)**	**2.39 ± 0.11 (0.84)**	**2.56 ± 0.14 (1.99)**	**2.30 ±0.13[j] (-2.95)**	**2.47 ± 0.12[k] (-1.59)**
HDL (mmol/L)	1.22 ± 0.03	1.17 ± 0.04	1.23 ± 0.04 (0.82)	1.20 ± 0.03 (2.56)	1.29 ± 0.04 (5.74)	1.30 ± 0.03[Li] (11.11)	1.22 ± 0.03[f] (0)	1.23 ± 0.03[c] (5.13)	1.20 ± 0.04[e] (-1.64)	1.21 ± 0.03[c] (3.42)

Values in columns are expressed as mean ± standard error of the mean (SEM) at different time points during the study and values in brackets indicates % difference when compred to 0 h (baseline). Abbreviations: CHOL = cholesterol; TRIG = triglyceride; LDL = low density lipoprotein, HDL = high density lipoprotein; PL= placebo; Rb = Rooibos. Inter-group = comparing time point differences between the two study interventions, intra-group = comparing time points differences within each intervention. **Inter-group comparison significance (p < 0.05) is indicated in red**. For intra-group comparison significant differences are as follow **d** (p < **0.0001**) compared to 0h placebo; **g** (p<**0.0001**), **h** (p = **0.061**), **L** (p = **0.041**) and compared to 0h Rooibos; **a** (p = **0.003**) and **i** (p < **0.0001**) compared to 1.5h Rooibos; **e** (p < **0.0001**), **f** (p = **0.002**) and **j** (p =**0.008**) compared to IAE placebo; **c** (p < **0.0001**), **b** (p = **0.027**) and **k** (p = **0.004**) compared to IAE Rooibos

5.3.8 Muscle damage and fatigue biomarkers

The effects of the two study beverages on participants' serum muscle damage and fatigue indicators is shown in Table 5.16, whereas, Figure 5.9 visualised the indicators with significant ($p < 0.05$) inter-group differences. When considering inter-group comparisons, the muscle damage indicator, LDH was decreased IAE and 1 h post-exercise, when rooibos was consumed, although this was not significant ($p > 0.05$), while the serum CK level was significantly ($p = 0.022$) decreased by 20.18% at 1.5 h when compared to placebo. The serum levels for the fatigue indicator, lactate, were increased IAE, 1 h and 24 h, but not significanly ($p > 0.05$) when Rooibos was consumed compared to placebo.

The intra-group comparisons show no significant ($p > 0.05$) change in serum LDH when Rooibos was consumed, but a significant ($p = 0.051$) increase occurred IAE when compared to baseline, with a significant ($p = 0.051$) decrease 1 h post-exercise when compared to IAE when the placebo beverage was consumed. No significant ($p > 0.05$) intra-group changes were noted for CK serum levels when placebo was consumed, whereas a significant ($p = 0.003$) decrease was shown 1 h post-exercise compared to IAE when Rooibos was consumed. Lactate serum levels was significantly ($p < 0.05$) increased and decreased at different time points when Rooibos was consumed, and similarly when the placebo beverage was consumed.

Table 5.16: Effect of the two study beverages on participant (n = 30) muscle damage and fatigue markers

	Different blood collection time points									
Marker	***0h PL***	***0h Rb***	***1.5h PL***	***1.5h Rb***	***IAE PL***	***IAE Rb***	***1h PL***	***1h Rb***	***24h PL***	***24h Rb***
LDH (U/L)	172 ± 40	181 ± 70	181 ± 40 (5.22)	175 ± 40 (-3.47)	191 ± 71a (11.20)	187 ± 52 (3.20)	175 ± 42 (1.75)	176 ± 53 (-2.85)	183 ± 54 (6.46)	179 ± 43 (-1.25)
CK (U/L)	287 ± 27	292 ± 28	**342 ± 29 (19.15)**	**273 ± 15 (-6.55)**	319 ± 23 (11.13)	293 ± 18 (0.31)	307 ± 23 (6.95)	279 ± 18^{c} (-4.48)	272 ± 23 (-5.24)	281 ± 22 (-3.78)
LAC *(U/L)*	3.20 ± 0.20	3.17 ± 0.16	3.33 ± 0.19 (4.06)	3.37 ± 0.20 (6.31)	14.21 ± 0.75^{de} (344.06)	15.08 ± 0.78^{ij} (375.71)	3.49 ± 0.24^{g} (9.06)	3.80 ± 0.23^{k} (19.87)	2.58 ± 0.18^{fgh} (-19.38)	2.69 ± 0.19^{kL} (-15.14)

Values in columns are expressed as mean ± standard error of the mean (SEM) at different time points during the study and values in brackets indicates % difference when compred to 0 h (baseline). Abbreviations: CK = Creatinine kinase; LAC = Lactate; LDH = Lactate dehydrogenase; PL = placebo; Rb = Rooibos. Inter-group = comparing time point differences between the two study interventions, intra-group = comparing time points differences within each intervention. **Inter-group comparison significance (p < 0.05) is indicated in red.** For intra-group comparison significant differences are as follow **a** (p = **0.051**) and **d** (p < **0.0001**) compared to 0h placebo; **i** (p < **0.0001)** compared to 0h Rooibos; **e** (p < **0.0001**) and **f** (p = **0.005**) compared to 1.5 h placebo; **j** (p < **0.0001)** compared to 1.5 h Rooibos; **b** (p = **0.051**) and **g** (p < **0.0001)** compared to IAE placebo; **c (**p = **0.003**) **k** (p<**0.0001**) compared to IAE Rooibos, **h** (p = **0.002**) compared to 1 h placebo; **L** (p = **0.001**) compared to 1 h Rooibos.

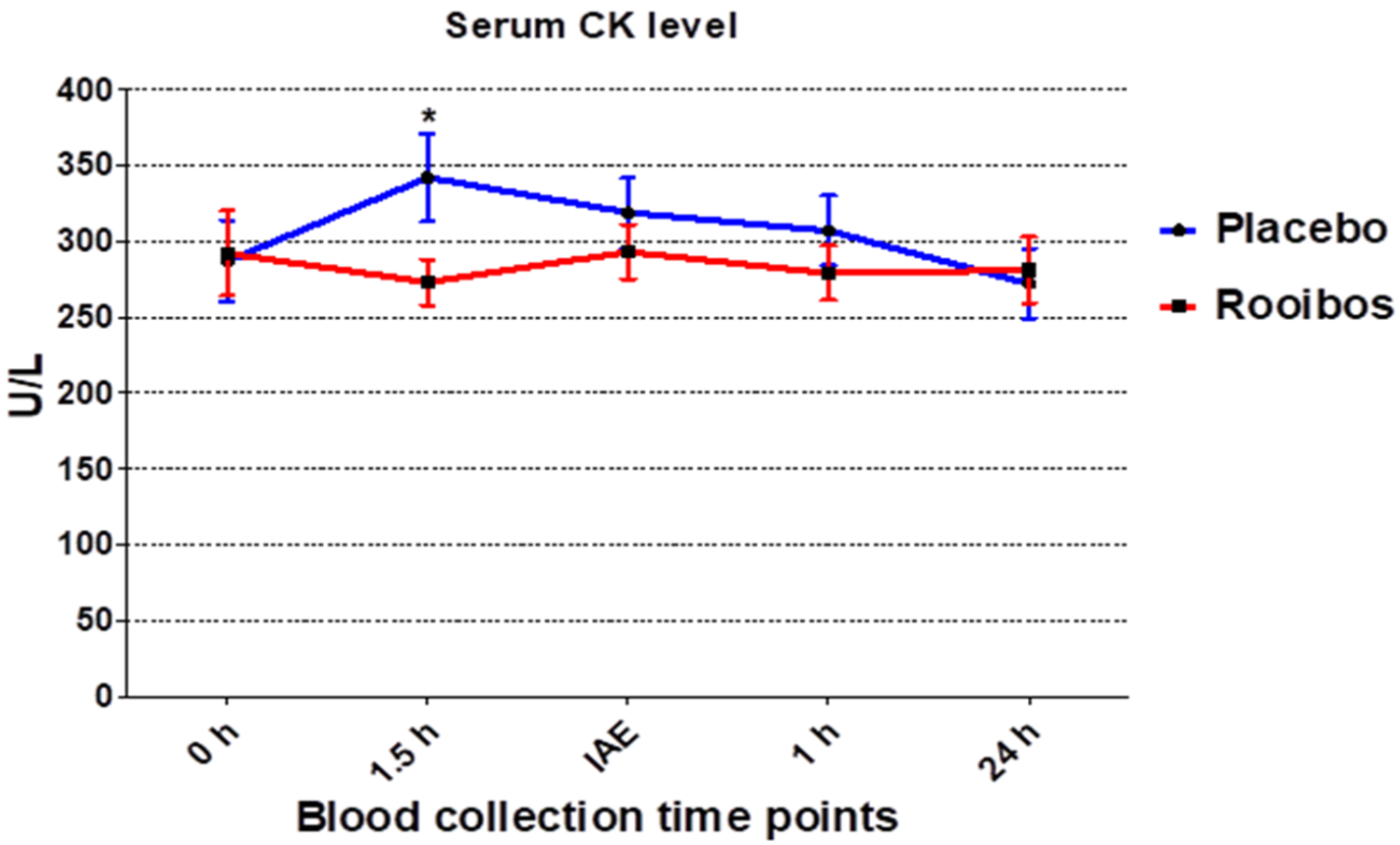


Figure 5.9: Effect of study beverages on participant (n = 30) serum creatinine kinase level.

Values are expressed as mean ± standard error of the mean (SEM). Abbreviations: IAE =Immediately after exercise; CK = Creatinine kinase, with * = $p < 0.05$ when comparing the two intervention beverages at the specific time point

5.3.9 Exercise performance and recovery

The effect of the intervention study beverages on participants' exercise performance during the sub-maximal (75 – 80%) ramp test on the Wattbike is displayed in Table 5.17. Results show important tendancies noted that during the submaximal phase of the test the participants, after consuming the rooibos beverage, exercised 14.96% longer (sec) and covered a 13.60% greater distance (m) when compared to the placebo session, although not significant. Participants also produced a significantly ($p = 0.047$) higher (2.35% increased) work (Kj) rate and cycled with 3.65% more power (W) during the rooibos session when compared to the placebo session. Figure 5.10 (a,b,c and d) provide a graphic indication of the submaximal exercise performance outcomes.

Table 5.17: Quantitative description of the participants (n = 30) submaximal performance parameters after consumption of the Rooibos and placebo beverages

	Placebo		Difference		Rooibos		
Variable	Mean ± SD	Sum	Mean %	P-value	Mean	(SD)	Sum
Time (sec)	254 ± 150	7605	14.96	0.36	292 ± 172		8755
Distance (metre)	2435 ± 1604	73048	13.60	0.42	2790 ± 1827		83709
Work (kilojoules)	30.26 ±14.97	16947.4	2.35	0.42	30.98 ± 15.65		19737.3
Power (Watts)	141 ± 43	78929	3.65	**0.047**	146 ± 48		93128

Values in columns are expressed as mean, standard deviation (SD) and sum of scores; along with percentage difference between mean scores and P-value for one-way ANOVA. Abbreviation: ANOVA = Analysis of variance. Inter-group comparison significance ($p < 0.05$) is indicated in red

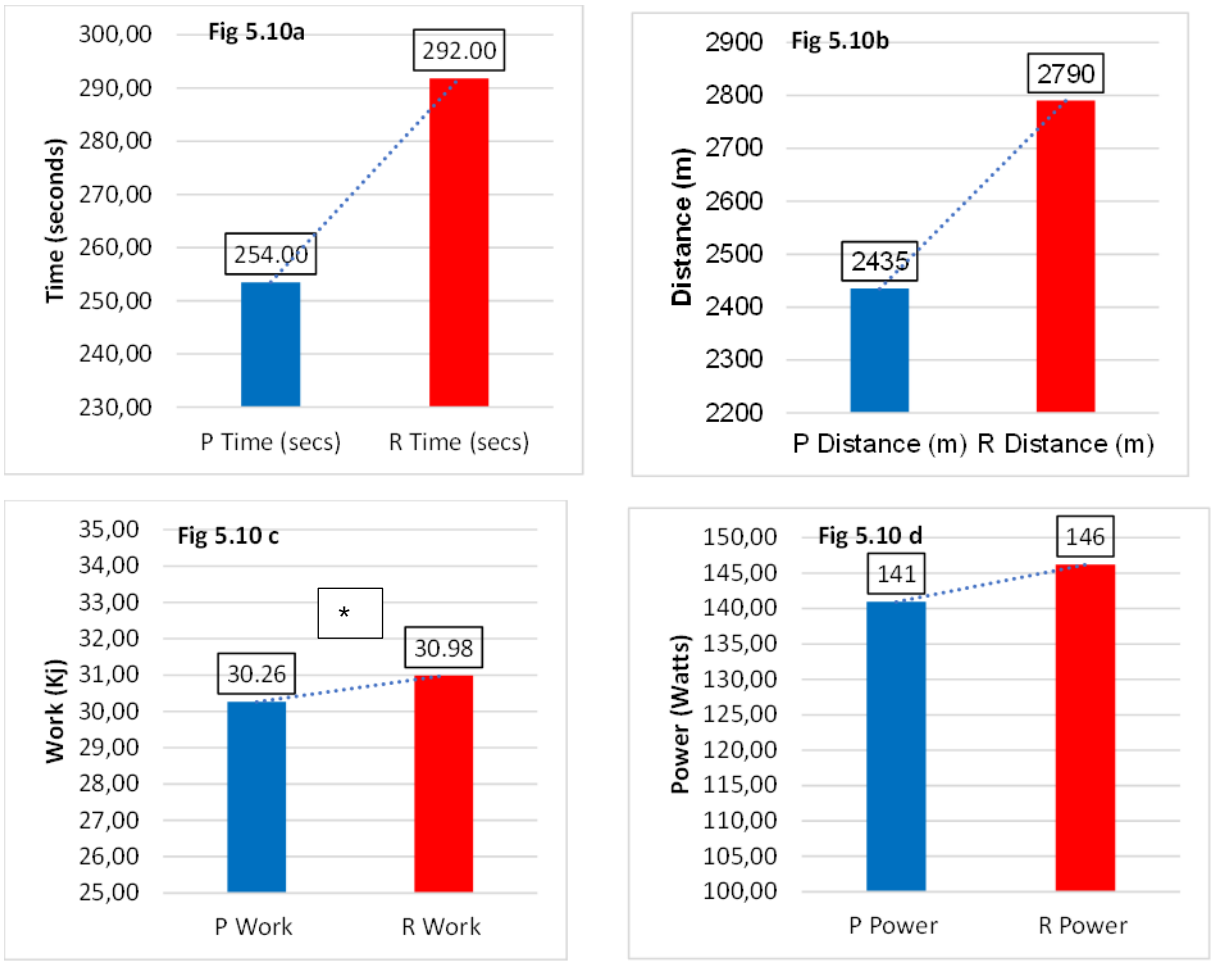


Figure 5.10: Participants' (n = 30) exercise performance parameters during the sub-maximal ramp test after the consumption of the Rooibos and placebo beverages

(a) Time to withdrawal; **(b)** distance covered; **(c)** work and **(d)** power. Units for time = seconds; distance = metres; work = Kilojoules; power = Watts. Abbreviation: P = Placebo; R = Rooibos. * = $p < 0.05$ when comparing the Rooibos with the Placebo intervention

Rooibos consumption also appears to have had an effect participants' cardiorespiratory performance as seen in Table 5.18 and Figure 5.11a and 5.11b. It was interesting to observe that following Rooibos ingestion, heart rate increased 2.47% which is less when compared to the increased time to withdrawal and distance completed, while relative oxygen uptake ($mL.min^{-1}.kg^{-1}$) increased 5.53%, perhaps demonstrating that rooibos had a beneficial effect on metabolic responses during exercise by possibly improving and/or optimising the efficiency of the oxidative phosphorylation system.

Table 5.18: Quantitative description of the effect of the study beverages, Rooibos and placebo on the cardiovascular response of the participants (n = 30).

	Placebo		Difference		Rooibos	
Variable	Mean (SD)	Sum	Mean %	P-value	Mean ± SD	Sum
Heart rate	131 ± 21.17	73511	2.47	**0.01**	135 ± 24.34	85714
Oxygen uptake	25.84 ± 9.37	39151.30	5.53	1.70	27.31 ± 9.96	46871.35

Values in columns are expressed as mean ± standard deviation (SD) and sum of scores; along with percentage difference between mean scores and p value for one-way ANOVA. Units for heart rate = beats per minute; oxygen uptake (relative) $mL.min^{-1}.kg^{-1}$. Inter-group comparison significance ($p < 0.05$) is indicated in red. Abbreviation: ANOVA = Analysis of variance.

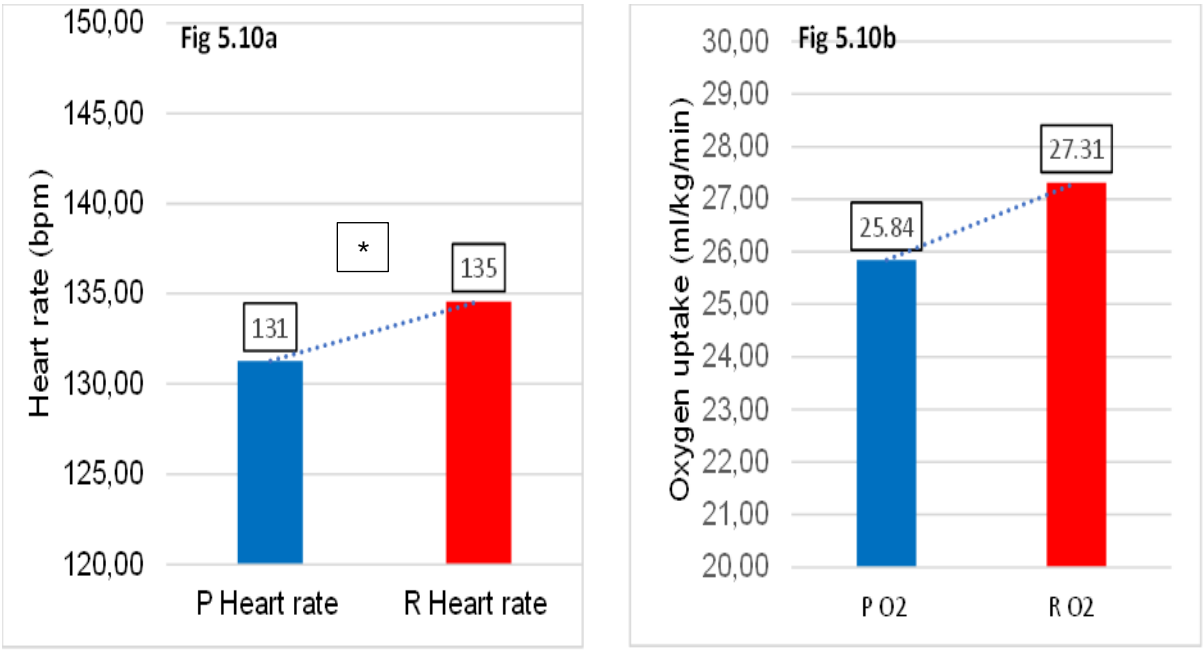


Figure 5.11: Participants' (n = 30) cardiovascular response during the submaximal exercise test **(a)** Heart rate; **(b)** oxygen uptake. Units for heart rate = beats per minute and relative oxygen uptake $mL/kg/min^{-1}$. Abbreviation: O_2 = Oxygen; P = Placebo; R = Rooibos. * = $p < 0.05$ when comparing the Rooibos with the Placebo interventions

5.4 Phase II – Discussion

Despite being well-known that exercise is beneficial to human health, it has been established that chronic or strenuous exercise or physical activity may lead to OS due to increased generation and formation of RONS (Ermakov et al., 2013; Phaniendra et al., 2015; Fuster-

Muñoz et al., 2016). During exercise or physical activity, the generation of RONS may also result in exercise-induced ailments such as fatigue, inflammation, muscle damage and overall poor exercise performances and prolonged recovery (Steinbacher & Eckl, 2015). Currently, besides the use of synthetic drugs, there is no other alternative approach/practice which is much safer and affordable to supplement or replace the use of synthetic drugs to prevent and/or mitigate exercise-induced ailments. Previous studies reported that consumption of polyphenolic-rich beverages/extracts can improve and/or raise plasma total phenolic content, and antioxidant capacity and improve exercise performance (Fuster-Muñoz et al., 2016; Sanguigni et al., 2017; de Lima Tavares Toscano et al., 2020; Moslemi et al., 2023). In this current study, we hypothesised that an acute intake of standardised fermented Rooibos beverage would modulate study participants' plasma total phenolic content, and antioxidant capacity and enhance their exercise performance outcomes, unlike placebo. The study recruited healthy young adult males with a mean age of 26.23 ±6.52 and a BMI of 24.45 ± 4.24 Kg/m^2. All participants completed the three-week study trial, (1st week = session one, 2nd week = wash out period and 3rd week = session two), and none reported any adverse effects resulting from the study beverages consumed. The ingestion of 375 mL, was to ensure participants ingest enough volume to remain well-hydrated during exercise, while 90 minutes before the submaximal Wattbike exercise regime was to ensure enough time is allowed for the absorption of phytochemical flavonols as reported by Mao-Jung et al. (2002) where participants consumed a single dose of green tea (394 mg polyphenols) and observed the highest plasma catechin content 1.3 –1.6 h after the intake.

The HPLC and spectrophotometer analysis of the two study beverages (Table 5.9) authenticates both Rooibos and placebo beverages, as indicated by the presence of phenolic compounds, polyphenolic content and antioxidant capacity in the Rooibos beverage and lack of an active antioxidant substance in the placebo beverage. Participants' plasma polyphenolic content and antioxidant capacity were determined with, three different assays TPC, FRAP and TEAC. When both study Rooibos and placebo beverages were consumed participant's plasma TPC was increased significantly IAE, but this did not last longer because 1 h post-exercise, plasma TPC level decreased and remained lower close to almost baseline level till 24 h post-exercise. This trend was also observed in the plasma antioxidant capacity, FRAP in particular, with a significant increase 1 h post-exercise when both beverages were consumed. Participants' plasma antioxidant capacity (FRAP and TEAC) level increased at 1.5 h and IAE respectively and remained high above the baseline values (0 h) throughout both beverages, but much higher the during Rooibos session, but none of these increases was statistically significant ($p > 0.05$) when compared to placebo. When compared the intra-group changes,

most significant ($p < 0.05$) changes in participants plasma TPC and FRAP levels were observed when both beverages were consumed, hence, this could not purely be attributed to the consumption of either of the beverages, but perhaps to the participants' endogenous antioxidant defence mechanisms. However, despite the observed intra-group increments at different time points when Rooibos was consumed, none of this had or made an overall significant ($p > 0.05$) difference in participants' plasma polyphenolic content and antioxidant capacity level when compared to placebo. These results support findings from previous studies where Marnewick et al. (2011) reported that consuming six cups of fermented Rooibos per day for six weeks failed to alter participant plasma antioxidant capacity whereas Morillas-Ruiz et al. (2006) reported a failure of polyphenolic antioxidant supplementation (acute dose of 2.3 g polyphenol/1600 ml) to improve cyclists' plasma antioxidant capacity. Both studies used the fermented Rooibos extract and it's known that the chemical oxidation process involved in the production of fermented rooibos decreases the herbal teas' final total polyphenol content (Joubert & Ferreira, 1996). Hence, this might be one of the factors contributing to this study beverage not significantly increasing or improving the overall participants' total polyphenolic content and antioxidant capacity when compared to the placebo beverage. Of course, another contributing factor could be that of dosage time (acute vs chronic) and bioavailability.

The use and role of dietary polyphenolic-rich beverages against lipids and protein damage are also well established (Belviranlı et al., 2012; Ajuwon et al., 2014; Fuster-Muñoz et al., 2016). Compared to the placebo, the consumption of the fermented Rooibos beverage, in the study did not significantly reduce exercise-induced oxidative lipid damage at any time point, although, intra-group results show promising trends of reduction in the two analysed lipid biomarkers, TBARS and CDs. Participants' plasma TBARS levels were reduced at 1.5 h and remained below baseline level throughout the study period and only started increasing to above baseline (0 h) level, 24 h post-exercise when Rooibos was consumed. This observation is not surprising because TBARS are the last end products of oxidative lipid damage, and only start appearing in the blood circulation 24 h post-exercise. Unlike the TBARS, the CDs which represent the early phases of oxidative lipid damage, was increased in participants' plasma IAE, and remained elevated till 24 h post-exercise when the placebo was consumed, but when Rooibos was consumed plasma CD levels were reduced IAE and 1 h post-exercise, although not significantly. Consumption of the Rooibos beverage also reduced, but not significantly, plasma PC levels at 1 h and 24 h post-exercise, indicative of the potential protective effect of Rooibos against protein oxidation during exercise. Furthermore, when participants consumed the placebo, their plasma CDs and PC levels increased IAE and remained high above the pre-

exercise levels, but this was not observed when Rooibos was consumed, hence, it could be an indication and/or affirming that the exercise test protocol (modified submaximal ramp test) has indeed induced oxidative stress damage in participants, and Rooibos consumption somehow protected against this induced damage, even though not significantly, and this was shown by the reduction in plasma lipid and protein damage biomarkers at different time points when Rooibos was consumed, unlike when placebo was consumed. Additionally, when both beverages were consumed, both lipid and protein damage biomarkers' plasma levels returned to almost baseline level at 1 h post-exercise. There was not an indication of possible ongoing oxidative stress damage, and this could be due to an enhanced general AOX defence system in the body serving as an additional mechanism supporting the bioactivate properties of the fermented Rooibos beverage. These results agree with the findings by Neubauer et al. (2008), that the body's endogenous antioxidant defence system was able to protect against an acute exercise-induced oxidative stress as indicated by the lipid and protein damage. Reduced glutathione (GSH) molecules which form part of the endogenous AOX defence system within a living cell does react with RONS - two molecules of GSH donate electrons - whenever oxidative stress conditions occur in an attempt to stabilise RONS and restore the antioxidant -– oxidant balance, resulting in the oxidation of GSH sulphydryl (-SH) group to form a disulfide compound (oxidised glutathione, GSSG). Theoretically, a decrease in GSH blood levels, an increase in GSSG levels, and a decreased GSH/GSSG ratio, is often associated with oxidative stress. In the current study, consumption of the fermented Rooibos beverage did significantly improve participants' blood tGSH and GSH levls at 1.5h and 24 h post-exercise, with no significant change in participants' blood GSSG or GSH/GSSG ratio when compared to placebo. The intra-group results also indicate that Rooibos consumption was able to significantly increase participant blood tGSH and GSH levels at 1.5 h and remained higher throughout all time points when compared to their baseline levels. Additionally, although the change was not significant, participants' blood GSSG levels increased sharply IAE and also significantly decreased 24 h post-exercise compared to baseline when placebo was consumed, but none when Rooibos was consumed. This could be interpreted as an indication of oxidation of GSH as a result of induced oxidative stress which could be another affirmation that the study exercise regime did indeed induced OS condition in the participant, and also further affirm the protective potential of the Rooibos beverage. The glutathione redox status in the current study agrees with previous studies where a similar conclusion was made regarding fermented Rooibos supplementation/ intake (Marnewick et al., 2011; Ajuwon et al., 2014). Furthermore, the strong significant trend or improvement in blood redox status (tGSH and GSH) observed in this study at 1.5 h and 24 h post-exercise when Rooibos was consumed might be also an indication of Rooibos's ability to upregulate the synthesis of GSH as suggested by Moskaug et al. (2005).

Throughout the study, no adverse effects were reported by any of the study participants. Additionally, serum analysis of the clinical chemistry analytes when the two study beverages were consumed particularly the liver and kidney function analytes, showed none of the markers were adversely affected. Even though when considering inter and intra-group results, some trends of significant decrease and increase were observed in some of the liver and kidney analytes (AST, ALT, ALP, GGT, TP, CREAT and UA) these changes were still within the normal clinical diagnostic ranges of these specific analytes. Current literature also positively reported on Rooibos' potential cardiovascular benefits, because of its potential effect on the lipid profile analytes (Hyunguk & Taesik, 2008; Marnewick et al., 2011). In the current study, a small but significant increase in total cholesterol, triglyceride and LDL was observed when Rooibos was consumed, and no significant change to the serum HDL cholesterol levels. This finding contradicts most of the current literature on Rooibos and other flavonoid polyphenolic-rich beverages (Marnewick et al., 2011; Jubayer et al., 2020), but it should be noted that in the other studies rooibos was consumed chronically (up to 6 weeks) whereas in the current study an acute dose of Rooibos was consumed and this could account for the contradictory results the changes in the lipid profile analytes were still within the normal clinical diagnostic ranges.

Exercise activities involve muscle contractions, which often provoke or induce exercise ailments such as muscle damage which eventually leads to overall poor performance, pain, and prolonged recovery. However, polyphenolic-rich beverages ought to prevent and/or mitigate exercise-induced ailments, mostly fatigue, and muscle damage and subsequently enhance exercise performance and recovery due to their various bioactive properties. Muscle damage often involves a temporary decrease in muscle function, an increase of intracellular proteins in the blood and muscular pain, which make it difficult or prolong athletes' recovery (Ferreira et al., 2020). Muscle damage is generally monitored by measuring enzymes such as CK, AST and LDH. Both CK and LDH are considered as specific biomarkers of muscle damage, however, just like CDs and TBARS, CK and LDH also appear at different phases of the muscle damage process (da Silva et al., 2018). The CK is an enzyme specific to the muscle and is responsible for catalysing adenosine triphosphate (ATP)-dependent phosphorylation of creatine while LDH is responsible for catalysing the anaerobic conversion of pyruvate to lactate, thus, elevation in serum/plasma CK levels is used as an early and/or indirect marker of exercise-induced muscle damage (Kim et al., 2019). In the current study, both LDH and CK serum levels increased IAE, but not significantly, but this gives an indication that damage to muscle fibres occurred and caused a disruption of the muscle cell membrane integrity

irrespective of the beverage consumed, although it was much higher during the placebo session. When compared to the placebo, consumption of the fermented Rooibos beverage prevented exercise-induced muscle damage as indicated by the significant decrease in participants' serum CK levels at 1.5 h and also significantly decreased 1 h post-exercise when compared to IAE serum levels when Rooibos was consumed. Intra-group results also showed that participants' serum LDH levels was significantly increased IAE compared to baseline serum level when placebo was consumed, but not when Rooibos was consumed. From these findings, one could suggest that the consumption of the fermented Rooibos beverage has the potential to mitigate possible exercise-induced muscle damage and/or ailments. Similar findings were also reported by da Silva et al. (2018), whereby the consumption of a green tea extract (polyphenolic-rich) lowered and minimised participants' serum CK activity after an exercise-induced delayed onset of muscle soreness exercise session. Da Silva and co-workers (2018) also argued that the lack of significant difference in the LDH activity in their study could have been influenced by the method, protocol or time point limitation, especially considering that LDH is a late muscle damage marker, hence, it could have been that LDH was still lower 48 h later when the last blood sample was collected, unlike the CK which is an early muscle damage marker. This argument could also be applied to our study considering that the last blood sample was collected 24 h post-exercise and could be a reason for the lack of LDH significant differences in this study. When compared to placebo, Rooibos consumption had no significant influence on the fatigue biomarker/indicator, lactate although intra-group results showed that participants' serum level was significantly reduced to almost that of the baseline level at 1 h and 24 h post-exercise when Rooibos was consumed, a similar pattern was also observed when placebo beverage was consumed, thus, these significant changes could not be wholly attributed to the Rooibos beverage. Additionally, both AST and ALT are mostly associated with liver function, however, when muscle damage occurs these enzymes tends to be released from muscles into the bloodstream as well, hence, proving to be a good indicator of tissue/muscle damage as well (Ferreira et al., 2020). Furthermore, compared to the liver, skeletal muscles have more AST and ALT due to a larger tissue mass, and in the case of muscular disorders or injury, AST and ALT levels may be elevated. In our study, both AST and ALT were slightly increased IAE regardless of the beverage consumed. This could be an indication that there was muscular injury/damage which also further affirmed the effectiveness of our exercise regime model used to induce oxidative stress as well as muscle damage. A study by Vecchio and colleagues (2017) highlighted a positive correlation between increased lipid peroxidation markers, CK and AST and suggested that biomarkers of muscle damage during exercise are indicative of oxidative stress. However, in our study, compared to the placebo, consumption of Rooibos significantly reduced participant serum AST at 1.5 h, IAE and 1 h post-exercise, and also reduced serum ALT levels at baseline (0 h), 1.5 h and

IAE respectively. These findings if viewed from a muscular damage point of view, could suggest that Rooibos intake was able to mitigate muscular damage in the study participants unlike the placebo, rendering the fermented Rooibos beverage to be considered as an ergogenic aid for sport athletes or individuals. A study by Ferreira and co-workers (2020) also concluded that a hydro electrolyte beverage (whey permeate with the phenolic extract of jabuticaba peel) attenuated markers of muscular and oxidative stress damage in trained individuals indicated by a significant reduction in AST, ALT and CK serum level.

Lastly, this study assessed whether Rooibos consumption improved submaximal exercise performance. Results show that consumption of the fermented Rooibos beverage influenced participants' exercise performances differently when compared to the placebo. When Rooibos was consumed participant exercise duration was improved (14.9% increase) which was accompanied by improved sustained exercise endurance, as participants covered a greater distance (13.6% difference). Particpants mean performance during the submaximal trial also resulted in more power (3.65%) and work done (2.35%) during the when compared to the placebo. Generally speaking one would have expected a concomittant increase during the rooibos trial in heart rate and oxygen uptake when referenced against the increase in endurance time (14.9%) and distance (13.6%). However, mean heart rate and oxygen uptake increased moderately (2.47%) and (5.53%) respectively, while cardiovascular responses closely matched incraesed mean power (3.65%) and work (2.35%).

The increased endurance performance (time and distance achieved) following ingestion of rooibos beverage may be indicative of its ability to optimise metabolic and cardiovascuar processes that support the aerobic metabolism. The delayed voluntary withdrawal following rooibos ingestion from the submaximal exercise test regime could also suggest potential anti-fatigue effects (delay in fatigue onset) as a result of rooibos consumption. These results corroborate previous human and animal studies (Watanabe et al., 2014; Davies et al., 2019) that reported on the potential anti-fatigue and ergogenic potential of rooibos beverage/extract. In summary, based on the results from the current study, one can optimistically propose that fermented Rooibos beverage/product could have beneficial effects for athletes and other individuals involved in sport and fitness activities, by delaying fatigue onset, improving exercise performance and/or enhancing cardiorespiratory functions, espcially at lower exercise intensities.

Note: *Tentative analyses of the study participant cohort based on their sprint performance, yielded a clear division into two distinct sub groups, i.e. high sprint performers (HSP) whom achieved 6 or more sprints during the exercise and average sprint performers (ASP) whom were able to complete 4 or less sprints during the exercise regime. These sub groups were incorporated in phase III of the study, i.e. that of the metabolomic analyses.*

5.5 Phase III – Results

5.5.1 Study participants whole group

When comparing placebo vs Rooibos at different time points (0 h, IAE and 24 h post-exercise), no metabolites were found to differ significantly in concentration when either of the metabolomic analytical platforms (LC-MS and NMR) was used. Multivariate PCA score plots (Figure 5.12 a, b, c and d and Figure 5.13. a, b, c and d) show no clear separation with overlapping clustering, which indicates similarity in the analysed metabolic profile during the placebo and Rooibos session.

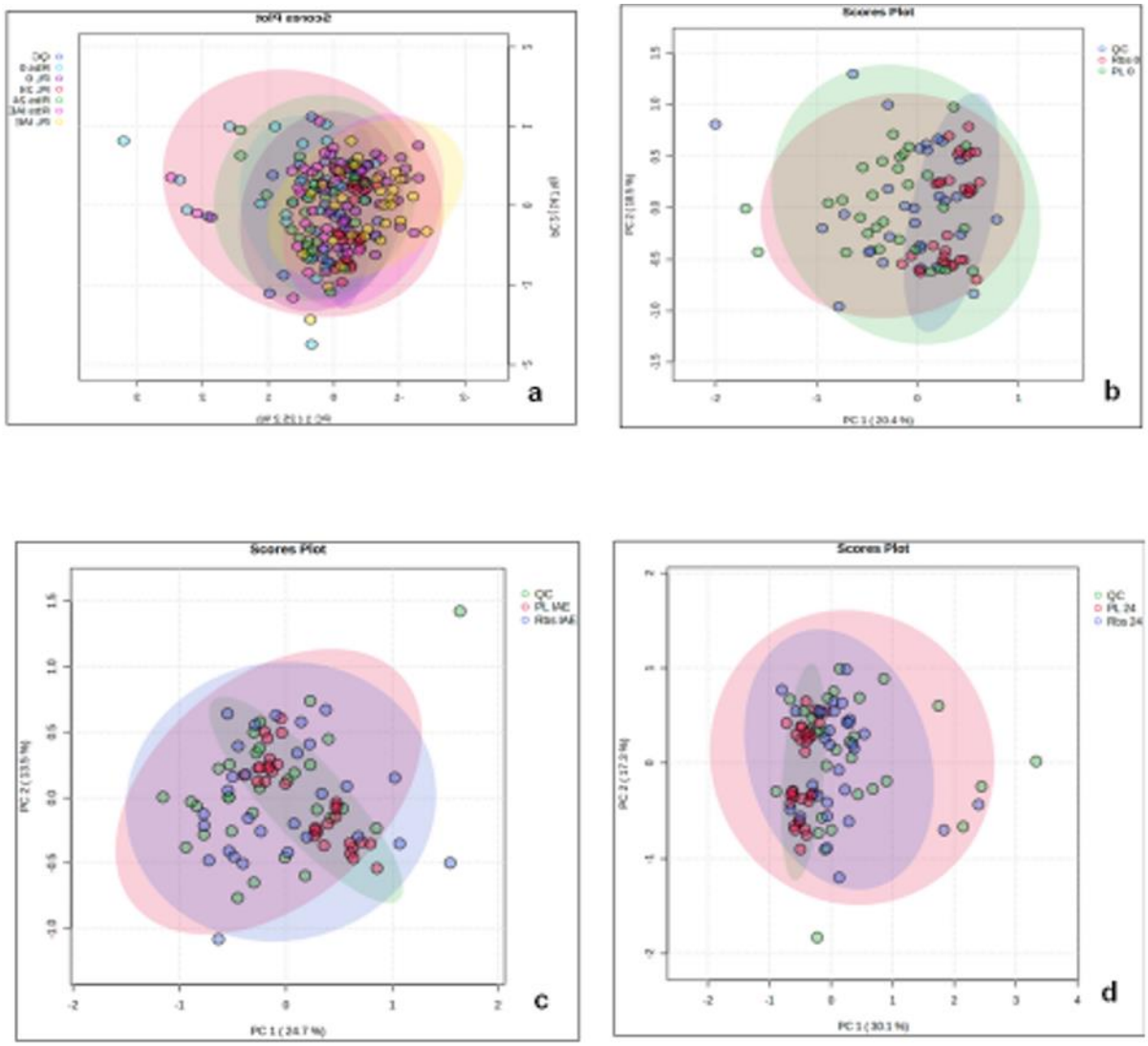
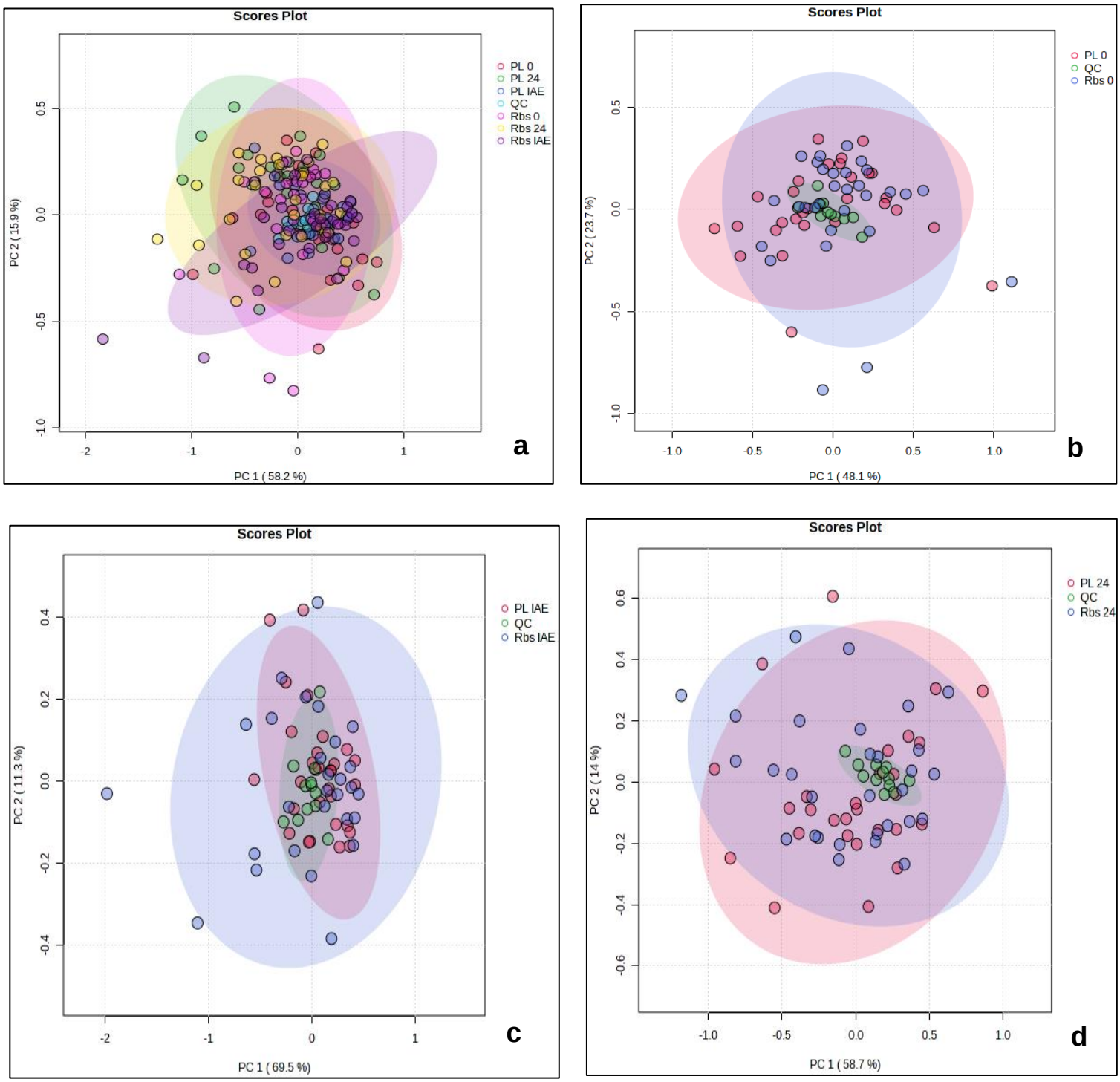


Figure 5.12: Multivariate analyses indicating clustering of metabolites from LC-MS analysed sample data of study participants (n = 30).

Principal component analysis (PCA) scores plots depict the clustering of placebo (n = 30, pink), Rooibos (n = 30, blue) and QC (n = 12, green) samples in **a**) all groups with all different time points, **b**) 0 h (baseline), **C**) IAE and **d**) 24 h post-exercise. Abbreviations: IAE = Immediately after exercise; LC-MS = Liquid chromatograph mass spectrophotometer; PL = Placebo; Rbs = Rooibos; QC = quality control.



. Figure 5.13: Multivariate analyses indicating clustering of metabolites from NMR analysed data of study participant (n = 30).

Principal component analysis (PCA) scores plots depict the clustering of placebo (n = 30, pink), Rooibos (n = 30, blue) and QC (n = 12, green) samples) in **a**: whole group all different time points, **b**: 0 h, **C**: IAE and **d**: 24 h. Abbreviations: IAE = Immediately after exercise; NMR = nuclear magnetic resonance; PL = Placebo; Rbs = Rooibos; QC = quality control.

5.5.2 Study participant subgroups

5.5.2.1 The average sprint performers (ASP) subgroup – immediately after exercise (IAE)

When comparing the ASP subgroup (ASP placebo vs ASP Rooibos) IAE data from the LC-MS analytical platform, only the tetradecanoyl carnitine (C14) metabolite was significantly (p 0.026 and q = 0.980) increased when Rooibos was consumed. However, when the NMR was used, no significant metabolite was identified. Multivariate analyses PCA score plots and hierarchical heatmap for LC-MS (Figure 5.14 a,b) and NMR analysed data (Figure 5.14 c,d) show overlapping clustering, which indicates similarity in ASP subgroup plasma metabolic profile at IAE when they consumed the placebo and Rooibos beverages. The univariate analyses (Figure 5.15) show the box plot of the significant metabolite.

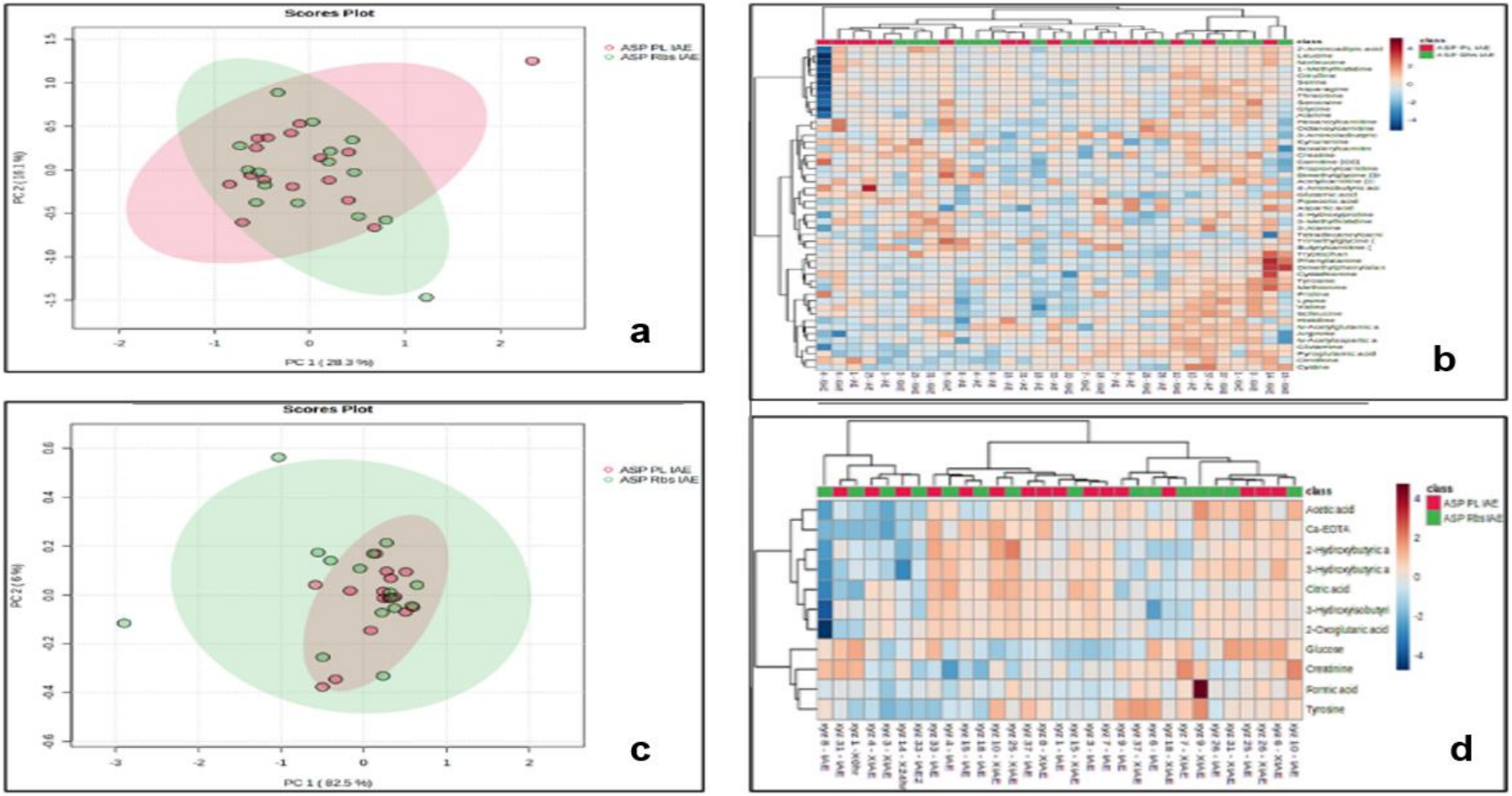


Figure 5.14: Multivariate analyses indicating clustering of the metabolites from LC-MS and NMR analysed samples of the ASP subgroup (n = 16)

The principal component analysis (PCA) scores plot and hierarchical heatmap depict the clustering of ASP PL IAE (n = 16, pink) vs ASP Rbs IAE (n = 16, green) samples after significant metabolite were selected ($p < 0.05$) in ASP study participants subgroup. Abbreviations: ASP = Average sprint performer; IAE = Immediately after exercise; LC-MS = Liquid chromatograph mass spectrophotometer; NMR = nuclear magnetic resonance; PL = Placebo; Rbs = Rooibos. The data was mean-centred scaled, and log transformed.

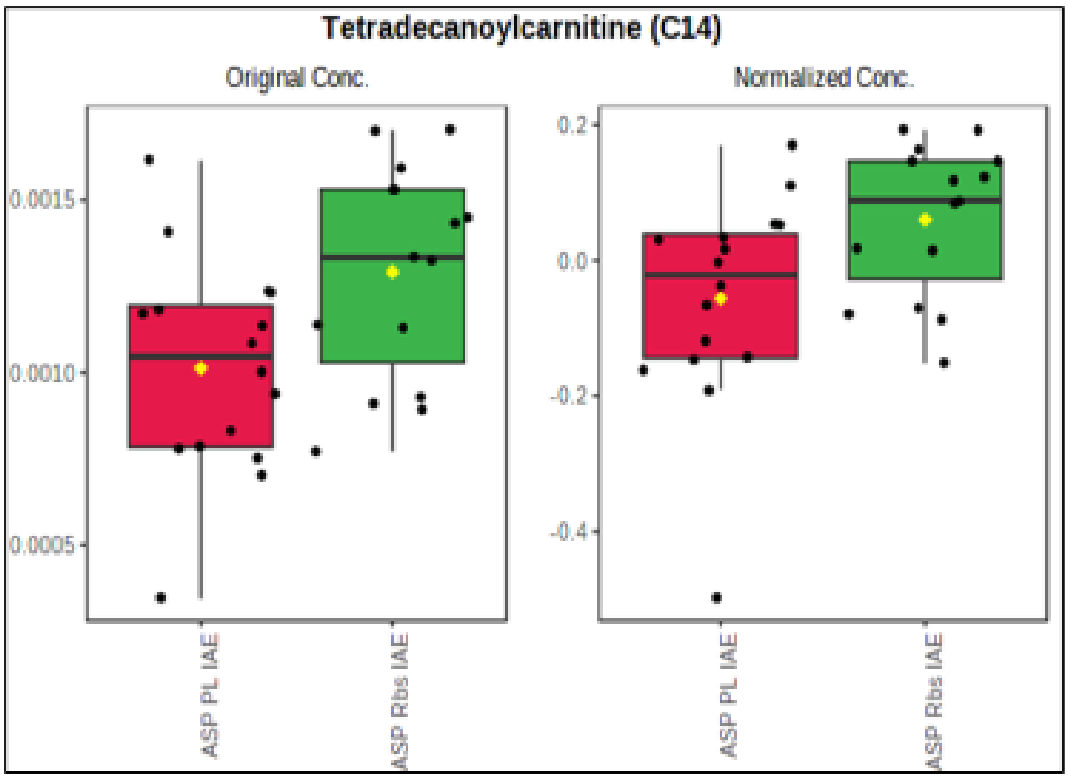


Figure 5.15: Univariate analyses showing the significantly changed plasma metabolite (tetradecanoylcarnitine) concentration in the average sprint performers (ASP, n = 16) subgroup at time point immediately after exercise (IAE) when comparing Rooibos (green) with placebo (red) intake

5.5.2.2 The average sprint performers (ASP) subgroup – 24 h post-exercise

When comparing the ASP subgroup data (ASP placebo vs ASP Rooibos) from the LC-MS analytical platform, only the acetylcarnitine (C2) metabolite was significantly ($p = 0.01729$, and $q = 0.70944$) increased 24 h post-exercise when Rooibos was consumed, while no metabolite was significantly changed and identified from the NMR generated data. Multivariate analyses PCA score plots and hierarchical heatmap for LC-MS (Figure 5.16 a,b) and NMR analysed data (Figure 5.16 c,d) show overlapping clustering, which is indicative of similarity in ASP subgroup plasma metabolic profile 24 h post-exercise when both the placebo and Rooibos beverages were consumed. The univariate analyses (Figure 17) show the box plot of the significant metabolite.

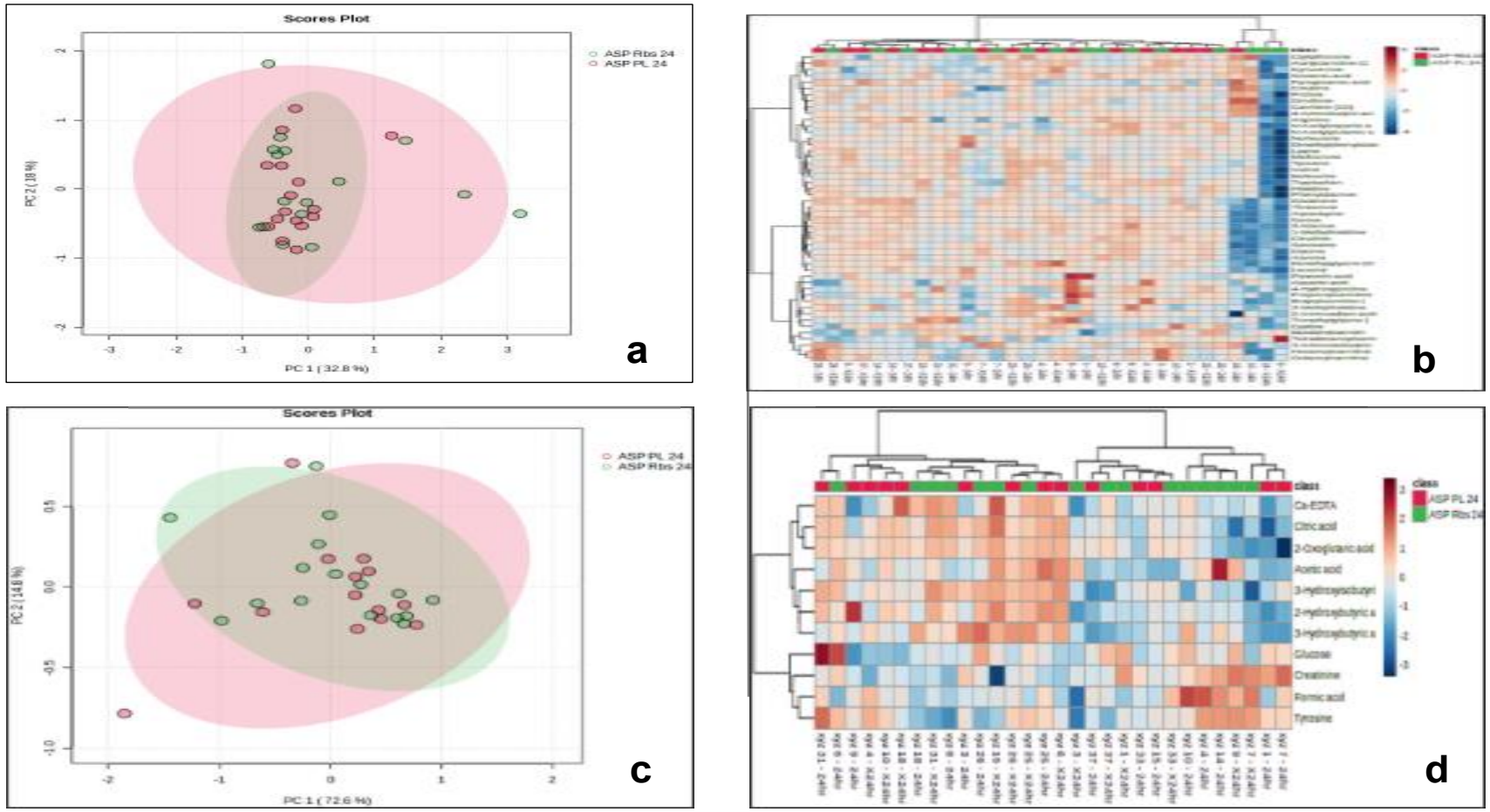


Figure 5.16: Multivariate analyses indicating clustering of the metabolites from LC-MS and NMR analysed sample of the ASP subgroup (n = 16).

The principal component analysis (PCA) scores plot and hierarchical heatmap depict the clustering of ASP PL 24 h (n = 16, pink) vs ASP Rbs 24 h (n = 16, green), samples after significant metabolite were selected ($p < 0.05$) in ASP study participants subgroup. Abbreviations: ASP = Average sprint performer; IAE = Immediately after exercise; LC-MS = Liquid chromatograph mass spectrophotometer; NMR = nuclear magnetic resonance; PL = Placebo; Rbs = Rooibos. The data was mean-centred scaled, and log transformed.

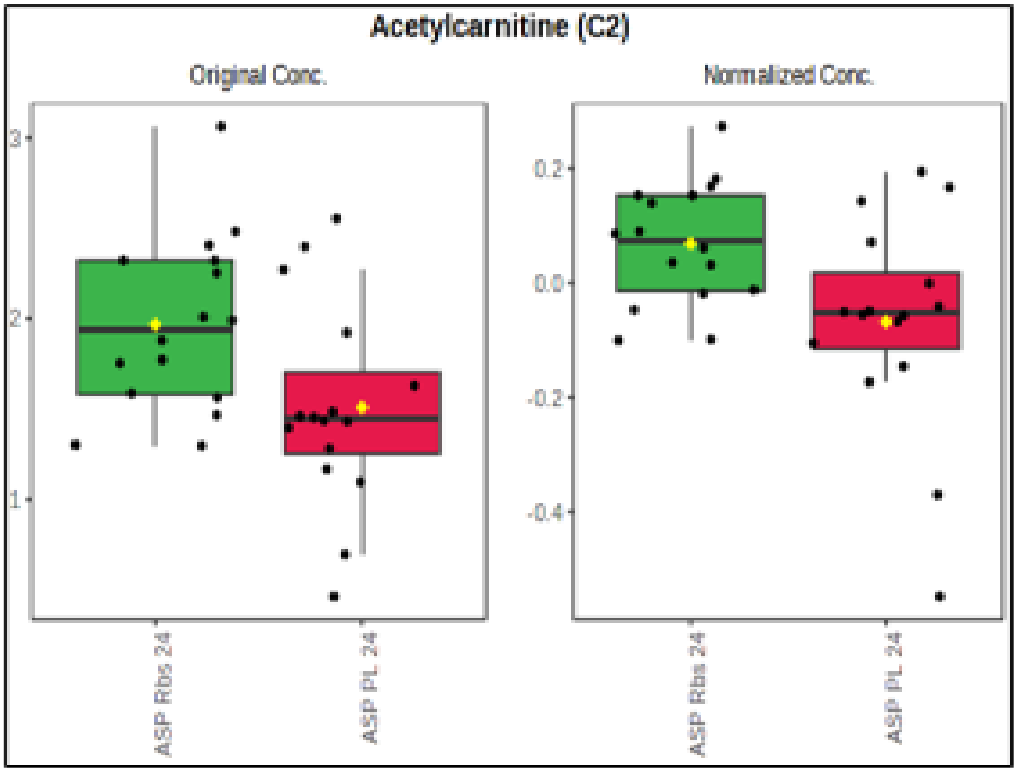


Figure 5.17: Univariate analyses showing the significantly changed plasma metabolite (acetylcarnitine) concentration in the average sprint performers (ASP, n = 16) subgroup at time point immediately after exercise (IAE) when comparing Rooibos (green) with placebo (red) intake

5.5.2.3 The high sprint performers (HSP) subgroup immediately after exercise (IAE)

When the IAE data from the LC-MS analytical platform for the HSP subgroup compared (HSP placebo vs HSP Rooibos), the aspartic acid metabolite was significantly ($p = 0.039311$, and $q = 0.95728$) decreased when Rooibos was consumed compared to placebo. Similar to the ASP subgroup, no significant metabolites were identified in the HSP subgroup either when the NMR platform generated data were analysed. Multivariate analyses PCA score plots and hierarchical heatmap for LC-MS (Figure 5.18 a,b) and NMR analysed data (Figure 5.18 c,d) show overlapping clustering, which indicates similarity in HSP subgroup plasma metabolic profile IAE when they consumed placebo and Rooibos intervention beverages. The univariate analyses (Figure 19) show the box plot of the significant metabolite.

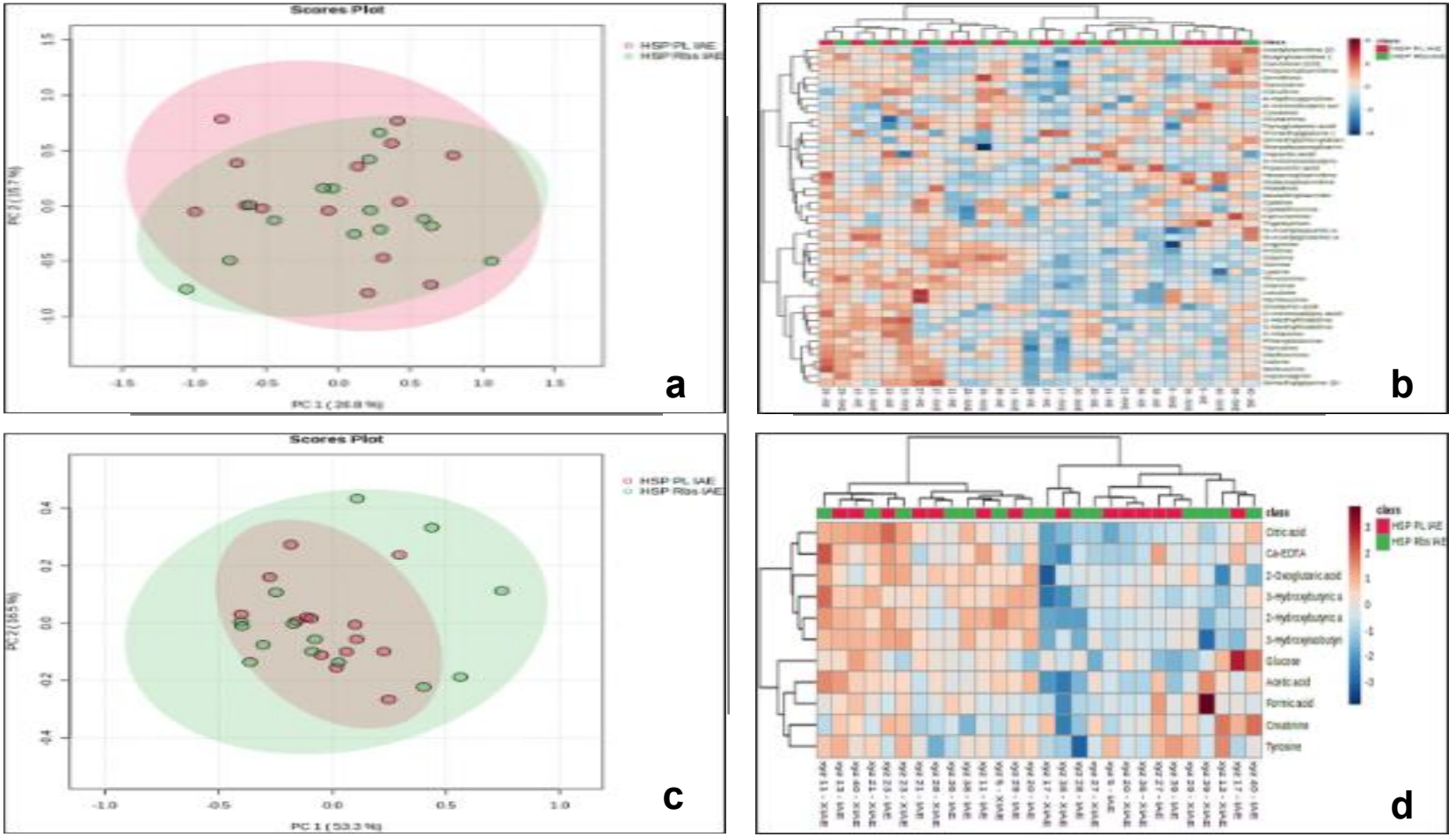


Figure 5.18: Multivariate analyses indicating clustering of the metabolites from LC-MS and NMR analysed samples of the HSP subgroup (n = 14)

The principal component analysis (PCA) scores plot and hierarchical heatmap depict the clustering of HSP PL IAE (n = 16, pink) vs HSP Rbs IAE (n = 14, green) samples after significant metabolite were selected ($p < 0.05$) in ASP study participants subgroup. Abbreviations: ASP = Average sprint performer; IAE = Immediately after exercise; LC-MS = Liquid chromatograph mass spectrophotometer; NMR = nuclear magnetic resonance; PL = Placebo; Rbs = Rooibos. The data was mean-centred scaled, and log transformed.

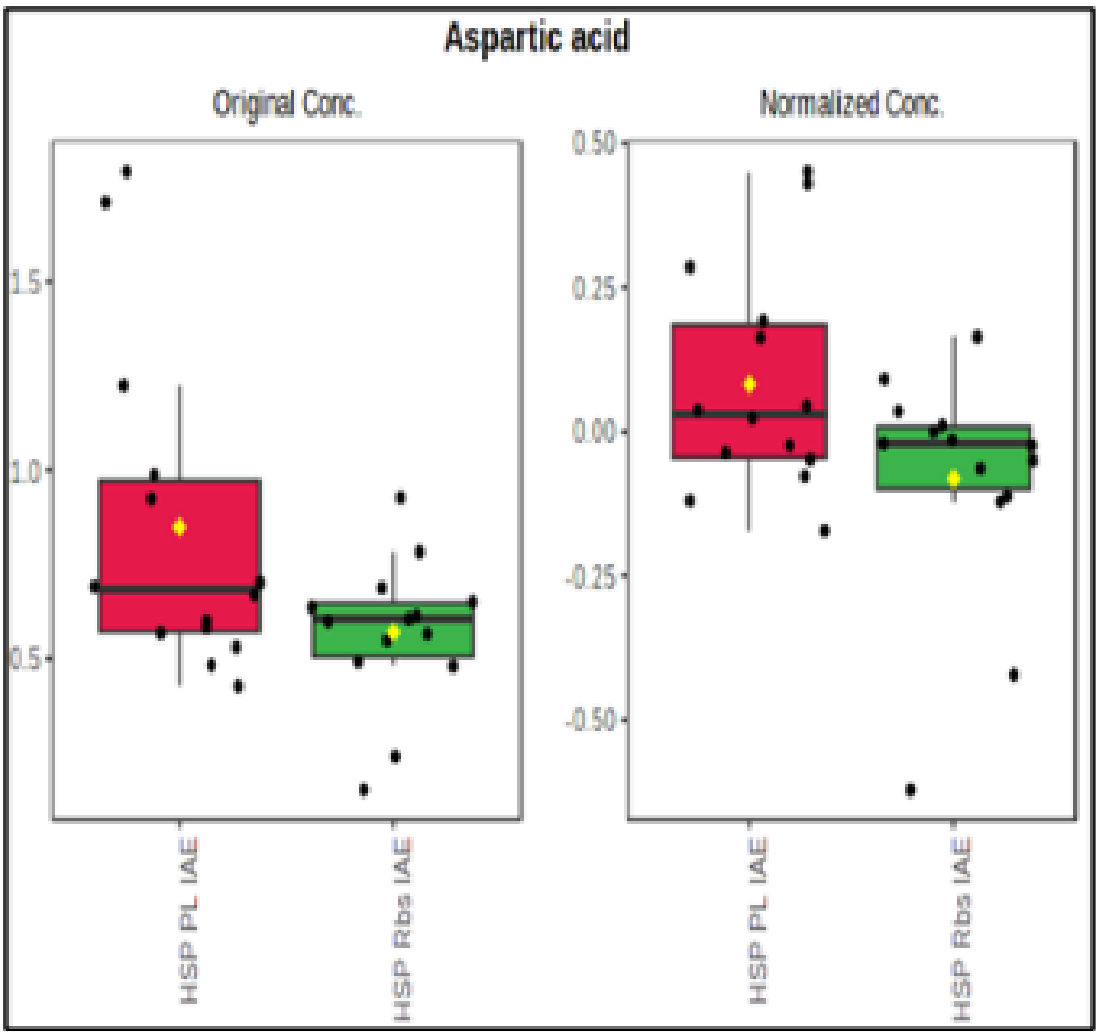


Figure 5.19: Univariate analyses showing the significantly changed plasma metabolite (aspartic acid) concentration in the high sprint performers (HSP,n = 14) subgroup at time point immediately after exercise (IAE) when comparing placebo (red) with rooibos (green) intake

5.5.2.4 The high sprint performers (HSP) subgroup - 24 hours post-exercise

From the LC-MS data, when compared the HSP subgroup (HSP placebo vs HSP Rooibos), pipecolic acid metabolite was significantly ($p = 0.04$, and $q = 0.92362$) increased, 24 h post-exercise when Rooibos was consumed, while no metabolite(s) differed significantly when the NMR data was analysed. Multivariate analyses PCA score plots and hierarchical heatmap for LC-MS (Figure 5.20 a,b) and NMR analysed data (Figure 5.20 c,d) showing overlapping clustering, which indicates a similarity in plasma metabolic profile of the HSP subgroup, 24 h post-exercise when they consumed either of the intervention beverages. The univariate analyses (Figure 21) show the box plot of the significant metabolite.

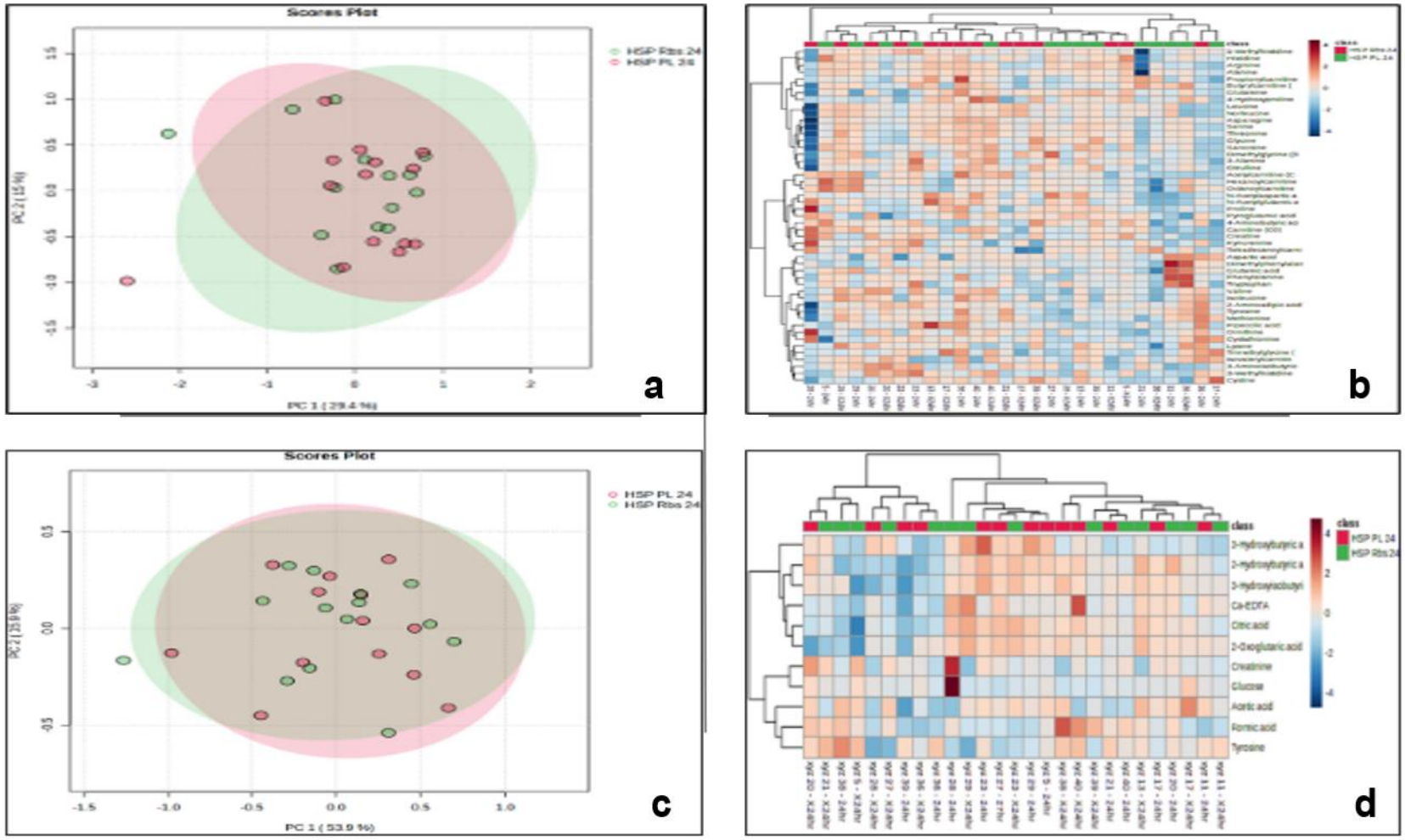


Figure 5.20: Multivariate analyses indicating clustering of the metabolites from LC-MS and NMR analysed samples of the HSP (n = 14) subgroup

The principal component analysis (PCA) scores plot and hierarchical heatmap depict the clustering of HSP PL 24 h (n = 14, pink) vs HSP Rbs 24 h (n = 14, green), samples after significant metabolite were selected ($p < 0.05$) in ASP study participants subgroup. Abbreviations: ASP = Average sprint performer; IAE = Immediately after exercise; LC-MS = Liquid chromatograph mass spectrophotometer; NMR = nuclear magnetic resonance; PL = Placebo; Rbs = Rooibos. The data was mean-centred scaled, and log transformed.

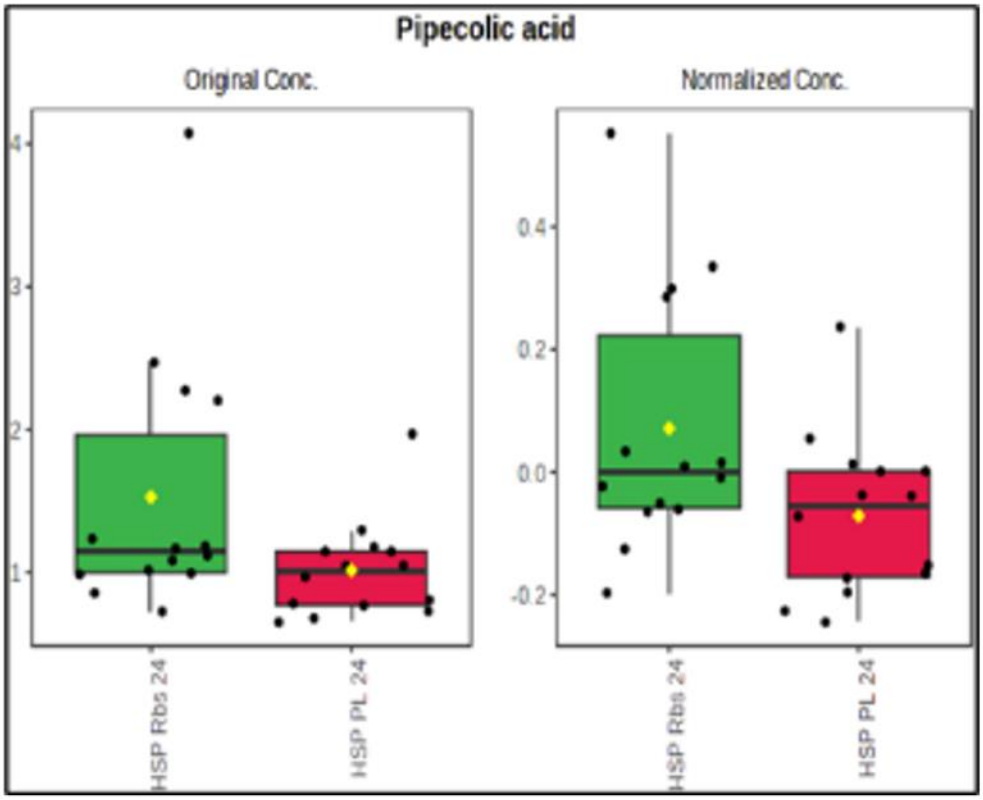


Figure 5.21: Univariate analyses showing the significantly changed plasma metabolite (pipecolic acid) concentration in the high sprint performers (HSP,n = 14) subgroup at time point immediately after exercise (IAE) when comparing placebo (red) with rooibos (green) intake

5.6 Phase III – Discussion

Metabolomics is a field of "omics" science which emerged in the late 1990s, but the use of NMR, GC, and MS techniques to analyse metabolites in biological samples dates from the early 1970s, while the LC-MS was only introduced later (Dettmer et al., 2007; Duarte et al., 2014; Cui et al., 2018; Khoramipour et al., 2022). Metabolites are end products of complex interactions occurring inside the cell and the environment and offer an ideal route to measure and understand both phenotype and physiology. In the recent past, the metabolomic analysis approach to measure metabolites has increasingly gained the attraction of many researchers in life sciences and exercise physiology fields due to its ability to simultaneously probe an individual's phenotype and physiology (Khoramipour et al., 2022). According to Khoramipour et al. (2022), exercise metabolomics research falls into five major categories: (**1**) exercise nutrition metabolism - research that seeks to examine the effect of any supplement or special diet on exercise metabolism; (**2**) exercise metabolism - research that seeks to examine metabolic responses/adaptations to a particular exercise protocol; (**3**) sport metabolism - research that seeks to examine metabolic responses to a particular sport or a specific exercise test; (**4**) clinical exercise metabolism - research that seek to examine the effect of exercise on

patients' metabolism; and (**5**) metabolome comparisons - research that seeks to compare athletes/patients/animals' metabolome. Based on these categories, this present study which aimed to assess the influence of both exercise and Rooibos beverage consumption on study participants' plasma metabolic changes/ metabolites shift resulting from a submaximal exercise regime, could fall under both categories 1 and 4.

In this study, when comparing the influence of both Rooibos and exercise on participant serum metabolites profiles as placebo vs Rooibos at different time points (0 h, IAE and 24 h post-exercise), no metabolites differed significantly between the groups when either of the metabolomic analytical platforms (LC-MS and NMR) was used. However, when data were further analysed, separating the study group into two subgroups of average sprint performers (ASP) and high sprint performers (HSP) and comparing them (i.e., ASP placebo vs ASP Rooibos and HSP placebo vs HSP Rooibos), a few significantly changed metabolites were observed. From the 60 metabolites identified and quantified (11 by NMR and 49 by LC-MS), only 4 were significantly altered/changed in the study participants' plasma metabolite levels. Furthermore, the four significantly changed metabolites were all identified from the LC-MS generated data. No significant metabolite(s) were identified when the NMR platform was used and this could be attributed to the NMR's low detection sensitivity, unlike the LC-MS which is considered to have high sensitivity and robust analytical technologies (Halama et al., 2021).

When comparing the LC-MS data for the ASP subgroup (ASP placebo vs ASP Rooibos) tetradecanoylcarnitine metabolite significantly increased IAE, while the acetylcarnitine was significantly increased 24 h post-exercise when Rooibos was consumed. Acetylcarnitine is also known as C2-carnitine and is the smallest short-chain acylcarnitine found in plasma and plays an important role in energy production. Furthermore, acetylcarnitine also provide acetyl groups for the synthesis of acetylcholine – a neurotransmitter that plays a crucial role in brain functioning (memory) and muscle contraction or movements functioning (Onofrj et al., 2013) The tetradecanoylcarnitine metabolite is also known as C14-carnitine and is one of the long-chain acylcarnitine metabolites which are generated as a product of L-carnitine esterification with long-chain fatty acids that are obtained from the diet or generated *de novo* from lipogenesis (Dambrova et al., 2022). Both C2 and C14 are acylcarnitine metabolites, which are bound to carnitine and are synthesised during fatty acid metabolism and also from degradation products of several amino acids such as lysine, valine, leucine, and isoleucine. Acylcarnitines metabolite's main biological function is to transport acyl groups from the cytosol into the mitochondrial matrix for β-oxidation resulting in energy production needed to sustain cell activity (Dambrova et al., 2022). Therefore, the significant increase of acylcarnitines

metabolites (C2- and C14-acylcarnintine) in this study participants could indicate Rooibos' influence on the ASP group plasma acylcarnitines metabolites level which may have subsequently led to more transportation of acyl groups β-oxidation in mitochondrial cytosol resulting to more energy production that enabled muscle contraction and sustaining endurance. Moreover, literature indicates that apart from being useful energy metabolism pathways metabolites, acylcarnitines are increasingly being identified as possible diagnostic and/or prognostic markers for metabolic disorders, cardiovascular diseases, diabetes, depression, and neurologic disorders (Onofrj et al., 2013; Dambrova et al., 2022).

When assessing the NMR data from the HSP group plasma samples, no metabolite(s) were identified that differ significantly between the two groups. However, a significant decrease in HSP participant plasma aspartic acid was observed IAE when Rooibos was consumed. Also, pipecolic acid was significantly increased 24 h post-exercise when Rooibos was consumed compared to placebo. Literature suggests that pipecolic acid metabolite is a product of lysine amino acid catabolism (Halama et al., 2021). Lysine amino acid belongs to the branch chain amino acid (BCAA) group which is known to play a crucial role in protein synthesis, improving cell proliferation and muscle recovery after exercise, as well as decreasing exercise-induced muscle damage (Atsumoto et al., 2009; Le Moyec et al., 2014; Sato et al., 2016). Furthermore, pipecolic acid has been found to stimulate protein synthesis rates and contribute to the reduction of muscle protein degradation (Sato et al., 2016) The significant increase of plasma pipecolic acid in the HSP group plasma in this study could be indicative of increased lysine amino acid catabolism to produce more pipecolic acid that would subsequently reduce protein degradation and exercise-induced muscle damage and improved muscle recovery time for the HSP group participants which lead to improved exercise performances when Rooibos was consumed as discussed in previous section (phase III). Additionally, this result point to Rooibos as a potential ergogenic aid/ nutraceutical supplement for athletes or fitness individuals especially those who participate in sports events that may not have enough time in between to allow full recovery. The results may also indicate possible metabolic shifts that may occur and underscore the positive effects of the Rooibos consumption. However, only a few metabolites were identified in this study that differed significantly ($p < 0.05$) between the placebo and Rooibos groups. When applying a false discovery rate (FDR) correction, all these metabolites lost significance ($q > 0.05$). This could be due to the small study sample size, especially when considering the subgroup sizes, i.e. ASP ($n= 16$) and the HSP ($n = 14$). Nonetheless, it could be speculated that Rooibos consumption in this study boasted study participants' metabolic pathways better than the placebo beverage. However, more research studies with a bigger sample size would be required to confirm these study findings in order

to reach and/or make a definite conclusion. It is also suggested that targeted metabolomics analyses be performed focussing only on the metabolites identified as perturbed in this study in order to quantify these metabolites better and more accurately. Lastly, these study results, apart from confirming the robustness and sensitivity of the LC-MS platform, also affirmed the importance of using more than one metabolomic analytical platform to avoid the possibility of missing some data that could be of significance to the study.

5.7 Phase IV – Results

5.7.1 Oxidative stress and muscle damage biomarkers

Plasma level of OS lipid and protein damage biomarkers as well serum biomarkers of muscle damages (Table 5.26) were assessed in study participants at baseline and 24 h post-exercise. No significant ($p > 0.05$) changes were noticed in any of the analysed blood biomarkers 24 h post-exercise when compared to baseline level (0 h).

Table 5.19: Blood biomarkers indicative of oxidative and muscle damage in study participant (n = 30) serum at baseline (0 h) and 24 h post-exercise

		Baseline	24 h post-exercise	P value
Oxidative stress damage biomarkers				
	TBARS **(nmol/ mL)**	10.37 ± 0.15	10.06 ± 0.16	1.000
	CDs **(nmol/ mL)**	13.98 ± 0.41	14.71 ± 0.37	0.452
	PC **(nmol/ mL)**	2.48 ± 0.12	2.14 ± 0.11	0.217
Muscle damage markers				
	CK (U/L)	287 ± 27	272 ± 23	1.000
	LDH (U/L)	172 ± 40	183 ± 54	0.508
	LAC (U/L)	3.20 ± 0.20	2.58 ± 0.18	0.074

Values in columns are expressed as mean ± standard error of the mean (SEM). Abbreviations: TBARS = Thiobarbituric acid reactive substance; CDs = Conjugated denies; PC = Protein carbonyl; CK = Creatinine kinase; LAC = Lactate; LDH = Lactate dehydrogenase.

5.7.2 Genetic background of study participants

The genetic background of different genetic variants analysed in the study participants, genotype distribution and allele frequencies are shown in Table 5.20. All the genes investigated in this study were selected based on previous published literature as the genes most associated with exercise-induced oxidative stress and muscle damage. In term of alleles and genotype distribution, MnSOD gene alleles (C and T) frequency in the study cohort was

0.43 and 0.57, with the heterozygous genotype (CT) mostly represented, found in 40% (n = 12), followed by the mutated homozygote genotype (TT) found in 36.7% (n = 11) and the wild-type genotype (CC) only found in 23.3% (n = 7). The TNF-α gene alleles (G and A) frequency in study participant was 0.80 and 0.20, with the wild-type genotype (GG) being largely represented 60% (n = 18), the heterozygote genotype (GA) found in 40% (12) while the mutated homozygote genotype (AA) was not found in any of the study participants. For the GDF5 gene alleles (C and T), only the C allele was represented 100% (n = 30), thus only the wild-type genotype (CC) was found in all study participants. The COL5A1 – rs1134170 variant allele T was more frequent than A allele (0.58 vs 0.42) with the heterozygote (AT) genotype found in half of the study participants 50% (n = 15) followed by the mutated homozygote genotype (TT) found in 33.3% (n = 10) and the wild-type genotype (AA) only found in 16.7% (n = 5). The MIR608 genes, G allele prevalence was higher in study participants than the C allele (0.57 vs 0.43) with the heterozygote (CG) genotype found in more than half of study participants 53.3% (n = 16), while the mutated homozygote genotype (GG) was present in 30% (n = 9) and the wild-type genotype (CC) was only present in 16.7% (n = 5). The CASP 8 - rs3834129 variant deletion (D) allele was present in more participants than the insertion (I) allele (0.55 vs 0.45) with the heterozygote genotype (I/D) being mostly represented in 63.4% (n = 19) followed by the mutated homozygote (D/D) genotype in 23.3% (n = 7) and the wild-type genotype only found in 13.3% (n = 4). The COL5A1 - rs16399 variant alleles (ATCT and D) frequency in study participant cohort was 0.42 vs 0.58, with the heterozygote genotype found in half of study participants with 50% (n = 15), followed by the wild-type genotype with 33.3% (n = 10) and then the mutated homozygote genotype with 16.7% (n = 5). Lastly, the COL5A1 - rs12722 gene variant C allele was present in most study participants than the T allele (0.82 vs 18), with the wild-type genotype (CC) highly represented in 66.7% (n = 20), followed by heterozygote genotype (CT) found in 30% (n = 9) and the mutated homozygote genotype (TT) was only found in 3.3% (n = 1) of the study participants.

Table 5.20: Relevant genetic variations and genotype distributions in study participant cohort

Symbol	Gene	Polymorphism/ SNP	dbSNP	Genotype and allele	Genotype and allele distribution/ frequency (%)
MnSOD	Mitochondrial superoxide dismutase	C > T	rs4880	CC CT TT C allele T allele	7 (2.3) 12 (40) 11 (36.7) 26 (43.3) 34 (56.7)
TNF- α	Tumour necrosis factor- Alpha	G>A	rs180029	GG GA AA G allele A allele	18 (60) 12 (40) 0 (0) 48 (80) 12 (20)
GDF5	Growth Differentiation Factor 5	T>C	rs143383	CC CT TT C allele T allele	30 (100) 0 (0) 0 (0) 60 (100) 0 (0)
COL5A1	Collagen 5 Alpha 1	ATCT>del	rs16399	ATCT/ATCT ATCT/D D/D ATCT allele D allele	10 (33.3) 15 (50) 5 (16.7) 25 (41.7) 35 (58.3)
COL5A1	Collagen 5 Alpha 1	A>T	rs1134170	AA AT TT A allele	5 (16.7) 15 (50) 10 (33.3) 25 (41.7)

				T allele	35 (58.3)
MIR608		C>G	rs4919510	CC CG GG C allele G allele	5 (16.7) 16 (53.3) 9 (30) 26 (43.3) 34 (64.7)
CASP8	Caspase 8	CTTACT>del	rs3834129	I/I I/D D/D I allele D allele	4 (13.3) 19 (63.4) 7 (23.3) 27 (45) 33 (55)
COL5A1		C >T	rs12722	CC CT TT C allele T allele	20 (66.7) 9 (30) 1 (3.3) 49 (81.7) 11 (18.3)

5.7.3 Genetic background and oxidative stress biomarkers

The genetic variants' influence on the blood oxidative stress damage biomarkers of lipids and protein are shown in Table 5.21. Result show that for the MnSOD- rs4880 SNP, study participants with the mutated homozygote (TT) genotype had increased plasma TBARS and CDs levels at baseline (0 h) and 24 h post-exercise when compared to those participants with the heterozygote (CT) and wild-type (CC) genotypes. Also, participants with the wild-type genotype had a higher plasma PC level at baseline (0 h) and 24 h post-exercise than those with the heterozygote and mutated genotypes. For the **TNF-α - rs180029** SNP, participants with the wild-type genotype (GG) had increased plasma CD and PC levels at baseline (0 h) and 24 h post-exercise when compared to those with the heterozygote (GA) genotype. However, those with the heterozygote genotype also had increased plasma TBARS levels at baseline and 24 h post-exercise than those with the wild-type genotype. For the **GDF5-rs143383** SNP, all study participants had the wild-type genotype (CC), and all showed higher plasma TBARS, CDs and PC levels at 24 h post-exercise than at baseline (0 h). Results also show that for the **COL5A1-rs1134170** SNP, participants with the mutated (TT) genotype had a higher increase in plasma TBARS and CDs level, 24 h post-exercise than those with heterozygote (AT) and wild-type (AA) genotype. For the **MIR608-rs4919510** SNP, study participants with the mutated homozygote (GG) and wild-type (CC) genotypes had increased plasma CDs and PC levels at 24 h post-exercise than those with heterozygote (CG) genotype when compared to their baseline (0 h) levels. Results on the **CASP8-rs3834129** SNP, show an increase in plasma levels for both lipid and protein OS damage biomarkers (TBARS, CDs and PC), 24 h post-exercise in participant with mutated homozygote (D/D) genotype when compared to those with the heterozygote (I/D) and wild-type (I/I) genotypes. For the **COL5A1-rs12722** SNP, study participants with the mutated homozygote genotype (TT) had a higher increase in plasma TBARS and CDs level at 24 h post-exercise than those with the heterozygote (CT) and wild-type (CC) genotypes when compared to their baseline (0 h) levels.

Table 5.21: Association between study participants' genetic background and selected blood oxidative stress damage biomarkers

Gene/genotype	Genotype and allele frequency (%)	TBARS		CD		PC	
MnSOD_r*s4880*		**Baseline**	**24 hr post-exercise**	**Baseline**	**24 hr post-exercise**	**Baseline**	**24 hr post -exercise**
CC	7 (2.3)	10.24 (1.48)	10.15 (1.09)	15.67 (2.38)	14.83 (3.56)	2.68 (0.64)	3.21 (1.07)
CT	12 (40)	10.48 (1.37)	10.39 (1.32)	14.78 (4.59)	14.45 (4.06)	2.43 (0.79)	2.49 (0.73)
TT	11 (36.7)	10.84 (1.10)	10.95 (0.87)	15.94 (2.63)	16.91 (3.07)	2.16 (0.60)	2.02 (0.90)
C allele	26 (43.3)						
T allele	34 (56.7)						
TNF-a_*rs180029*							
GG	18 (60)	10.51 (1.30)	10.40 (1.28)	15.75 (3.62)	15.50 (3.46)	2.48 (0.71)	2.73 (0.97)
GA	12 (40)	10.64 (1.30)	11.15 (0.61)	14.90 (3.23)	15.35 (4.12)	2.26 (0.69)	2.11 (0.85)
AA	0 (0)	/	/	/	/	/	/
G allele	48 (80)						
A allele	12 (20)						
GDF5_*rs143383*							
CC	30 (100)	10.56 (1.28)	10.70 (1.11)	15.41 (3.44)	15.44 (3.67)	2.39 (0.70)	2.49 (0.96)
CT	0 (0)	/	/	/	/	/	/
TT	0 (0)	/	/	/	/	/	/
C allele	60 (100)						
T allele	0 (0)						
COL5A1_*rs1134170*							
AA	5 (16.7)	10.25 (0.98)	10.60 (0.75)	17.22 (3.82)	16.78 (2.20)	2.16 (0.40)	2.07 (0.88)
AT	15 (50)	10.73 (1.24)	10.46 (1.16)	15.53 (3.63)	15.47 (4.22)	2.33 (0.57)	2.61 (1.15)
TT	10 (33.3)	10.45 (1.50)	11.11 (1.14)	14.32 (2.81)	14.73 (3.44)	2.59 (0.95)	2.51 (0.68)
A allele	25 (41.7)						

T allele	35 (58.3)						
MIR608_rs*4919510*							
CC	5 (16.7)	10,9423	10,8964	16,5662	17,406	1,93561	1,96409
CG	16 (53.3)	10,4057	11,1797	15,443	14,953	2,57637	2,44891
GG	9 (30)	10,619	9,74576	14,7089	15,2112	2,31086	2,84141
C allele	26 (43.3)						
G allele	34 (56.7)						
CASP8_*rs3834129*							
I/I	4 (13.3)	10,1991	11,0491	16,2811	15,1718	2,56553	2,12027
I/D	19 (63.4)	10,5595	10,5775	15,6987	15,6763	2,3951	2,59055
D/D	7 (23.3)	10,7638	10,8428	14,1286	14,9488	2,27554	2,41061
I allele	27 (45)						
D allele	33 (55)						
COL5A1_*rs16399*							
ATCT/ATCT	10 (33.3)	10,45	11,11	14,32	14,73	2,59	2,51
ATCT/D	15 (50)	10,73	10,46	15,53	15,47	2,33	2,61
D/D	5 (16.7)	10,25	10,60	17,22	16,78	2,16	2,07
ATCT allele	25 (41.7)						
D allele	35 (58.3						
COL5A1 _*rs12722*							
CC	20 (66.7)	10,4368	10,5098	15,6837	15,5669	2,39689	2,52341
CT	9 (30)	10,9602	11,1384	14,9436	14,7741	2,34571	2,43973
TT	1 (3.3)	9,39667	10,6267	14,1343	18,8726	2,64848	2,15
C allele	49 (81.7)						
T allele	11 (18.3)						

Abbreviations: TBARS = Thiobarbituric acid reactive substance; CDs = Conjugated denies; PC = Protein carbonyl; MnSOD = Mitochondrial superoxide dismutase; TNF α-Tumour necrosis factor- Alpha; GDF-5 = Growth Differentiation Factor 5; COL5A1 = Collagen 5 Alpha 1; CASP 8 = Caspase 8

5.7.4 Genetic background and muscle damage markers

The influence of genetic variation/ SNPs on the extent of muscle damage and fatigue in the study participant is presented in Table 5.22. When considering the **MnSOD - rs4880** SNP, study participants with the mutated homozygote (TT) genotype had a higher increase in serum CK and decreased serum LDH and lactate levels 24 h post-exercise when compared to those with the heterozygote (CT) and wild-type (CC) genotypes. For the **TNF-α - rs180029** SNP, those with the heterozygote (GA) genotype had a higher decrease in serum CK levels than those with the wild-type (GG) genotype. Those who possess the wild-type genotype also had higher increased serum LDH levels, however their serum lactate levels were lower than those with the heterozygote genotype 24 h post-exercise when compared to their baseline (0 h) levels. For the **GDF5-rs143383** SNP, all study participants had the wild-type genotype (CC), and their serum CK and lactate levels were decreased 24 h post-exercise when compared to their 0 h levels, while the LDH level increased. For the **COL5A1_rs1134170** SNP, all study participant serum CK levels decreased 24 h post-exercise but the decrease was more pronounced in those with the wild-type genotype (AA), followed by those with the homozygote (TT) and then by those with the heterozygote (AT) genotype. Also, those with the wild-type genotype had decreased serum LDH levels, while those with the heterozygote and mutated genotypes had increased serum LDH levels with a higher increase in those with the heterozygote genotype 24 h post-exercise when compared to the baseline level. Serum lactate levels also drastically decreased in participants with the heterozygote and wild-type genotypes, but increased in those with the mutated genotype 24 h post-exercise when compared to the baseline level. For the **MIR608_rs4919510** SNP, when compared to baseline serum levels, results showed that all participants had decreased CK levels 24 h post-exercise, but with more pronounced decreases in those with the wild-type genotype (CC) and least pronounced decreases in those with the mutated homozygote (GG) genotype. For the serum LDH levels, those with the mutated genotype showed a higher increase and those with the heterozygote (CG) genotype, the least increase 24 h post-exercise. Serum lactate levels increased in those with the wild-type genotype 24 h post-exercise compared to baseline, while decreased in those with the heterozygote and mutated homozygote genotypes with a more pronounced decrease in those with the mutated homozygote genotype. Similar to the MIR608_rs4919510 SNP, when considering the **CASP8-rs3834129** SNP, serum CK and lactate levels decreased in all participants 24 h post-exercise but the decrease was more pronounced in those with the wild-type (I/I) genotype, and least pronounced in those with the heterozygote (I/D) genotype. The LDH only decreased in those with wild-type genotype, while increased in those with the heterozygote and mutated homozygote (I/D and D/D) genotypes, with higher increases in those with the mutated genotype. Results on the **COL5A1-rs16399** SNP, show that all participant serum CK levels decreased 24 h post-exercise but the decrease

was more pronounced in those with the mutated homozygote (D/D) genotype, and least in those with heterozygote (ATCT/D) genotype. Study participants with heterozygote and wild-type (ATCT/ATCT) genotypes also had increased serum LDH levels with a higher increase in those with the heterozygote genotype, while those with the mutated homozygote (D/D) genotype showed decreased serum LDH levels 24 h post-exercise when compared to the baseline level. Serum lactate levels decreased in participants with the heterozygote and mutated homozygote genotypes, while increased in those with the wild-type genotype 24 h post-exercise when compared to the baseline level. Lastly, for the **COL5A1- rs12722** SNP, results showed that participants with the wild-type (CC) and heterozygote (CT) genotype had decreased CK serum levels with more pronounced decreased levels in those with the wild-type, while those with the mutated (TT) genotype had a high increase in serum CK levels 24 h post-exercise when compared to the baseline level. Results also indicate that all participants had increased serum LDH levels, with more pronounced increases in those with the mutated homozygote genotype and least in those with the heterozygote genotype. Participant serum lactate levels decreased in those with wild-type and mutated homozygote genotype, with more pronounced decreases in those with the wild-type genotype and least in those with the mutated genotype, but it increased in those with the heterozygote genotype 24 h post-exercise when compared to the baseline level.

Table 5.22: Association between participants' genetic background and selected muscle damage markers

Gene/genotype	Genotype distribution and allele frequency (%)	CK		LDH		LAC	
MnSOD_rs*4880*		**Baseline**	**24 hr post-exercise**	**Baseline**	**24 hr post-exercise**	**Baseline**	**24 hr post-exercise**
CC	7 (2.3)	376.43 (238.80)	329.52 (152.06)	185.62 (30.62)	188.33 (39.32)	2.57 (1.48)	2.37 (0.71)
CT	12 (40)	556.98 (661.42)	547.23 (595.42)	187.04 (53.40)	209.42 (56.88)	2.65 (1.03)	2.50 (0.87)
TT	11 (36.7)	420.71 (413.76)	544.33 (633.27)	182.01 (53.57)	178.67 (41.06)	3.21 (1.71)	2.42 (1.32)
C allele	26 (43.3)						
T allele	34 (56.7)						
TNF-a_*rs180029*							
GG	18 (60)	539.09 (544.69)	503.19 (489.51)	193.26 (50.52)	203.85 (56.81)	2.85 (1.57)	2.37 (0.79)
GA	12 (40)	466.89 (621.42)	370.31 (401.62)	172.28 (41.86)	177.28 (25.98)	2.82 (1.16)	2.54 (1.29)
AA	0 (0)	/	/	/	/	/	/
G allele	48 (80)						
A allele	12 (20)						
GDF5_*rs143383*							
CC	30 (100)	510.21 (567.18)	450..04 (453.91)	184.87 (47.65)	193.22 (48.20)	2.84 (1.39)	2.44 (1.00)
CT	0 (0)	/	/	/	/	/	/
TT	0 (0)	/	/	/	/	/	/
C allele	60 (100)						
T allele	0 (0)						
COL5A1_*rs1134170*							
AA	5 (16.7)	711.96 (882.29)	575.42 (537.29)	209.11 (60.65)	205.00 (44.57)	3.86 (2.19)	2.31 (0.64)
AT	15 (50)	412.03 (557.16)	378.70 (415.23)	181.97 (45.82)	197.13 (43.21)	3.36 (3.37)	2.16 (0.69)
TT	10 (33.3)	556.60 (408.21)	494.35 (497.97)	177.09 (44.80)	181.48 (58.68)	2.62 (1.22)	2.92 (1.38)
A allele	25 (41.7)						

T allele	35 (58.3)						
MIR608_rs*4919510*							
CC	5 (16.7)	570,419	473,49	186,361	199,747	2,788	3,062
CG	16 (53.3)	394,896	328,303	171,314	169,922	2,77813	2,295
GG	9 (30)	681,765	653,429	208,13	231,026	2,97	2,34444
C allele	26 (43.3)						
G allele	34 (64.7)						
CASP8_*rs3834129*							
I/I	4 (13.3)	594,513	437,033	204,443	185,619	2,91	2,29
I/D	19 (63.4)	422,226	377,208	175,779	186,896	2,86684	2,49105
D/D	7 (23.3)	700,855	655,153	198,345	214,748	2,71571	2,37714
I allele	27 (45)						
D allele	33 (55)						
COL5A1_*rs16399*							
ATCT/ATCT	10 (33.3)	556,60	494,35	177,09	181,48	2,62	2,92
ATCT/D	15 (50)	412,03	378,70	181,97	197,13	2,65	2,16
D/D	5 (16.7)	711,96	575,42	209,11	205,00	3,86	2,31
ATCT allele	25 (41.7)						
D allele	35 (58.3						
COL5A1 (rs12722							
CC	20 (66.7)	621,892	550,87	192,365	203,89	3,135	2,388
CT	9 (30)	303,591	256,609	173,693	174,168	2,05889	2,40778
TT	1 (3.3)	136,155	174,284	135,45	151,41	3,89	3,7
C allele	49 (81.7)						
T allele	11 (18.3)						

Abbreviations: TBARS = CK = Creatinine kinase; LAC = Lactate; LDH = Lactate dehydrogenase; MnSOD = Mitochondrial superoxide dismutase; TNF α- Tumour necrosis factor- Alpha; GDF-5 = Growth Differentiation Factor 5; COL5A1 = Collagen 5 Alpha 1; CASP 8 = Caspase 8.

5.8 Phase IV – Discussion

To date, it is still unknown why some individuals suffer and/or experience a higher magnitude of muscle damage than other, despite participating in the same exercise or physical activity. This inter-individual variation in response to exercise-induced muscle damage is attributed to genetic variability which apparently play a key role in this phenomenon (Del Coso et al., 2020; Baumert et al., 2022). Genetic variants are known to influence individuals' responses to exercise, however, a variety of environmental, psychological, lifestyle and epigenetic factors may also influence exercise responses (Harvey et al., 2020).

The term pathology support genetic testing (PSGT) was coined by Kotze and Van Rensburg (2012) and this approach has been used previously to develop risk reduction strategies for breast cancer (Okunola et al., 2023) and multiple sclerosis (van Rensburg et al., 2021; Johannes et al., 2023). In the main, this concept is aimed at transitioning the risk stratification of the population (existing knowledge on the selected SNPs and their environmental triggers to cause biochemical abnormalities due to gene-gene and gene-environment interaction) to the individual. This concept was now explored for the first time in the current study but within an exercise-induced oxidative stress environment. In the present study, analysed genes were selected based on current literature as the most common genes associated with exercise-induced oxidative stress and muscle damage. This study utilised the PGST to evaluate the possible genotype-phenotype relationship, by analysing some selected SNPs and exercise-induced oxidative stress and muscle damage biomarkers. The study further tries to understand the study participants' likelihood or susceptibility to exercise injuries by analysing known genetic variants/SNPs associated with skeletal muscle injury in collected blood samples.

Superoxide dismutase (SOD) is an endogenous antioxidant enzyme encoded by the nuclear SOD2 gene and it protects cells and mitochondria from free radical and other RONS damage by converting the anion superoxide into hydrogen peroxide (Pourvali et al., 2016). But the MnSOD-rs4880**,** Ala16Val C>T SNP causes conformational change in the target sequence of MnSOD which affects MnSOD activity in mitochondria and/or deficiency in SOD production (Synowiec et al., 2021). This causes the accumulation of superoxide radicals and other RONS leading to increased damage of mitochondrial membranes and cell apoptosis. The T allele of this SNP has been also associated with muscle damage susceptibility (Akimoto et al., 2010). The present study observed a higher level of plasma TBARS and CDs at baseline and 24 h post-exercise in study participants with a mutated homozygote (TT) genotype of **MnSOD - rs4880** SNP, than those with the heterozygote (CT) and wild-type (CC) genotypes. This may be an indication of increased lipid damage in study participants with the mutant genotype than those with the heterozygote and wild-type genotypes. This study also observed a higher level

of plasma PC at baseline and 24 h post-exercise in study participants with the MnSOD-rs4880 wild-type (CC) genotype. When considering the muscle damage and fatigue biomarkers, participants with the MnSOD - rs4880 SNP, the mutated homozygotes (TT) genotype exhibited a higher increase of serum CK and decrease of LDH levels at 24 h post-exercise while those with the heterozygote (CT) and wild-type (CC) genotype had lower serum CK levele. These results suggest higher susceptibility to muscle damage in participants with the mutant homozygote genotype and better resistance for those with the heterozygote and wild-type genotypes. Similar findings have been also reported in a study by Minlikeeva et al. (2016) that the mutated homozygote genotype (TT) was more associated with increased TBARS levels compared to the wild-type and heterozygote genotypes. While Vecchio et al. (2017) reported that elite water polo players with MnSOD - rs4880, heterozygote and mutated homozygotes genotypes exhibited a significant increase in plasma LDH, CK, and myoglobin levels post-exercise in comparison with those harbouring the wild-type genotype. Also, Jówko et al. (2017) noted a significant decrease in serum CK and LDH levels post-training in carriers of the CC genotype (wild-type genotype). Both Minlikeeva et al. (2016) and Vecchio et al. (2017) further suggested that screening for gene variants of antioxidant enzymes could be useful to assess individual susceptibility to oxidative stress and muscle damage.

The Tumour Necrosis Factor (TNF-α) gene encodes for the TNF-α pro-inflammatory cytokine which is associated with conditions such as apoptosis, proliferation and inflammation. Many TNF-α SNPs have been researched and identified in human promoter regions, and the genetic variant at position 308G/A has a potential to cause structural changes within the regulatory site which may subsequently affect the function and/or regulation of TNF-α production (Yamin et al., 2008; Umapathy et al., 2018). In their study, Yamin and co-worker (2008) reported a milder association between TNF α -308G/A SNP and serum CK activity post an eccentric exercise regime. In the present study, no mutant homozygote (AA) genotype of the **TNF-α - rs180029** SNP was identified in the study participants. However, participants with the wild-type (GG) genotype exhibited high levels of plasma CDs and PCs, while those with the heterozygote (GA) exhibited high levels of plasma TBARS. When considering the muscle damage markers, study participants with the heterozygote (GA) genotype appeared not to have suffered similar muscle damage, unlike those with the wild-type (GG) genotype as indicated by the more pronounced decrease in their serum CK levels and less decrease in those with the wild-type genotype at 24 h post-exercise when compared to baseline levels. Those with the wild-type genotype showed serum LDH levels more increased than those with the heterozygote genotype at 24 h post-exercise when compared to baseline levels. At 24 h post-exercise, serum lactate levels decreased in all participants, but it was more pronounced in participants with the wild-type genotype. This could also suggest that participants with the TNF-α -

rs180029 SNP wild-type genotype were less susceptible to fatigue onset than those with the heterozygote genotype.

This study also analysed a set of collagen genes known to be associated with muscle damage and/or injuries. Collagen are proteins found almost in every tissue and helps with maintaining tissue structural integrity (Collins & Posthumus, 2011). The Collagen V (COL5) gene is the most researched collagen gene and form a crucial structural component of connective tissues in the musculoskeletal system. This gene encodes alpha-1 chain type V collagen (COL5A1) gene which has many genetic variations [COL5A1 A>T (rs1134170), COL5A1 ATCT (rs16399) and COL5A1 C>T (rs12722)] which are all associated with skeletal muscle damage and soft tissue injury risk, especially the mutant homozygote genotype (McCabe & Collins, 2018). Participants in our study with the **COL5A1_rs1134170** SNP, mutant homozygote (TT) genotype exhibited higher TBARS and CDs plasma levels unlike those with the heterozygote (AT) and wild-type (AA) genotypes. Participants with the wild-type genotype also had more pronounced decreased serum CK, LDH and lactate levels while those with heterozygote and mutated homozygote genotypes had increased serum LDH lactate levels at 24 h post-exercise when compared to baseline level. These results indicate those individuals bearing the mutant homozygote genotype to be more prone to oxidative lipid damage and muscle damage than those bearing the wild-type, but not the heterozygous and mutant genotypes. For the **COL5A1-rs12722** SNP, serum CK levels in the study participants with the wild-type (CC) and heterozygote (CT) genotypes decreased but increased in those with the mutant homozygote (TT) genotype. Additionally, participants with COL5A1- rs12722 SNP, mutant homozygote genotype also had increased serum LDH levels and the least decreased serum lactate levels, while those with the wild-type genotype showed high decreased serum lactate and the least increase LDH levels. When assessed, the COL5A1-rs12722 SNP, influences the oxidative damage biomarkers with participants harbouring the mutant homozygote genotype exhibiting higher levels of plasma TBARS, CDs and PC at baseline and 24 h post-exercise than those with the wild-type and heterozygote genotypes. This result indicates that study participants with the mutant homozygote were more susceptible to oxidative damage of lipids and protein macromolecules, while those with the wild-type and heterozygotes genotypes did not suffer much induced oxidation damage to these important cellular macromolecules. Unlike for other COL5A1 genes SNPs, participants with the **COL5A1-rs16399** SNP, the mutant homozygote (D/D) genotype, were able to resist the induced muscle damage and fatigue onset as indicated by the more pronounced decrease in their serum CK, LDH and lactate levels when compared to those with the wild-type (ATCT/ATCT) and heterozygote (ATCT/D) genotypes. For the **MIR608-rs4919510** SNP, which is another genetic variant associated with skeletal muscle injuries, participants with the wild-type (CC) and mutant homozygote (GG) genotypes, both exhibited higher plasma CDs and PC levels, which is an indication of increased lipid and

protein oxidative damage in these study participants. When assessing the MIR608-rs4919510 SNP influence on the muscle damage biomarker, CK, all study participants had decreased serum levels, but those with the wild-type genotypes showed more resistance to muscle damage as indicated by the more pronounced decrease in their serum CK levels, followed by those with the heterozygote genotype and then last by those with the mutant homozygotes genotype. These results indicate that the wild-type genotype might have protected participants against induced muscle damage, unlike those with the heterozygote and mutated genotypes.

The study also analysed the growth differentiation factor 5 (GDF5) gene, which encodes the growth differentiation factor 5 protein that regulates the growth and maintenance of tendons, muscles, and bones (McCabe & Collins, 2018). However, genetic variation, T>C, rs143383 affects the GDF5 expression in the connective tissues. A study by Harada et al. (2007) concluded that, GDF5 deficiency in tendons could lead to the inability to develop intra-articular ligaments and consequently weaker tendons due to a 40% lack of collagen. McCabe and Collins (2018) also indicated that unlike the C allele of the GDF5-rs143383 SNP, the T allele is associated with muscle and soft tissue injury risk and osteoarthritis due to poor or less production of the GDF5 protein. In our study, all participants had the wild-type (CC) genotype of **GDF5-rs143383** SNP and were resistant to the exercise-induced muscle damage and fatigue as indicated by the low serum CK and lactate levels at 24 h post-exercise when compared to the baseline levels. Caspase-8 gene is also another gene analysed in this study. This gene encodes genetic variant (CASP8-rs3834129) within the structural and regulatory components of tendons and ligament ECM which often predispose individuals to musculoskeletal injuries (Seale et al., 2020). In the present study, participants with **CASP8-rs3834129** SNP, the wild-type (I/I) genotype showed more pronounced decreases in serum CK and lactate levels, unlike those with mutant homozygote (D/D) genotypes. Participants with mutant homozygotes also had high increased serum LDH levels, while it decreased in those with the wild-type genotype and heterozygote genotypes. Participants with the mutated homozygote (D/D) genotype also exhibited higher levels of plasma TBARS, CDs and PC at baseline and 24 h post-exercise. These results further suggest a positive effect or influence of the wild-type genotypes in the study participants and also indicate the detrimental effect of the mutant homozygotes genotype as participants with mutant genotypes for most genes exhibited higher level of plasma lipids and protein oxidative damage biomarkers as well as serum muscle damage markers.

Based on these study results, as much as exercise yields lot of health benefits, understanding the genetic influence that contribute to this or could compromise this is important and could be helpful in the development of personalised exercise therapeutic/ nutraceutical interventions (Harvey et al., 2020). The current study utilised the PSGT approach to compliment clinical

testing methods to assess the blood biochemistry markers. This approach has a high potential to improve the quality of medical care and/or intervention strategies through a more comprehensive testing approach which involves genetic and biochemical testing. These current study results provide an insight into the potential injury susceptibility of the healthy adult male population who participated in this study. Additionally, these results indicate a potential of PSGT in combination, with other blood oxidative damage biomarkers and muscle damage markers that could be used or applied to better understand and manage intervention strategies to prevent injury risk.

Furthermore, these results may be useful to sports scientists on how an individuals' genetic make-up may influence exercise science and how the use of genomics may support or influence decisions pertaining to performance analysis, coaching, personalising nutrition, rehabilitation, and sports medicine, as well as the potential to prompt new research for future scientific investigations. However, although, genetic testing plays a crucial role in identifying gene abnormalities that underpin several diseases, September et al. (2012) cautioned that genetic test should not be considered diagnostic in nature but should be used in conjunction with other intrinsic and extrinsic factors to determine risk for specific injury. Hence, the PSGT approach can be considered to have therapeutic and prognostic value provided that the testing is performed and interpreted by an expert in the field (Gray & Semsarian, 2020). Castelletti et al. (2022), further cautioned that despite genetic testing benefits, there are a number of limitations and pitfalls associated with genetic testing that needs to be recognised, especially ethical matters.

Overall, the current study results are in line with the recommendation by September et al. (2012) not to use SNP analysis as a diagnostic test in the context of sport injury genetics, and further, suggesting that wild-type genotypes may have positive influence or serve a protective function against exercise-induced muscle damage, while the mutated homozygote genotype for most genes SNPs negatively influence the extend of muscle damage. Hence, it is reasonable to hypothesis that study participants who possess mutated homozygote genotypes were more susceptible to oxidative damage and muscle damage as they had increased plasma and serum TBARS, CDs, PC, CK, LDH and lactate levels when compared to those with wild-type and heterozygote genotypes. However, it should be noted that these results are for only a few SNPs associated with oxidative stress, inflammation, and exercise injury risks, therefore, interpretation of the present results should be in that context. Lastly, it is important to note that all the SNPs are context dependant as they are common in the general population and thus considered as normal variation like eye or hair colour. It is only when someone with a risk-associated SNP (mutated allele) enters or are exposed to a high-risk environment (like exercise in this study) that the SNPs become clinically relevant.

CHAPTER SIX
SUMMARY AND CONCLUSION

6.1 Study summary and conclusion

The goal of this multiphase study was to establish a human exercise model inducing oxidative stress and use it to evaluate the possible health advantages of dietary herbal therapies, such as Rooibos as an ergogenic aid for athletes. These therapeutic interventions should be able to modulate the exercise-induced oxidative stress effects and optimise exercise performance outcomes without inducing adverse effects and increasing injury risk. A number of investigations were performed on the blood samples drawn to further evaluate the model and intervention effects (Figure 6.1).

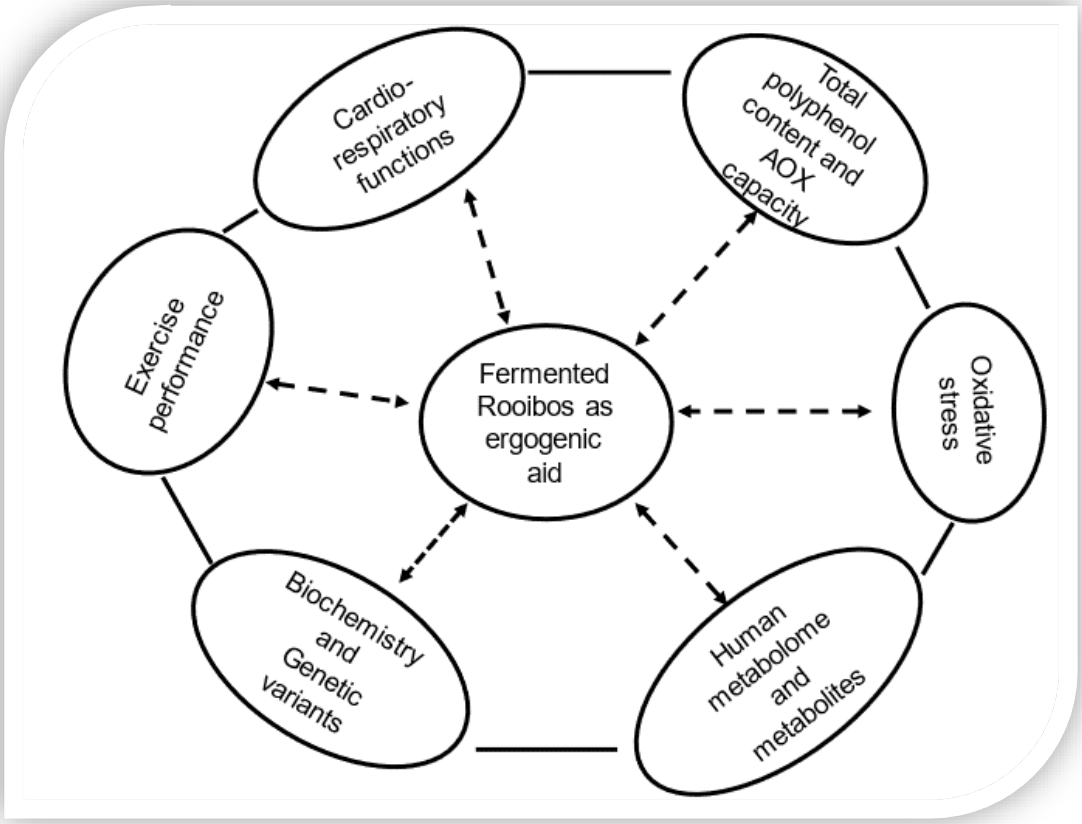


Figure 6.1: Different aspects evaluated to assess the modulatory role of herbals, such as Rooibos using the exercise-induced oxidative stress model established in human participants.

The controlled exercise regime employed in this study was able to reliably and repeatably induce oxidative stress in human participants. This was achieved in an environment that has a low likelihood of causing adverse events given that participants were able to determine the point of withdrawal from the exercise based on their perception of a high degree of exertion and/or near maximal heart rate and/or relative oxygen uptake. The use of herbal interventions such as Rooibos did not induce any complications in the generation of oxidative stress, nor did

it cause any adverse events in the study participants. This confirmed that herbal interventions can be successfully introduced along with the exercise-induced oxidative stress model. In the current study, the Rooibos intervention was able to modulate oxidative stress markers, showing that the antioxidant effects of herbal infusions can be successfully tested in this exercise-induced oxidative stress model. We would thus propose that this may be a viable model for the study of mild oxidative stress in human participants, within the context of exercise and sport applications.

When compared to the placebo beverage, consumption of the standarised fermented Rooibos beverage improved submaximal endurance performance, notably in terms of the mean withdrawal point. In other words it showed that rooibos supplementation enabled participants to cycle longer and consequently achieve a greater distance when compared to the placebo beverage. This suggests that fermented Rooibos may have a beneficial effect on the efficiency of the aerobic metabolism during submaximal exercise. Therefore, it is suggested that the use of Rooibos may be beneficial in reducing exercise-induced oxidative stress damage effects via the imporvement of the redox status of the individual and as a result improving endurance performance at submaximal (<80%) exercise levels. The study findings imply that fermented Rooibos could offer athletes and individuals involved in sport and fitness activities, bettter performance and recovery by delaying fatigue onset, improving exercise performance and/or enhancing cardiorespiratory functions, espcially at lower exercise intensities. Additionally, the metabolomic analyses highlighted possible metabolic shifts that occurred to underscore the positive effects of Rooibos consumption and point to the ergogenic aid potential of fermented Rooibos to improve exercise performance and recovery as metabolites involved in energy production as well as exercise muscle recovery were significantly increased when Rooibos was consumed. From the study results, it can be proposed that Rooibos consumption augmented study participants' metabolic pathways which could subsequently lead to improved exercise performance outcomes. Analyses of genetic variations associated with exercise/muscle injury risks were linked to blood biochemistry results in this study, highlighting the potential of using a pathology supported genetic testing approach as the bridging science to link genes (predisposition or tendency for a relevant pathology) with clinical/physiological outcomes, with the potential to improve the quality of care/intervention through a comprehensive testing approach.

As oxidative stress is at the core of sport and exercise due to the increased demand for oxygen, it is important that this phenomenon be studied in human models to understand the role of free radicals and antioxidants in physiology, but also for possible development of preventative and/or therapeutic interventions. This study is the first to use an exercise-induced oxidative stress model to assess the ergogenic potential of a fermented Rooibos beverage while

including metabolomic and genetic analyses. Results from this study provide new knowledge and enhance our current understanding of the role fermented Rooibos can play in oxidative stress, exercise performance, and how it affects metabolite shifts and processes within the context of exercise performance and/or recovery.

6.2 Study limitations and recommendations

In this study, a relatively small sample of participants was recruited, which may have contributed to increased variability of significant results. This should be expanded upon in future studies in order to produce higher powered outcomes. In addition to this, the participants recruited appeared to have been at different levels of physical fitness. This may have served as a confounder to the results of this study, given that the production of waste products and radicals may differ in individuals of varying levels of fitness (Furrer et al., 2023). It would thus be recommended in future that when participants are recruited, a control mechanism be included to ensure they are of similar fitness or participants of different levels of fitness can be allocated into different experimental groups. This may be necessary as the fitness level would impact on both the generation of oxidative stress and the body's response thereto. In this study, targeted metabolomics analyses focused only on the metabolites identified and it is recommended that in order to better understand the metabolomic responses additional research is needed in order to quantify these metabolites better and more accurately. Thus, in summary it is recommended that future research should assess these same variables with a larger number of study participants, while also considering the variable fitness levels of the participants.

Ultimately, oxidative stress does have a role to play in exercise and sport as part of the conditioning of an athlete, but it should be closely monitored in conjunction with genetic vaiability as not to be causing cell damage with resultant increased risk of injury or fatigue. Part of the monitoring and optimising process should include the effect of dietary antioxidant interventions in these athletes.

CHAPTER SEVEN

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CHAPTER EIGHT
APPENDICES

Appendix 8.A: Endpoint genotyping and sanger sequencing.

Due to the great volume of genotyping results generated from this study only one sample batch of each assay is illustrated in this section. This is to demonstrate the results obtained using the Roche Light Cycler 480 II machines, and respective Sanger sequencing results as performed by the Central Analytical Facility (CAF) of Stellenbosch University.

1. GDF 5 Assay

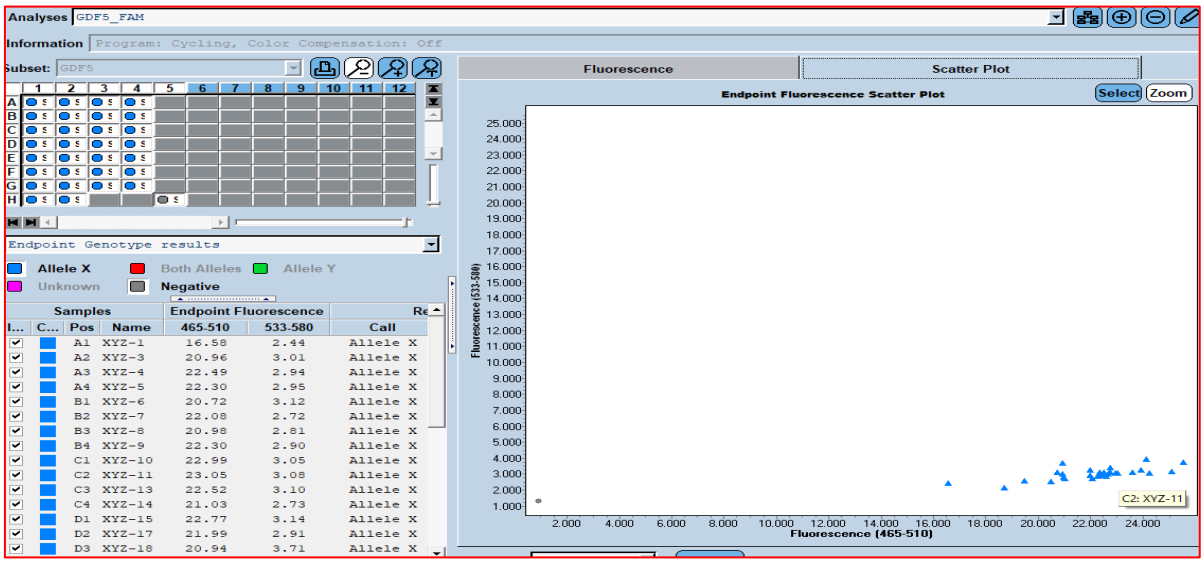

Figure 8.1: Genotypes grouped by scatterplot analysis (FAM) fluorescence vs. VIC fluorescence) of the Roche Light Cycler 480 II, TaqMan endpoint genotype assay. [Allele X (C - FAM labelled) vs Allele Y (T- VIC labelled)]. Upper left cluster = mutant homozygous GG, middle cluster = heterozygous GT, bottom left cluster = wild-type homozygous CC, bottom right corner = Negative control.

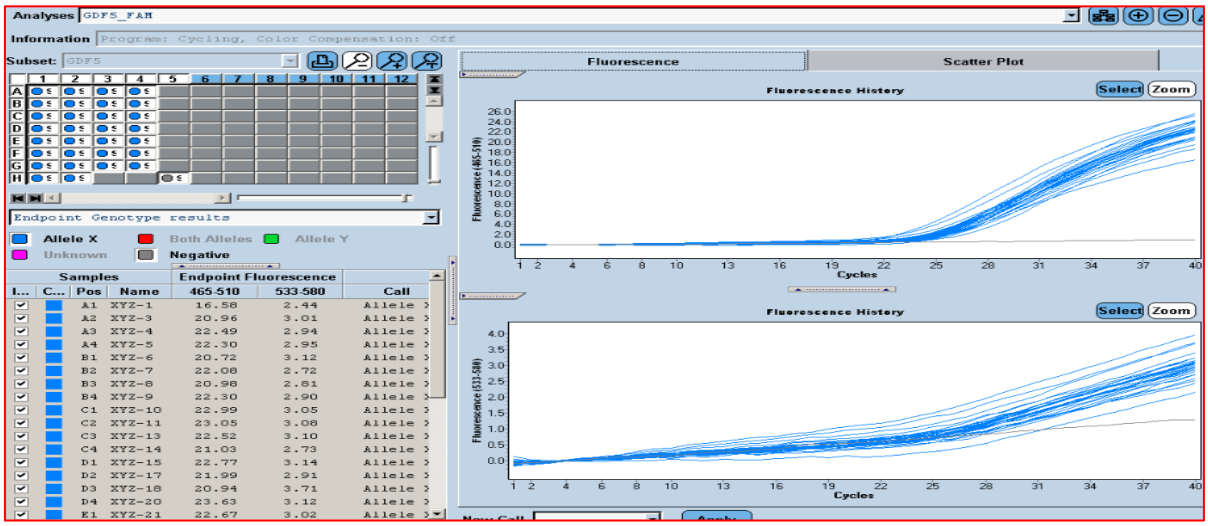

Figure 8.2: Amplification curve of GDF5 assay, showing fluorescence vs number of cycles, performed on the Roche Light Cycler 480 II.

2. COL5A1-rs12722 assay

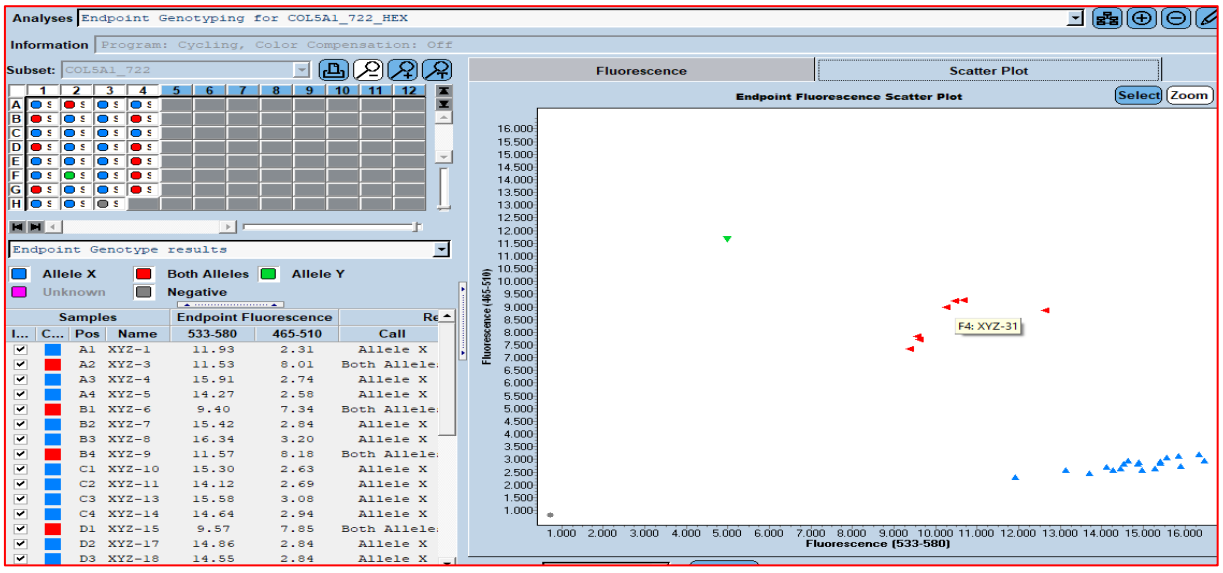

Figure 8.3: Genotypes grouped by scatterplot analysis (FAM™ fluorescence vs. VIC fluorescence) of the Roche Light Cycler 480 II, TaqMan endpoint genotyping assay. [Allele X (C - FAM labelled) vs Allele Y (T- VIC labelled)]. Upper left cluster = mutant homozygous TT, middle cluster = heterozygous CT, bottom left cluster = wild-type homozygous CC, bottom right corner = Negative control.

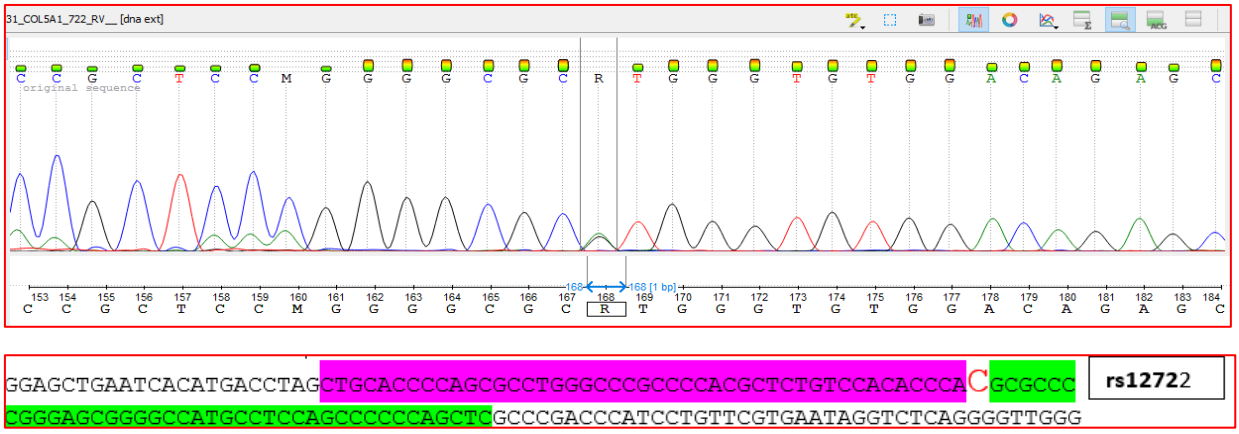

Figure 8.4: Electropherogram illustrating the reverse sequencing reaction of an amplified PCR product for COL5A1_rs12722. The nucleotide position of the SNP is indicated by the highlighted red region on the reference sequence and corresponds to a genotype of C, representing a heterozygote genotype.

3. COL5A1-rs16399

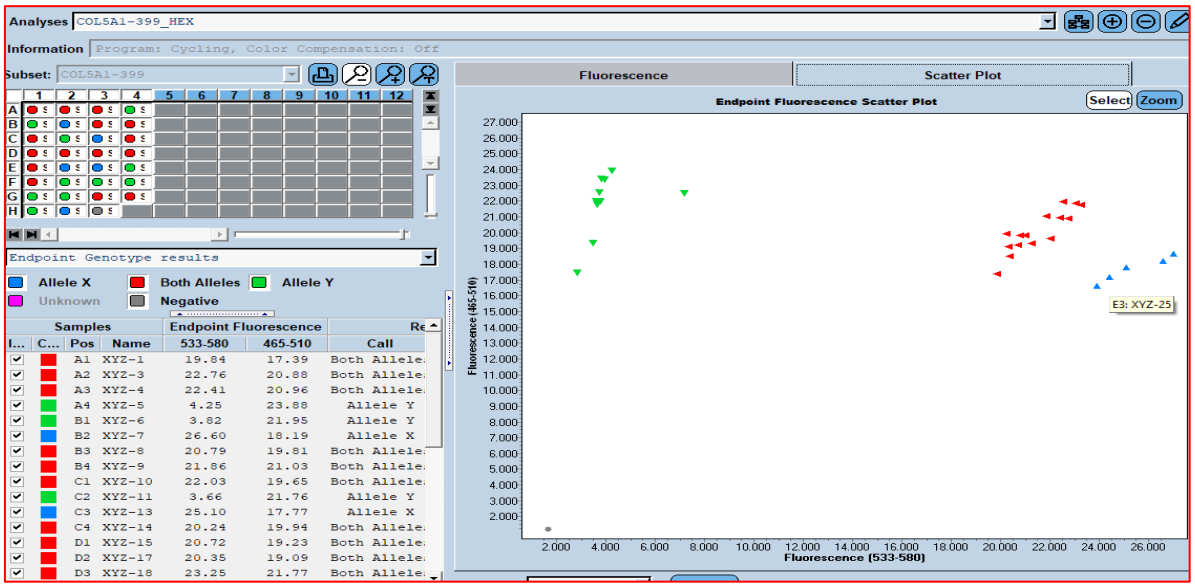

Figure 8.5: Genotypes grouped by scatterplot analysis (FAM™ fluorescence vs. VIC fluorescence) of the Roche Light Cycler 480 II, TaqMan® endpoint genotyping assay. [Allele X (ATCT- FAM labelled) vs Allele Y (D - VIC labelled)]. Upper left cluster = mutant homozygous D/D, middle cluster = heterozygous ATCT/D, bottom left cluster = wild-type homozygous ATCT/ ATCT, bottom right corner = Negative control.

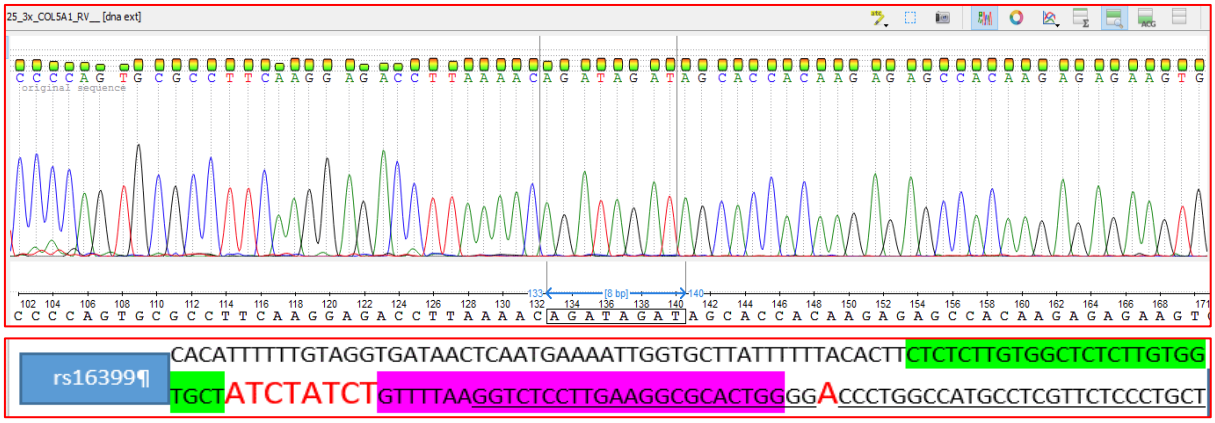

Figure 8.6: Electropherogram illustrating the revere sequencing reaction of an amplified PCR product for COL5A1-rs16399. The nucleotide position of the SNP is indicated by the highlighted red region on the reference sequence and corresponds to a genotype of ATCTATCT, representing a wild-type genotype.

4. COL5A1_rs1134170

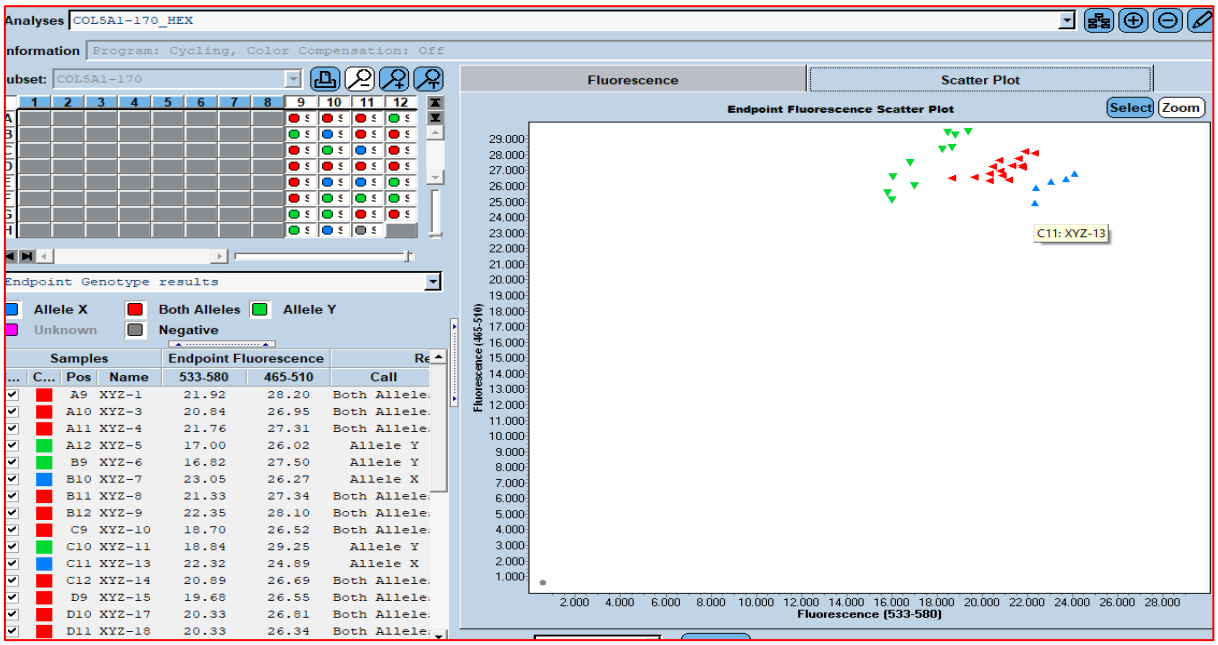

Figure 8.7: Genotypes grouped by scatterplot analysis (FAM™ fluorescence vs. VIC fluorescence) of the Roche Light Cycler 480 II, TaqMan endpoint genotyping assay. [Allele X (A - FAM labelled) vs Allele Y (T- VIC labelled)]. Upper left cluster = mutant homozygous TT, middle cluster = heterozygous AT, bottom left cluster = wild-type homozygous AA, bottom right corner = Negative control.

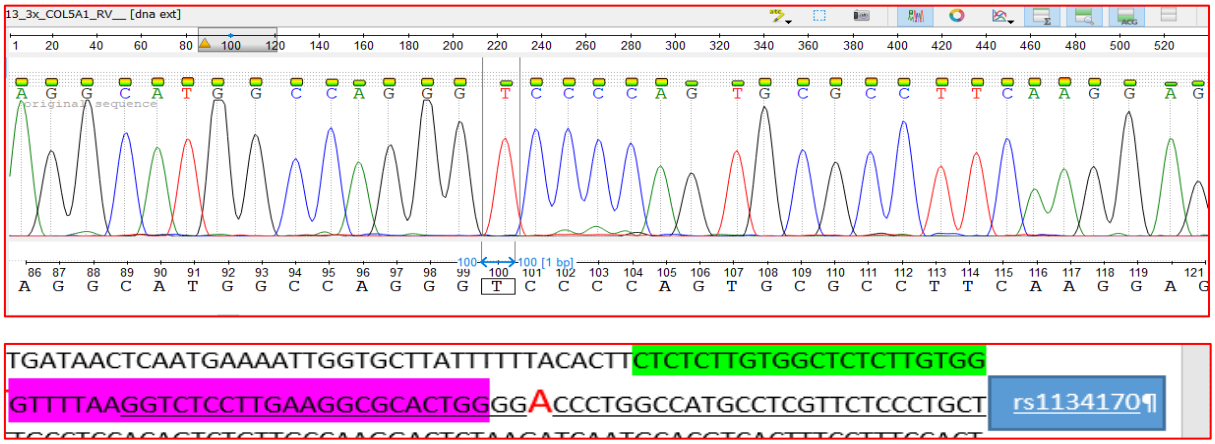

Figure 8.8: Electropherogram illustrating the reversing sequencing reaction of an amplified PCR Product of COL5A1_rs1134170. The SNP position is indicated by the highlighted red region on reference sequence, corresponds to a genotype of AA, representing a wild-type genotype.

5. CASP8_rs3834129

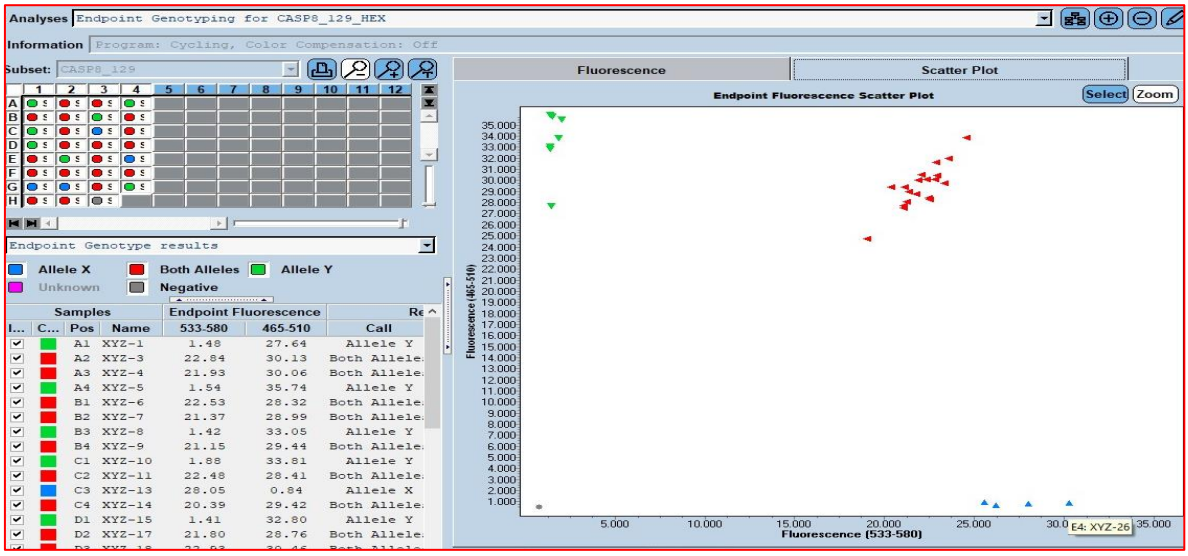

Figure 8.9: Genotypes grouped by scatterplot analysis (FAM™ fluorescence vs. VIC fluorescence) of the Roche Light Cycler 480 II, TaqMan endpoint genotyping assay. [Allele X (I- FAM labelled) vs Allele Y (D- VIC labelled)]. Upper left cluster = mutant homozygous D/D, middle cluster = heterozygous I/D, bottom left cluster = wild-type homozygous I/I, bottom right corner = wild-type.

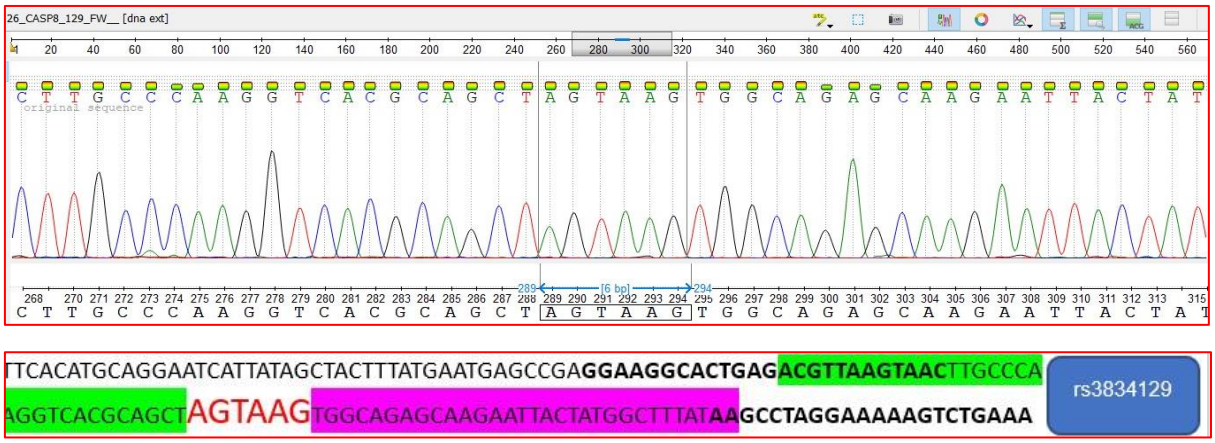

Figure 8.10: Electropherogram illustrating the forward sequencing reaction of an amplified PCR product of the CASP8_rs3834129. The SNP position is indicated by the highlighted red region on reference sequence, corresponds to a genotype of I/I, representing a wild-type genotype.

6. MIR608_rs4919510

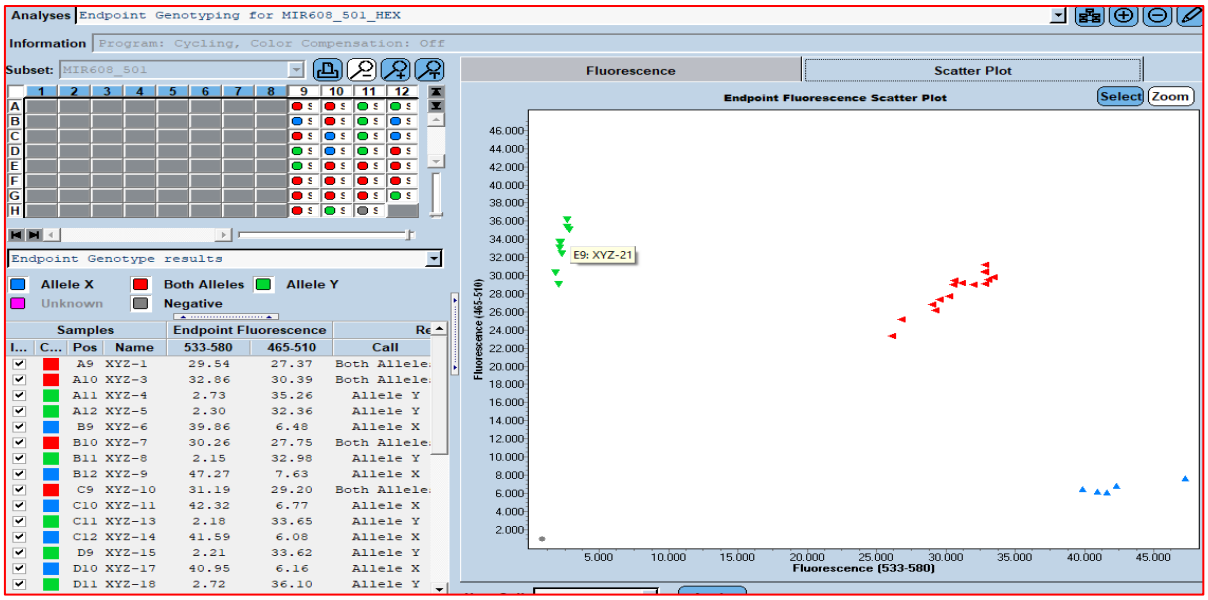

Figure 8.11: Genotypes grouped by scatterplot analysis (FAM™ fluorescence vs. VIC fluorescence) of the Roche Light Cycler 480 II, TaqMan endpoint genotyping assay. [Allele X (C- FAM labelled) vs Allele Y (G - VIC labelled)]. Upper left cluster = mutant homozygous GG, middle cluster = heterozygous CG, bottom left cluster = wild-type homozygous CC, bottom right cluster = Negative control.

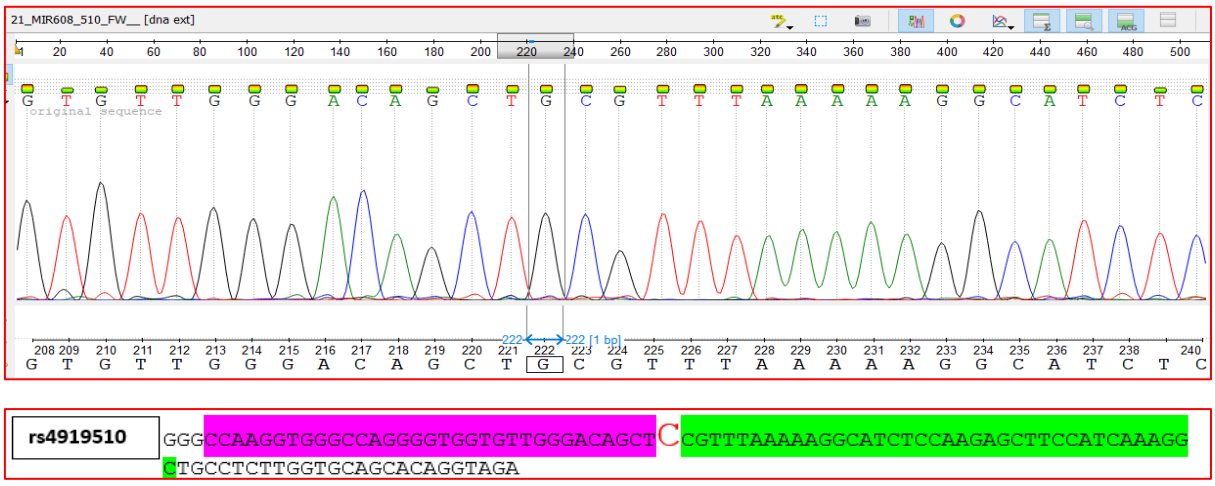

Figure 8.12: Electropherogram illustrating the forward sequencing reaction of an amplified PCR product of MIR608_rs4919510. The SNP position is indicated by the highlighted red region on the reference sequence and corresponds to a genotype of GG, representing a mutant homozygote genotype.

7. MnSOD_rs4880

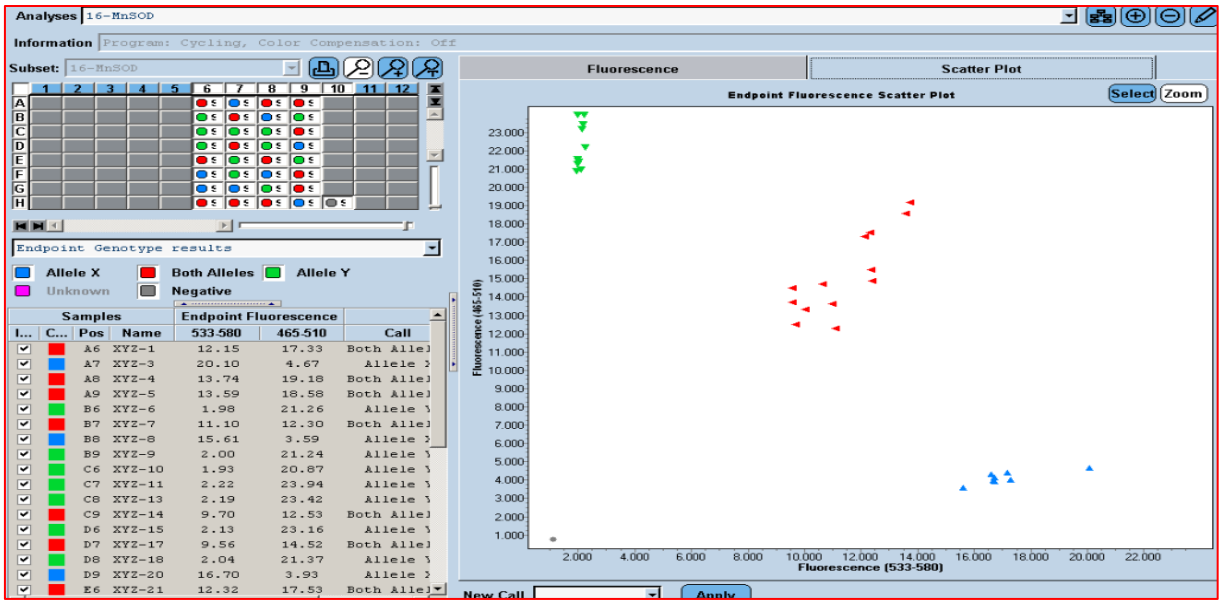

Figure 8.13: Genotypes grouped by scatterplot analysis (FAM™ fluorescence vs. VIC fluorescence) of the Roche Light Cycler 480 II, TaqMan endpoint genotyping assay. [Allele X (C - FAM labelled) vs Allele Y (T- VIC labelled)]. Upper left cluster = mutant homozygous TT, middle cluster = heterozygous CT, bottom left cluster = wild-type homozygous CC, bottom right corner = Negative control.

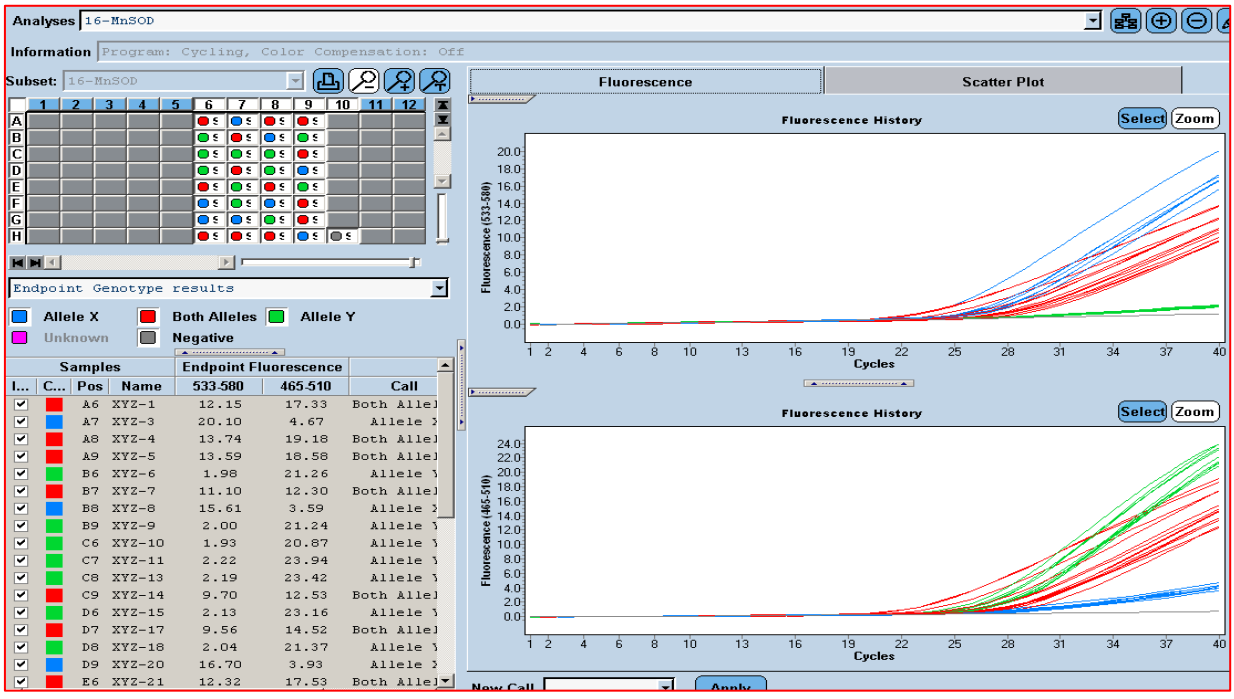

Figure 8.14: Amplification curve of MnSOD assay showing fluorescence vs number of cycles, performed on the Roche Light Cycler 480 II.

8. TNF-a _rs180029

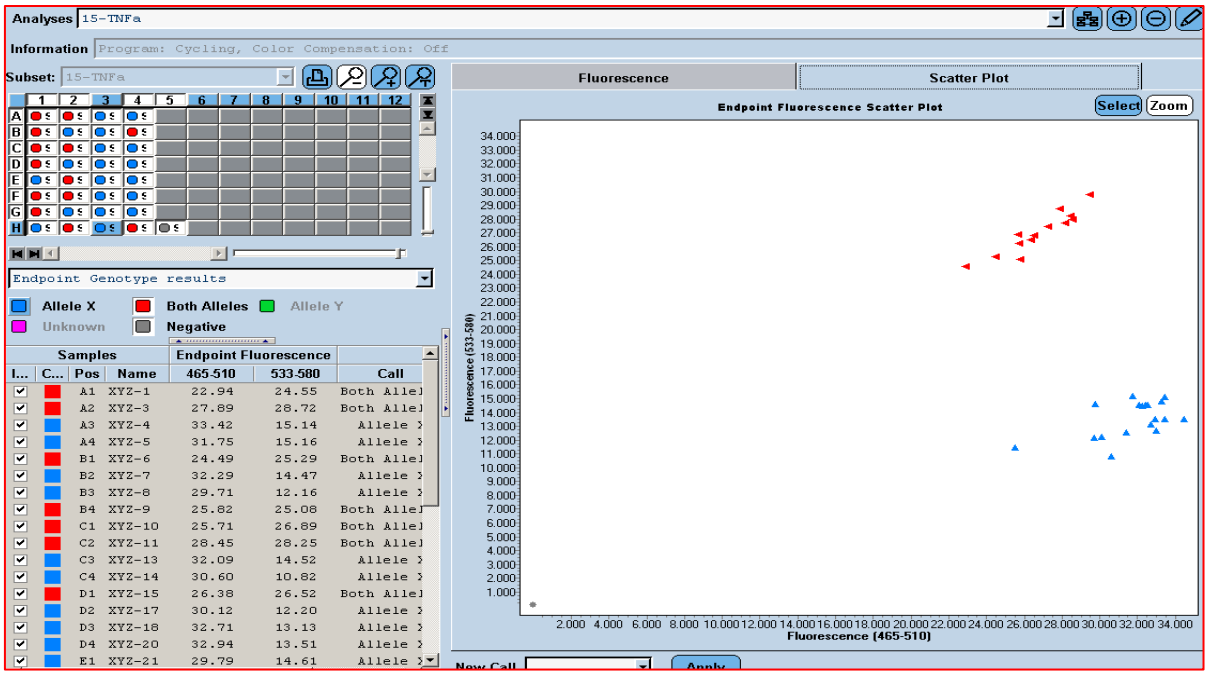

Figure 8.15: Genotypes grouped by scatterplot analysis (FAM fluorescence vs. VIC fluorescence) of the Roche Light Cycler 480 II, TaqMan endpoint genotyping assay. [Allele X (G - FAM labelled) vs Allele Y (A - VIC labelled)]. Upper left cluster = mutant homozygous AA, middle cluster = heterozygous GA, bottom left cluster = wild-type homozygous GG, bottom right corner = Negative control.

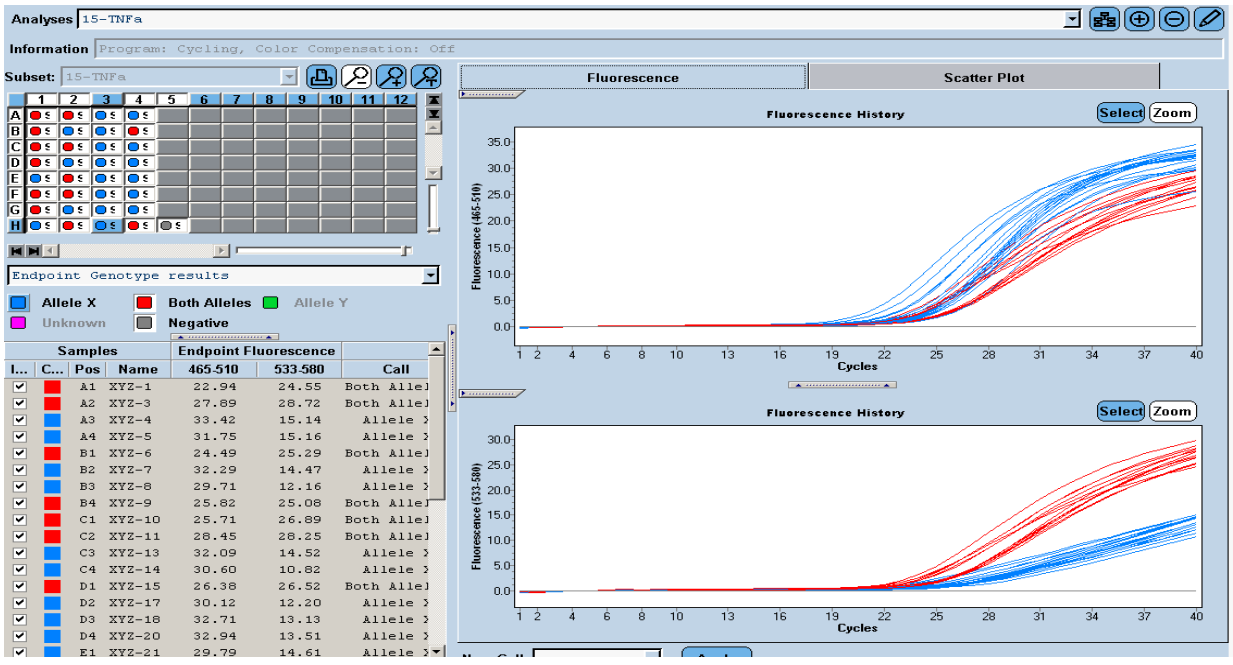

Figure 8.16: Amplification curve of TNF-α assay showing the fluorescence vs number of cycles, performed on the Roche Light Cycler 480 II.

Appendix 8.B: Participant information sheet and informed consent form.

OXIDATIVE STRESS RESEARCH CENTRE, CAPE PENINSULA UNIVERSITY OF TECHNOLOGY, BELLVILLE CAMPUS, CPUT, SOUTH AFRICA
DEPARTMENT OF SPORT MANAGEMENT (HUMAN PERFORMANCE LABORATORY), MOWBRAY CAMPUS CPUT, SOUTH AFRICA

Participant Information Sheet and Informed Consent Form

This Informed Consent Form is for male participants who we are inviting to participate in the research study on Rooibos and its potential health benefits. The title of our research project is "Modulation of exercise-induced oxidative stress using Rooibos (*Aspalathus linearis*) in adult males."

Name of Study leader: Mr Oiva Kamati

Name of Organization: Cape Peninsula University of Technology (CPUT)

Name of Sponsor: South Africa Rooibos Council

Name of Project proposal: Modulation of exercise-induced oxidative stress using Rooibos (*Aspalathus linearis*) in adult males.

This Informed Consent Form has two parts:

- **Information Sheet (to share information about the research with you)**
- **Certificate of Consent (for signatures if you agree to take part)**

You will be given a copy of the full Informed Consent Form

PART I: Information Sheet

Introduction

I am Oiva Kamati, an MSc student, registered at CPUT within the Faculty of Health and Wellness Science, Biomedical Technology Department. We are doing research to establish the health promoting properties of Rooibos, which is an indigenous herbal tea in South Africa. I am going to give you information and invite you to be part of this research study. There may be some words that you do not understand. Please ask me to stop as we go through the information and I will take the time to explain it. If you have questions later, you can ask them of me, or any member of the study team present here today. You do not have to decide today whether or not you will participate in the research study.

Purpose of the research

Exhaustive exercise may cause the redox imbalance in humans with a resultant induced oxidative stress (OS) and potential damage to important cellular molecules such as lipids, DNA and proteins, impacting the normal cellular function which may lead to impaired exercise outputs (such as performance and recovery). The body possesses an endogenous antioxidant defence system, but in the case of overtraining, exercise-induced OS can overwhelm this defence system. Therefore, the need of dietary antioxidant supplements would be of great importance, especially those exhibiting strong antioxidant properties and previously have shown a potential to alleviate ailments associated with exercise-induced oxidative stress as well as improving exercise performance and recovery**.** The current research study is proposed to address this issue by focusing on the indigenous herbal tea, Rooibos, as an innovative measure to reduce the oxidative stress-induced damage in your body during exercise.

"A good case can be made for the notion that health depends on a balance between oxidative stress and antioxidant defenses." – quote by Dr Andrew Weill, M.D.

Type of Research Intervention

The research will entail an experimental study and will follow a randomised, placebo-controlled crossover design. After an overnight fast, participants will report to the study site in the morning where baseline measurements and fasting blood samples will be taken. Thereafter a standardised breakfast snack and a single dose of either the Rooibos or placebo drink will be consumed. Ninety minutes after ingestion of the beverage and snack, blood samples will be drawn again before the commencement of the modified Wattbike Submaximal Ramp Test protocol. The two test sessions (Rooibos & placebo) will be 7 days apart and during exercise protocol, participants will be also asked to complete ten sets of 10-sec sprints, separated by 15 s of passive recovery rest periods and will have their breath by breath analysis done on the CosMed Metabolic System at both sessions. Both tests will involve similar start times in the morning.

Participant selection

We are inviting all males within the age range of 18 to 60 years old who meet certain criteria to take part in the research study. The inclusion criteria for participants will include a consumption of less than 2 alcoholic beverages/day, following a conventional diet and not a special diet (i.e., vegetarian), not smoking, absence of cardiovascular disease, diabetes, renal, hepatic and endocrine disorders and not taking any medication, vitamins or dietary supplements with established antioxidant properties and with no muscular or skeletal injury.

Voluntary Participation

Your participation in this research study is entirely voluntary. It is your choice whether to participate or not. Even if you have agreed to take part in the study, you may still stop participating at any time you want without any consequences to you.

Procedures and Protocol

This project forms part of a human intervention study to determine the effect of Rooibos consumption on antioxidant /oxidative stress, inflammation and performance output measures in volunteers and recovery. There are no known risks to participate in this study. If you volunteer to take part in this study, you will be asked to do the following:

1) Answer questions and complete questionnaires about your demography, health, diet/food intake, dietary supplements, and physical activity. The study nurse will also take your body measurements, (such as weight) during the study.
2) Take part in a study approximately over a 3 (three) week period which includes the completion of a self-administered dietary record, based on your usual dietary intake for the first week. During this 1-week period (known as the *wash-out period*) you will be requested to follow a flavonoid-restricted diet where you omit/restrict certain antioxidant-containing food and beverages from your diet and continue to complete the dietary records. The following week you will be required to consume either the single dose of Rooibos or placebo beverage (375 millilitres) before doing the modified Wattbike exercise as described above. Blood will be taken on five occasions on the exercise day and will equal a volume of 50 millilitres (approximately 6 tablespoons). Hereafter you will have a rest period of 7 days and then return to the study site to consume the alternate beverage and proceed with the Wattbike exercise and also breath by breath analysis done during the protocol on the CosMed Metabolic System. You will receive training at the beginning of the study on how to complete the dietary records, health and fitness questionnaires. The modified Wattbike Submaximal Test will be explained to you, and you will have an opportunity to ask questions. Thereafter you will be habituated in terms of riding on the Wattbike.
3) If you are willing, a qualified phlebotomist or nursing sister will take samples of your blood from your forearm and take your blood pressure measurements. The first blood sample (1 blood tube, approximately two teaspoons in volume) to be drawn will be for screening purposes before the study starts to determine if you are eligible to take part in the study, while the remaining blood samples (five) will be drawn after you have completed the exercise regime. Your blood samples will be analysed to determine your general health, antioxidant/oxidative stress status, inflammatory status and induced muscle damage.
4) The questions and blood tests are not for diagnostic purposes, your blood will not be tested for HIV-AIDS. Should the study doctor deem it necessary after your blood test results are known, he will refer you to your personal physician or local clinic doctor.
5) Someone from the study may call to clarify your information.

All these above procedures will probably be familiar to you as most of them occur when you visit your general practitioner.

Randomization

Because we do not know if the Rooibos drink will be better, we need to compare it to a placebo drink (containing no antioxidants). To do this, we will put people taking part in this research into two groups. The groups are selected by chance as if tossing a coin, also called *randomization.* Participants in one group will be given the Rooibos drink while participants in the other group will be given the placebo drink. The placebo or inactive beverage does not contain the active Rooibos substances. Then, we will compare which of the two has the best results.

Side Effects

We do not anticipate any side effect you may experience, but should you experience any unease or feel unsure during the study please contact the study supervisors Prof Marnewick (082 897 9352) or Prof Davies (083 235 3914) or WhatsApp study leader Mr Kamati (+264 81 408 3006/ oivaviety@gmail.com) immediately and they will advise and guide you in this regard.

Risks

No risk is expected but you may experience some discomfort when your blood is drawn or when completing the respective questionnaire about your health, nutrition, physical activity and smoking habits. The risks of drawing blood from your arm include the unlikely possibilities of a small bruise or localized infection and bleeding. These risks will be reduced by using a qualified phlebotomist or nursing sister to draw the blood.

Benefits

Apart from getting to know certain of your health and fitness indicators such as blood pressure, there are not any direct benefits for you, but your participation is likely to help us find the answer to the research question.

Costs

You will not be given any money or gifts to take part in this research study nor will you be charged any costs for the results of the brief health report, which will include your blood pressure and body weight.

Confidentiality

Your personal information we collect during this research study will be kept confidential, it will be stored in a locked filing cabinet and/ or a password protected a computer to which only the study leader, Mr Kamati and study supervisor Prof Marnewick and Prof Davies will have access. Any information about you will have a number on it instead of your name. Only the study leader will know what your number is, it will not be shared with or given to anyone else except the study doctor, if and when deemed necessary.

Sharing the Results

The knowledge that we will get from doing this research will be shared at public meetings such as conferences and we will also publish the results in order that other interested people may learn from our research, a study findings information leaflet will be also sent here (Human Performance Laboratory). Confidential information will not be shared. You should note that this is a long process and may take up to 2 years after the study has been completed.

Right to Refuse or Withdraw

You do not have to take part in this research if you do not wish to do so. You may also stop participating in the research at any time you choose. It is your choice, and all of your rights will still be respected.

Who to contact.

If you have any questions, you may ask them now or later, even after the study has started. If you wish to ask questions later, you may contact study leader Mr Kamati (+264 81 408 3006 / 083 466 2069 oivaviety@gmail.com) and study supervisor Prof Marnewick (082 897 9352) or Prof Davies (083 235 3914).

This research study has been reviewed and approved by Faculty of Health and Wellness Research Ethics Committee (CPUT/HW-REC 2018/H29), a committee whose task it is to make sure that research participants are protected from harm. If you wish to find out more about the H&W-REC, contact The Chairperson, H&W-REC, Tel: +27 21 959 6917; email: sethn@cput.ac.za.

PART II: Certificate of Consent

Declaration by study participant:

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked to have been answered to my satisfaction. I consent voluntarily to participate as a participant in this research. I may choose to leave this study at any time and will not be penalised or prejudiced in any way. I may be asked to leave the study before it has finished if the study doctor or study leader feels it is in my best interest or if I do not follow the study plan as agreed to.

Place_____

Print Name of Participant_____

Signature of Participant _____

Date _____

Day/month/year

Signature of witness_____

Declaration by the investigator:

I declare that I have explained the information in this document to the study participants and encourage them to ask questions and took adequate time to answer them. I am satisfied that he/she adequately understand all aspects of the research study as discussed above. I did/did not use an interpreter. (If an interpreter is used the interpreter must sign the declaration below).

Place_____

Print Name of Investigator_____

Signature of Investigator _____

Date _____

Day/month/year

Signature of witness_____

Declaration by the interpreter:

I declare that I assisted the investigator to explain the information in this document to the participant using the language medium of Afrikaans/Xhosa/Zulu or another language. We encouraged him/her to ask questions and took adequate time to answer them. I conveyed a factual correct version of what was related to me. I am satisfied that the participant fully understands the content of this information and informed consent document and has had all his/her questions satisfactorily answered.

Place_____

Print Name of Interpreter_____

Signature of Interpreter _____

Date _____

Day/month/year

Signature of witness_____

Thumbprint of participant

Appendix 8.C: Advertisement call for study participants.

EXERCISE & OXIDATIVE STRESS RESEARCH STUDY

OXIDATIVE STRESS RESEARCH CENTRE, CAPE PENINSULA UNIVERSITY OF TECHNOLOGY, BELLVILLE CAMPUS, SOUTH AFRICA

DEPARTMENT OF SPORTS MANAGEMENT (HUMAN PERFORMANCE LABORATORY), MOWBRAY CAMPUS, SOUTH AFRICA

MALE VOLUNTEERS NEEDED FOR RESEARCH STUDY

Are you a fit, healthy male and would like to join us and participate in a "first of its kind" research study investigating the possible modulating role of the popular South African herbal tea, **ROOIBOS** in exercise performance & recovery. Rooibos contains components referred to as polyphenols that are strong antioxidants and have been suggested to lessen oxidative stress, inflammatory response, muscular damage, fatigue, and soreness experienced during exercise which all result in declining performance and recovery. We, therefore, hypothesise that Rooibos may minimise some of, or perhaps all these factors and possibly enhance your exercise performance and recovery. The study aims to investigate this hypothesis.

Participation requirements

- Age between 18 and 60 years
- Healthy with no significant clinical abnormalities of the liver, kidneys, or blood
- Be physically active with cycling experience (and/or able to habituate on WattBike)
- Not on any chronic medication
- Not on any anabolic, ergogenic or antioxidant nutritional supplements

All volunteers interested in this study will be screened beforehand at no cost to determine if they meet the requirements to partake in the study. Please feel free to contact us even if you do not know or are uncertain about your health and fitness status requirements for this study.

Research Intervention

The research will entail an experimental study and will follow a randomised, placebo-controlled crossover design. In this 3-week study, participants will visit the Human Performance Laboratory, Mowbray Campus of CPUT once a week. They will be required to omit/restrict certain foods and beverages from their diet, participate in 2 exercise days when they will consume a standardised Rooibos beverage or placebo beverage before completing a modified submaximal ramp test (Wattbike) & ten sets of 10-sec sprints, while having a breath-by-breath analysis done on the CosMed Metabolic System at both sessions. Participants will also be required to keep a 3-day dietary food record. Blood samples will be taken during each of the visits to measure various makers.

Benefits

- Participants will learn more about their own health and fitness status at no cost at the end of the study.
- The research is conducted at no cost to the participants.
- The study beverages and all study materials will be provided without any costs.

All interested participants please submit your contact details via email or WhatsApp:

Mr Oiva Kamati (oivaviety@gmail.com/ +264 81 408 3006/ +27 83 466 2069) or Mr Raeeq (gamieldienr@cput.ac.za /+27814482536) or Prof Simeon Davies (daviess@cput.ac.za +27 83 235 3914). This study has been approved by the Cape Peninsula University of Technology, Faculty of Health and Wellness Science Research Ethics Committee (CPUT/HW-REC 2018/H29) of CPUT (Tel: +27 21 959 6917; email: sethn@cput.ac.za)

Appendix 8.D: Demographic, health and fitness questionnaire.

STUDY TITLE

Modulation of exercise-induced oxidative stress using Rooibos (*Aspalathus linearis*) in adult males.

Demographic questionnaire

Section A: General

1.1 Participant number			
1.2 Date	D	M	Y

Contact telephone number: _____

Please mark your response with an "X" in the appropriate response box

		Response
1.4 Your gender?	Male	1
	Female	2

1.5 Your age in years? (Please indicate)			
1.6 Your date of birth? (Please indicate)	D	M	Y

1.7 Your first language?	1.7.1 Afrikaans	1
	1.7.2 English	2
	1.7.3 Xhosa	3
	1.8.4 Zulu	4
	1.7.5 Other	5

1.8 Your second language?	1.8.1 Afrikaans	1
	1.8.2 English	2
	1.8.3 Xhosa	3
	1.8.4 Zulu	4
	1.8.5 Other	5

		Response
1.9 Your marital status?	1.9.1 Never married	1
	1.9.2 Married	2
	1.9.3 Divorced	3
	1.9.4 Widowed	4

1.10 What is your occupation?	
1.10.1 **Legislator, senior official and manager** *(e.g., CEO, president/vice president, general manager, divisional head, postmaster, superintendent, dean, school principal)*	1
1.10.2 **Professional** *(e.g., engineer, architect, lawyer, biologist, geologist, psychologist, accountant, medical doctor, town planner)*	2
1.10.3 **Associate professional and technician** *(e.g., computer programmer, nurse, physio/occupational therapist, actor, photographer, illustrating artist, product. designer, translator, pilot, broker, quality inspector)*	3
1.10.4 **1.10.4 Clerk** *(e.g., bookkeeper, teller, cashier, messengers and office helper, typist, telephone operator, secretary, reception clerk, library clerk)*	4
1.10.5 **Service and sales worker** *(e.g. nurses' aid, hairdresser, guide, housekeeper, childcare, fire-fighter, advertising agent, real estate agent, sales clerk, shop attendant)*	5
1.10.6 **Skilled agricultural and fishery worker** *(e.g., farmer, grower, planter, winemaker, horticultural worker, fisherman/woman)*	6
1.10.7 **Craft and related trades e.g.,** *miner, quarry worker, bricklayer, carpenter, plasterer, plumber, electrician, painter, mechanic, locksmith)*	7
1.10.8 **Plant and machine operator** *e.g., truck driver, bus driver, taxi driver, sound and video recorder, textile worker, production machine worker)*	8
1.10.9 **Elementary occupation** *e.g., news vendor, garage attendant, car washer, gardener, farm labourer, garbage collector, sweeper)*	9
1.10.10 **Learner/student**	10

1.11 Your highest level of education?	1.11.1 Secondary school/High school	1
	1.11.2 Matric (St.10/Grade 12)	2
	1.11.3 Certificate	3
	1.11.4 Diploma	4
	1.11.5 Degree	5
	1.11.6 post-graduate degree (Masters/Doctoral)	6

Section B: Lifestyle

2. Smoking		**Response**
2.1 Do you smoke?	2.1.1 Never smoked	1
	2.1.2 Former smoker	2
	2.1.3 Current smoker	3
2.2 If you currently smoke, please indicate your selection	2.2.1 Cigarettes	1
	2.2.2 Tobacco/pipe	2
	2.2.3 Cigar	3

3. Physical activity		
3.1 Are you physically active?	3.1.1 Yes	1
	3.1.2 No	2
3.2 If yes, how regularly do you exercise (mark the appropriate section)?		
	3.2.1 1x/week	1
	3.2.2 3x/week	2
	3.2.3 > 3x/week	3
3.3 Would you describe your physical activity level as:		
3.3.1 Moderately active - walking briskly (about 5½ km per hour); hiking; gardening; dancing; golf (walking and carrying clubs); bicycling (less than 16 km per hour); weight training (general light workout)?		1
3.3.2 Vigorously active running/jogging (8 km per hour); bicycling (more than 16 km per hour); Swimming (freestyle laps); aerobics; walking very fast (7 km per hour); Heavy work, such as chopping wood; Weight lifting (vigorous effort)?		2

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Health questionnaire

Participant number:	**Date:**
Date of birth:	**Age:**

	Yes	No	Unsure
1. Do you have high blood pressure?			
2. Do you smoke?			
3. Do you have low blood sugar/diabetes?			
4. Do you have a family history of heart disease?			
5. Do you have elevated cholesterol levels?			
6. Do you drink alcohol?			
If yes, please indicate type of alcohol and frequency:			
6.1 Wine			
6.1.1) Red wine			
6.1.2) White wine			
6.2 Frequency 6.2.1 Not more than two (2) glasses per day			
6.2.3 More than two (2) glasses per day			
6.3 Beer			
6.4 Frequency 6.4.1 Not more than two (2) drinks per day			
6.4.2 More than two (2) drinks per day			
6.5 Spirits			
6.5.1 Brandy			
6.5.2 Whiskey			

6.5.3 Rum			
6.6 Frequency 6.6.1 Not more than two (2) drinks per day			
6.6.2 More than two (2) drinks per day			
	Yes	**No**	**Unsure**
7. Do you use life sustaining medication for conditions such as:			
7.1 Cholesterol?			
7.2 Blood pressure?			
7.3 Diabetes?			
7.4 Other (Please specify)			
8. Are you physically active? (>30 minutes moderate intensity exercises most days of the week)			
9. Did your body weight remain stable for the past 6 (six) months?			
10. Do you use dietary supplements?			
a. If yes, state frequency			
b. Describe the dietary supplements used:			
11. Do you follow any diet specific diet such as:			
a. Vegetarian or vegan diet?			
b. High protein diet?			
c. Healthy eating habits incorporated in diet?			
d. Other (Please specify)			
12. Do you have any kidney problems?			
13. Do you have any liver diseases or problems?			

Appendix 8.E: Anthropometry and blood pressure form.

Anthropometry and Blood Pressure

Subject number:	**Date:**
Date of birth:	**Age:**

	Date:				**Date:**			
	Screening:				**Intervention 1 completed:**			
Measurement:	1	2	3		1	2	3	
Blood pressure (mm Hg)								
Weight (kg)								
Height (cm)								
Body Mass Index (kg/m^2)								
Waist circumference (cm)								

Anthropometry and Blood Pressure

Subject number:	**Date:**
Date of birth:	**Age:**

	Date:				**Date:**			
	Intervention 2 completed:				**Additional date:**			
Measurement:	1	2	3		1	2	3	
Blood pressure (mm Hg)								
Weight (kg)								
Height (cm)								
Body Mass Index (kg/m^2)								
Waist circumference (cm)								

Appendix 8.F: Dietary record booklet.

DIETARY RECORD BOOKLET

Code number: ……

Period: ……

Days and dates: ……

(1) Please write down everything that you eat and drink on the days/date as indicates above. Two record pages are included for each day.

(2) Your next appointment is on: ……

Please use the instruction for keeping a food intake record as a guideline for record keeping

OXIDATIVE STRESS RESEARCH CENTRE, CAPE PENINSULA UNIVERSITY OF TECHNOLOGY, BELLVILLE CAMPUS, SOUTH AFRICA

department of sport MANAGEMEN (human performance laboratory)

Mowbray campus, South Africa

Building a health nation

Through research

Nutritional Intervention

Research Unit

FOOD INTAKE RECORD

Period:……

Date:……

Day of the week:……..

Time	What did you eat/drink?	How was it prepared?	How much?

Appendix 8.G: List of restricted food and beverages for the Rooibos and exercise study.

Dietary restrictions for the Rooibos & Exercise Intervention study 2019

Study participants are kindly asked to strictly adhere to the following beverage and food intake restrictions during the study, starting after the 1-week of screening.

1. Beverages

Avoid the intake of the following flavonoid-containing beverages while participating in this study, as it may influence the outcome of the study.

1. Coffee (all brands: Pure and instant; Caffeinated and decaffeinated; Filter, percolated or plunger)
2. Tea (all brands: Black, green, herbal, ice, flavoured and unflavoured)
3. Cocoa drinks (all brands e.g. Hot chocolate, Milo, Ovaltine)
4. Red wine; Rosé (all labels)
5. Fruit juices – 100% pure juices and blends (all brands of red grape, orange, apple and berry juices along with red Grapetizer and Appletizer)

The following beverages may be taken in **restricted quantities** per day:

1. Soda drinks: Coca cola, Coke Light, Tab, Fanta, Cream Soda, Sprite, Lemon Twist (one 340 mL can per day)
2. Two glasses of white wine (120 mL per glass) or one beer (340 mL) or one spirit drink (1 tot / 25 mL) per day

The following beverages may be taken **as usual/freely** per day:

1. Fruit juices (specific flavours: Litchi, White grape, Hanepoot), fruit nectars (≤ 50% fruit juice), dairy fruit blends and fruit juice and yoghurt blends
2. Diluted base drinks (specific brands: OROS, Jive, Tang, Drink-O-Pop, Sweet-O)
3. Water / Bottled / Mineral (all brands: Still and sparkling; Flavoured and unflavoured)

2. Fruits

Restrict the number of portions consumed **per day** of the following fruits:

1. Apples (one apple per day)
2. Oranges or nartjies (one orange or nartjie per day)
3. Black/Red grapes or berries (one cup per week)

All other fruits (banana, pears, etc.) may be consumed **as usual/freely**.

3. Dark Chocolate

Restrict the intake of dark chocolate to a maximum of three **40g** (about 6 blocks) portions **per week**.

All other chocolate (milk) may be consumed **as usual/freely**.

4. Dietary supplements

No vitamin C **supplementation** or any other antioxidant supplement (capsules or powder) should be taken during the study period as it may influence the outcome of the study.

***NOTE**: Study participants are also kindly asked not to change any other aspect regarding their food and beverage intakes during the study period compared to before the study*.

Appendix 8.H: Study ethical clearance certificate.

Cape Peninsula University of Technology

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)
Registration Number NHREC: REC- 230408-014

P.O. Box 1906 • Bellville 7535 South Africa
Symphony Road Bellville 7535
Tel: +27 21 959 6917
Email: sethn@cput.ac.za

5 December 2018
REC Approval Reference No:
CPUT/HW-REC 2018/H29

Dear Mr Oiva Kamati - 217283101

Re: APPLICATION TO THE HW-REC FOR ETHICS CLEARANCE

Approval was granted by the Health and Wellness Sciences-REC to Mr Kamati for ethical clearance on 30 November 2018. This approval is for research activities related to student research in the Department of Biomedical Sciences.

TITLE: **Modulation of exercise – induced oxidative stress using rooibos (Aspalathus linearis) in adult males**

Supervisor: Prof J Marnewick

Comment:

Approval will not extend beyond 6 December 2019. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an **annual progress report** that should be submitted to the HWS-REC in December of that particular year, for the HWS-REC to be kept informed of the progress and of any problems you may have encountered.

Kind Regards

Dr Navindhra Naidoo
Chairperson – Research Ethics Committee
Faculty of Health and Wellness Sciences

Cape Peninsula University of Technology

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HWS-REC)
Registration Number NHREC: REC- 230408-014

P.O. Box 1906 • Bellville 7535 South Africa
Symphony Road Bellville 7535
Tel: +27 21 959 6917
Email: sethn@cput.ac.za

29 April 2021
REC Approval Reference No:
CPUT/HW-REC 2018/H29 (renewal)

Faculty of Health and Wellness Sciences – Biomedical Sciences

Dear Mr Oiva Kamati,

Re: APPLICATION TO THE HWS-REC FOR ETHICS RENEWAL

Approval was granted by the Health and Wellness Sciences-REC on 30 November 2018 to Mr Kamati for ethical clearance. This approval is for research activities related to student research in the Department of Biomedical Sciences at this Institution.

TITLE: Modulation of exercise-induced oxidative stress using rooibos (*Aspalathus linearis*) in adult males

Supervisor: Prof J Marnewick

Comment:

Approval will not extend beyond 30 April 2022. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an **annual progress report** that should be submitted to the HWS-REC in December of that particular year, for the HWS-REC to be kept informed of the progress and of any problems you may have encountered.

Kind Regards

Ms Carolynn Lackay
Chairperson – Research Ethics Committee
Faculty of Health and Wellness Sciences