The antioxidant and anti-adipogenic properties of phenolic acids identified in Mageu

by

Nompumelelo Saule

Thesis submitted in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE

in the

FACULTY OF HEALTH SCIENCES

Department of Anatomy

University of Pretoria

Supervisor: Prof JC Serem

Co- Supervisor: Prof MJ Bester

2024

Abstract

The increasing burden of non-communicable diseases (NCDs), such as obesity and cardiovascular diseases, poses significant public health challenges across African countries. Identifying functional foods with health-promoting properties has become a priority in addressing these issues. Mageu, a fermented maize-based beverage native to Southern Africa, is of particular interest due to its widespread consumption and cultural significance. Beyond its traditional role in the diet, Mageu has been shown to possess bioactive properties, including cellular antioxidant, anti-adipogenic, and anti-inflammatory effects, largely attributed to its phenolic compounds namely, ferulic acid (FA), caffeic acid (CA), 3,4-dihydroxybenzoic acid (3,4-DHBA), 4-hydroxybenzoic acid (4-HBA), and p-coumaric acid (p-CA).. Simulated *in vitro* digestion studies have revealed increased concentrations of FA, 3,4-DHBA and 4-HBA. Given the prevalence of NCDs in Southern Africa, this study aimed to evaluate the antioxidant, anti-adipogenic, cytotoxicity and the related polyphenol-polyphenol interactions of 3,4-DHBA, 4-HBA, and FA, the most abundant phenolic acids identified in Mageu. This research is crucial for understanding the potential health benefits of Mageu and its role in mitigating the growing health crisis in the region.

The effective concentration at which 3,4-DHBA, 4-HBA, and FA neutralized reactive oxygen species (ROS) by 50% (half maximal effective concentration (EC50)) was determined with the oxygen radical absorbent capacity (ORAC) assay. Using this information the antioxidant properties at the EC25 and EC50, (EC50(2X) and EC50(5X) for some assays) were determined for each phenolic acid and combinations thereof with the Folin-Ciocalteu (F-C) assay, Trolox equivalent antioxidant capacity (TEAC) and ORAC assays. Then inhibition of oxidative damage was demonstrated using the dichlorofluorescein diacetate (DCFH-DA) cellular (Caco-2 cells) antioxidant assay. Dosage dependent differences in activity was observed in most instances. Although not significant, FA consistently had the highest antioxidant activity, when compared with 3,4-DHBA and 4-HBA. Structural differences, particularly the arrangement and number of hydroxyl groups on the phenolic ring, contributed to these differences in antioxidant capacity. Synergism was observed at the EC25 with the F-C assay for 3,4-DHBA/4-HBA and 3,4-DHBA/FA for the TEAC assay, and with the ORAC assay for 3,4-DHBA/4-HBA/FA. With the DCFH-DA assay synergism was observed at the EC50 for 3,4-DHBA/4-HBA, 4-HBA/FA and 3,4-DHBA/4-HBA/FA, at EC50(X2) for 3,4-DHBA/4-HBA/FA and at EC50(X5) for 3,4-DHBA/4-HBA and 3,4-DHBA/4-HBA/FA.

The efficacy of these polyphenols and their combinations in mitigating lipid accumulation in 3T3-L1 differentiated to adipocytes cells assessed using Oil Red O (ORO) and Nile Red (NR) assays. Phenolic acids demonstrated significant dose-dependent anti-adipogenic effects at elevated

concentrations relative to control cells in both assays. At EC25, EC50, and EC50(X2) concentrations, polyphenol-polyphenol interactions were predominantly additive. Notably, synergistic interactions were consistently observed in triple combinations and at higher concentrations. Synergy was particularly evident in the ORO assay at EC25 and EC50(X5) for the combination of 3,4-DHBA/4-HBA/FA. In the NR assay, synergy was observed at EC25 for 3,4-DHBA/4-HBA/FA, at EC50(X2) for 3,4-DHBA/FA, 4-HBA/FA, and 3,4-DHBA/4-HBA/FA, and at EC50(X5) for 3,4-DHBA/FA and 3,4-DHBA/4-HBA/FA. These phenolic acids also synergistically inhibited the differentiation of preadipocytes, thereby preserving their fibroblast morphology. Finally, the cytotoxicity was determined in the Caco-2 and 3T3-L1 cell lines with the Crystal Violet (CV) assay. At the EC25 in the Caco-2 cell, the combination of 4-HBA and FA significantly reduced the percentage cell number to 83.7 ± 1.88% after 24 hours, but not at 48 and 72 hours. Similarly, 3,4-DHBA with 4-HBA and FA reduced the % cell number to 81.23 ± 4.69% after 24 hours, with no significant effects at 48 and 72 hours. At the EC50, 3,4-DHBA, 4-HBA, and FA were not cytotoxic, but combinations of 3,4-DHBA + 4-HBA, 3,4-DHBA + FA, and 4-HBA + FA decreased the percentage cell biomass after 24 hours, with lesser effects after 48 and 72 hours. Differences between the EC25 and EC50 groups were not statistically significant. The cytotoxic effects of phenolic acids on preadipocyte mouse fibroblasts, 3T3-L1 cells were time- rather than dose-dependent, with mild cytotoxicity observed at 72 hours. The observed anti-adipogenic effects were not directly linked to cytotoxic activity. Notably, mixtures containing 4-HBA, particularly at lower concentrations, inhibited the proliferation of the Caco-2 and 3T3-L1 cells.

In conclusion, Mageu derived phenolic acids possess both antioxidant and anti-adipogenic properties, with their effects modulated by concentration and influenced by additive and synergistic interactions. These elucidate the potential health benefits of Mageu and its phenolic acid constituents, contributing to the understanding of Mageu as a source of bioactive compounds with therapeutic potential.

Declaration

I, Nompumelelo Saule hereby declare that this research dissertation is my own work and has not been presented for any degree of another University

Signed:

Date: May 2024

Department of Anatomy, School of Medicine, Faculty of Health Sciences,

University of Pretoria

South Africa

Acknowledgements

I would like to extend my sincerest gratitude and acknowledgements to all who contributed to the completion of this dissertation.

Firstly, I'd like to thank my Supervisors Profs JC Serem and MJ Bester for their guidance, unwavering support, and insightful feedback throughout the duration of this research project. Their expertise, encouragement, and dedication have been instrumental in shaping the direction and outcomes of this study.

I'm deeply thankful to my fellow colleagues and friends, who were always supportive and ready to help.

A special appreciation goes to the Cell Biology lab assistants and Prof JC Serem for providing the necessary resources, and the department of Anatomy for providing the facilities, and opportunities for me to accomplish my academic and research pursuits.

I'm truly indebted and would like to express my heartfelt gratitude to my family for their unwavering love, encouragement, and understanding during this academic journey. Their encouragement and support have given me the strength and inspiration to persevere and succeed.

Finally, I thank God, without whose divine intervention I would never have been able to complete this research project.

This endeavour would not have been feasible without the collaborative assistance and encouragement of all the individuals listed above. Thank you for taking part in this trip.

Table of Contents

List of Figures

List of Tables

List of abbreviations and chemical formulae

3,4-DHBA	3,4-dihydroxybenzoic acid
3T3-L1	murine fibroblast cell line
4-HBA	4-hydroxybenzoic acid
%	percentage
˚C	degrees centigrade
µL	microliter
µL/mL	microliter per milliliter
µM	micromolar
A	
AAPH	2,2'-azo-bis(2-amidinopropane) dihydrochloride
ABTS	2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
ADME	absorption, distribution, metabolism, and excretion
AMPK	5′ AMP-activated protein kinase
ATGL	adipose triglyceride lipase
AUC	area under the curve
B	
BMI	body mass index
C	
C	contribution
C/EBPα	CCAT/enhancer-binding protein α
CA	caffeic acid
CAA	cellular antioxidant activity
Caco-2	human colon adenocarcinoma cell line
CAT	catalase
CHD	coronary heart disease
CO_2	carbon dioxide
CV	Crystal Violet
CVD	cardiovascular disease
D	
ddH_2O	double distilled water
DCFH-DA	dichlorofluorescein diacetate
DEX	dexamethasone
DMEM	Dulbecco’s modified Eagle’s media
DNA	deoxyribonucleic acid
E	
EC25	quarter maximal effective concentration
EC50	half maximal effective concentration
Em	emission wavelength
ET	electron transfer
EtOH	ethanol
Ex	excitation wavelength
F	
FA	ferulic acid
Fasn	fatty acid synthase
FBS	fetal bovine serum
F-C	Folin-Ciocalteu
FCS	fetal calf serum
G	
GA	gallic acid
GAE	gallic acid equivalence

GNA	*Galanthus nivalis* agglutinin
GPx	glutathione peroxidase
H	
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
I	
IBMX	isobutylmethylxanthine
K	
$K_2S_2O_8$	potassium persulfate
L	
L929	mouse fibroblast non-cancerous cell line
LDL	low density lipoprotein
Lpl	lipoprotein lipase
M	
MAPK	mitogen-activated protein kinase
MTT	3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
N	
NaCl	sodium chloride
Na_2CO_3	disodium carbonate
$NaHCO_3$	sodium hydrogen carbonate
NaOH	sodium hydroxide
$Na_2(PO_4)_3$	disodium triphosphate phosphate
NCDs	non-communicable diseases
Nile red	9-diethylamino-5H-benzo[a]phenoxazine-5-one
O	
O_2	oxygen
OH-	hydroxyl radical
ORAC	oxygen radical absorbent capacity
ORO	Oil Red O
P	
p-CA	p-coumaric acid
PI3K/Akt	phosphoinositide 3-kinase
PlasDIC	polarisation-optical transmitted light differential interference contrast
PPAR γ	peroxisome proliferator activated receptor γ
PBS	phosphate buffered saline
R	
rpm	revolutions per minute
RNS	reactive nitrogen species
ROS	reactive oxygen species
S	
Scd1	stearoyl-CoA desaturase 1
SEM	scanning electron microscopy
SOD	superoxide dismutase
T	
T2D	type 2 diabetes mellitus
TE	Trolox equivalence
TEAC	Trolox equivalent antioxidant capacity
TEM	transmission electron microscopy
U	
UCP1	uncoupling protein 1
W	
WHO	World Health Organisation

Chapter 1. Introduction

Previously, African diets consisted largely of whole grains, cereals, legumes, and leafy vegetables. Presently, there has been a significant shift in diet patterns, as African households consume more energy dense foods containing calorie-based sweeteners, fats, oils, and products that are high in saturated fats such as, animal-based products. These diets are classified as “Western diets” and have led to an increase in adiposity, while a decrease in the intake of high fibre plant-based foods, has led to a deficiency/imbalance in essential nutrients (Mbogori & Mucherah, 2019).

Africa is particularly plagued with the double burden of malnutrition, the coexistence of undernutrition and obesity. This poses a burden on communities, households, and individuals, where for example, there are households with obese parents and undernourished children, or obese individuals with micronutrient deficiencies. Children who suffer from nutritional deficiencies from infancy, often have developmental challenges such as, stunting (WHO, 2018, Mbogori & Mucherah, 2019). Malnutrition, under- and overnutrition, is the cause of chronic metabolic and degenerative diseases such as diabetes type 2 (T2D), obesity, cardiovascular disease (CVD), cancer and osteoporosis. In addition, the associated diets lack essential molecules, such antioxidants, that scavenge ROS and prevents oxidative stress that contributes to chronic metabolic and degenerative diseases including T2D (Liu e*t al.,* 2018).

Dietary intervention can prevent many of these diseases from developing and can delay the requirement of pharmacotherapy intervention. As such functional foods, that contain nutritional ingredients with the ability to affect bodily functions and reduce risk of disease, reinforce the ‘prevention’ rather than ‘therapy’ strategy of improving human health (Minatel *et al.,* 2017; Proestos, 2018). Traditional diets contain functional foods, and the reintroduction of these foods would have health benefits. However scientific evidence is lacking on the health benefits and the associated bio-active molecules present in these foods. Processing of foods such as fermentation and digestion further alters the levels of these functional molecules and interactions between bioactive molecules would further affect the biological activity (Nathu *et al.,* 2021; Knez *et al.,* 2023). Such a product is Mageu, a fermented sour beverage indigenous to southern Africa. In a study by Nathu *et al.*, 2022, Mageu was identified to have cellular antioxidant, anti-adipogenic and anti-inflammatory properties. Identified phenolic acids potentially contributing to activity were FA, CA, 3,4-DHBA, 4-HBA and p-CA with 3,4-DHBA, 4-HBA, and FA being the most abundant following simulated digestion. The aim of this study was to evaluate the antioxidant, anti-adipogenic, cytotoxicity and the related polyphenol-polyphenol interactions of 3,4-DHBA, 4-HBA, and FA.

Chapter 2: Literature review

2.1. Introduction

Food is a critical need for human survival and for centuries, humans have struggled to find and consume enough food to satisfy their caloric and nutritional requirements. The aim was not only to ensure survival, but also to optimize reproduction and productivity. In the past century however, with increased access to inexpensive food and a decreased burden of communicable diseases, there has been an increase in adiposity amongst the human species (Caballero, 2012). Global dietary trends have shown decreased consumption in fruits, vegetables and cereals, and increased consumption of refined grains, sugars, and animal food products. This has resulted in a global epidemic; where 39% of the adult population is overweight and 13% are obese, inevitably the risk of non-communicable diseases (NCDs) such as, T2D, CVD and cancer is increased (Caballero, 2012; Nishida *et al*., 2017).

The relationship between nutritional status and adult NCD has been observed through epidemiological studies; indicating that early life under- and overnutrition are important risk factors for diabetes, CVD, and respiratory disorders (Caballero, 2012). In traditional African communities, the rates of CVD are significantly lower than those seen in the African American populations. The differences in rates seen among urban vs rural, high-income vs low-income countries indicate that the main determinant of disease is not genetics but rather environmental factors such as diet and lifestyle (Camps & García-Heredia, 2014). As the commonest cause of morbidity and mortality in the world, studies have indicated that these diseases share common pathophysiological manifestations. Both inflammation and oxidative stress intricately contribute to the onset and development of NCDs. For this reason, nutritional manipulation of oxidants and inflammation, potentially can significantly reduce NCD associated morbidity and mortality. This is substantiated by a randomized epidemiological study, that evaluated the impact of dietary changes on the risk of diabetes in high-risk adults. It was found that those assigned to a dietary and weight loss program, lowered their risk of T2D by 50% (Willett *et al*., 2006).

Although it is well established that dietary alterations can have positive effects on health, the relationship between specific diets/ foods such as functional foods, and NCDs are increasingly being investigated (Adefegha, 2018; Ramezani-Jolfaie, & Mohammadi, 2023). Six dietary changes that have beneficial health implications are firstly that saturated and trans fats should be replaced with unsaturated fats. Secondly, a generous portion of fruits and vegetables, with enough folic acid intake should be consumed. Furthermore, consumption of sugar, sodium consumption and an excessive intake of calories must be limited. Lastly, the consumption of functional foods, such as cereal products, in the high-fibre form should be encouraged (Willett *et al*., 2006).

South Africa, much like other developing countries, is battling with the double burden of malnutrition that includes overnutrition and undernutrition and the continued increase in the prevalence of obesity, it can be concluded that existing therapeutic strategies are insufficient and/or inefficient in mitigating this challenge (Seyedsadjadi & Grant, 2020). This literature review will thus aim to evaluate the association between malnutrition and oxidative stress and the development of NCDs. In addition, the role of functional food products, such as Mageu in addressing these issues will be discussed.

2.2. Malnutrition

Malnutrition is an umbrella term used to address several different conditions and generally is imbalances in an individual's nutrient or energy intake. It is associated with wasting, stunted growth, inadequate vitamins or minerals, obesity, and other diet related NCDs. Thus, malnutrition can be divided into four sub-groups; these are undernutrition, micronutrient- related malnutrition, overnutrition and diet-related NCD (WHO, 2018). Malnutrition is a significantly greater concern in developing countries where both undernutrition and overnutrition co-exists and is known as the double burden of malnutrition. In South Africa, due to socio-economic factors, there is an increase in the prevalence of this 'double burden' phenomenon. In a study conducted in the North-West province including 1040 rural black women, it was found that most of them had a lower body mass index (BMI) compared to urban black women. This was due to their consumption of a less fatty diet, possibly due to a lower income and increased physical activity. In contrast, among the urban population it was found that 56.4% and 49.3% of white and black men respectively were either overweight or obese. For urban woman, this was 74.6% and 42% respectively for black and white woman. For Asian men and women, the obesity prevalence of 35% and 37% respectively (Kruger *et al.,* 2005).

2.2.1. Forms of malnutrition

2.2.1.1. Undernutrition

Undernutrition is defined as the insufficient intake of nutrients and energy required to meet an individual's caloric and nutrient needs, to maintain good health (Saunders *et al.,* 2015). There are several indicators used to determine the nutritional status of an individual. These include the measurement of physical function, dietary intake, body composition, metabolic processes, and biochemical compounds. The most used indicators include the anthropometric measurements of weight and height in relation to sex and age and are then used to describe and classify individuals or populations according to their nutritional status (Maleta, 2006). These are used to classify individuals as follows. Low weight for height, also referred to as wasting, indicates recent and severe weight loss attributed to either an insufficient intake of food or a condition such as diarrhoea, that causes a dramatic weight loss. Low height for age or stunting, is chronic undernutrition, associated with poor maternal care due to poor socioeconomic conditions and or frequent illness. Lastly low weight for age individuals is known as underweight and may be due to wasting, stunting or both (Maleta, 2006; WHO, 2018).

2.2.1.2. Overnutrition

Overnutrition is defined as a form of malnutrition arising from the excessive nutrient intake which leads to increased accumulation of fat that eventually impairs health (Pant & Vaidya, 2018). The BMI is a measure of weight to height and is used as an indicator to determine whether an individual is overweight or obese. This parameter is not an accurate measure of adiposity, because it includes muscle mass. However, epidemiological studies have shown that all or most cardio-metabolic conditions, cancer and cardiovascular morbidity starts to rise when the BMI is over 25 (Flier & Maratos-Flier, 2018). The risk of over nutrition/obesity is dependent on and varies with regards to urbanization, economic growth, food security and living conditions. Globally individuals consume foods high in fats and sugars and engage in less physical activity, have a higher risk for NCDs such as, T2D and hypertension (WHO, 2018; Mathur & Pillai, 2019).

2.2.1.3. Diet-related non-communicable diseases

Diet-related non-communicable disease (NCDs) are diseases which are caused by common modifiable risk factors such as, poor nutrition, unhealthy diets, obesity, and a lack of physical activity. These diseases include diabetes, cancer, CVDs, and respiratory diseases and are the leading cause of premature death; accounting for a third of the world's deaths. In 2016 it was reported that NCDs contributed to 16 million global deaths and was predicted to worsen in the future. The World Health Organisation (WHO) predicted based on the forecast of the year 2016, that by 2020 NCDs would contribute to 73% of global deaths and 60% the burden of disease (Spiers *et al*., 2016; WHO, 2018). This is supported by the present data which shows that in the year 2022, NCDs kill 41 million people every year, contributing to 74% of global deaths (WHO, 2022).

Double burden of malnutrition

In 2014, 1.9 billion adults aged 18 years and older were reported to be overweight with 600 million being obese. In contrast, 462 million of the world's population are underweight. Of the overweight population, 42 million children under the age of 5 were reported to be overweight/ obese while 156 million were classified as underweight and were affected by stunting. This is referred to as the double burden of malnutrition; where, under- and overnutrition and associated NCDs coexist in populations due to the changing global nutrition landscape which, is influenced largely by various socioeconomic conditions (WHO, 2018).

The double burden of malnutrition can occur at an individual, household or population level. At the individual level, an individual may be classified as obese and yet have a deficiency of several nutrients or may be obese yet during childhood suffered from undernutrition and was stunted. At the household level, a mother and child may be over- and underweight respectively. Lastly, at the population level, over and undernutrition are prevalent in the same region or nation (WHO, 2018).

2.3. Reactive oxygen species related diseases

Chronic oxidative stress due to an overproduction of ROS has been implicated in the development of atherosclerosis, gastrointestinal dysfunction, osteoporosis, diabetes mellitus, chronic obstructive pulmonary disease, CVDs, hypertension, neurological diseases such as Alzheimer's and Parkinson's disease, cancer, and rheumatoid arthritis (Mohamed, 2015). Studies have shown that patients who present with malnutrition and antioxidant deficiencies have increased risk of disease development and poor treatment outcomes. This is attributed to the fact that antioxidant deficiency and malnutrition – a deficiency in essential nutrients- renders the individuals more vulnerable and susceptible to oxidative stress than their healthier counterparts, thus increasing their risk of disease occurrence (Mohamed, 2015; Liu, 2018).

2.3.1. Reactive oxygen species

Reactive oxygen species are the undesirable by-products of essential metabolic processes. The reduction of molecular oxygen by electron-transfer reactions results in ROS production and includes free radicals such as the hydroxyl radical (OH-), superoxide anion (O_2); as well as non-radicals such a hydrogen peroxide (H_2O_2). Free radicals are molecules which, possess highly reactive unpaired electrons thus making them unstable and as such can either donate or receive/ accept an electron from other molecules; thus, behaving as either oxidants or reductants. Reactive oxygen species, free radicals, and reactive nitrogen species (RNS) have both beneficial and detrimental effects. Low to medium concentrations of ROS are needed for the regulation of cellular responses to infections, mitogenic response induction and in the regulation of various cellular signalling pathways. The presence of excess free radicals causes oxidative stress and structural changes to biologically essential molecules such as proteins, DNA, lipids, and carbohydrates; and this leads to cellular dysfunction contributing to the development of disease (Lobo *et al.,* 2010; Sharma *et al.,* 2012; Mohamed, 2015).

Antioxidants regulate ROS levels which are either produced endogenously or externally supplemented via the diet (Lobo *et al.,* 2010; Sharma *et al.,* 2012; Mohamed, 2015). Therefore, oxidative stress is the term used to describe the oxidative damage that occurs because of an imbalance between the production of free radicals and antioxidant defences.

2.3.2. Antioxidants

Antioxidants are compounds, which play an important role in the regulation of cellular oxidation and act to either prevent or delay cellular damage. Antioxidants protect cells against cell oxidation and free radical damage by scavenging ROS. The balance between pro-oxidants and antioxidants alleviates oxidative stress and helps prevent degenerative diseases (Mohamed, 2015; Masisi *et al.,* 2016).

The mechanisms of action of antioxidants consists of three lines of defence. The first is referred to as the preventative defence, where antioxidants suppress the formation of free radicals. This is achieved through the reduction of H_2O_2 to water and oxygen without the generation of free radicals. The second line of defence involves scavenging of active radicals and the third line of defence involves the repair and *de novo* antioxidants, which remove oxidatively modified proteins and prevent the accumulation of oxidised proteins (Lobo *et al.,* 2010).

Dietary antioxidants play a significant role in supplying and reducing the depletion of endogenous antioxidants. The concentration and bioavailability of exogenous antioxidants can be affected by handling, storage, and processing. Different antioxidants have varying effects, some have additive while others have synergistic effects (Mohamed, 2015; Masisi *et al.,* 2016; Liu, 2018).

2.3.2.1. Polyphenols

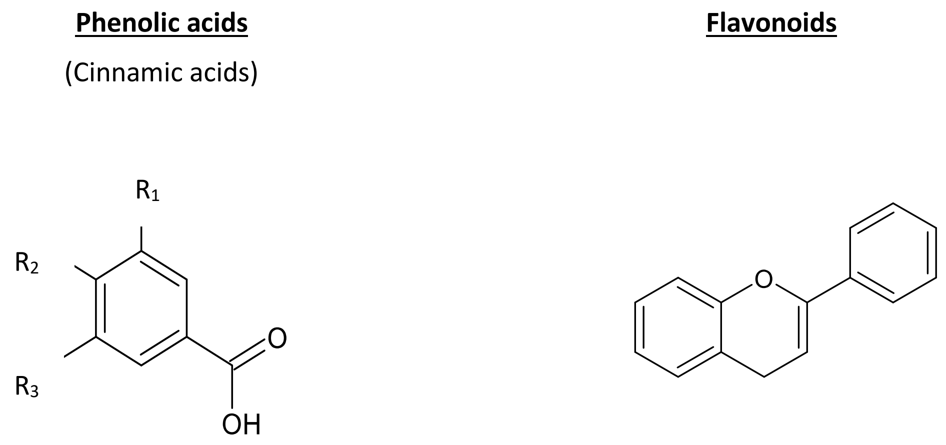
Polyphenols are important phytochemicals naturally produced in plants with a phenolic ring structure. These molecules arise from secondary metabolism in plants and are responsible for the protection of plants from diseases and degenerative damage. Dietary sources (Table 2.1) include vegetables, fruits, cereals, seeds, oils, and coffee (Mohamed, 2015). Polyphenols are strong antioxidants with the ability to prevent oxidative stress by scavenging and eliminating free radicals produced in the body; thereby, preventing and or decreasing the risk of diabetes, cancer, CVD, osteoporosis, and inflammation. In addition, polyphenols are multifunctional and have anticancer, antithrombotic, anti-infection, antimicrobial, immunity enhancing and liver-protecting properties (Amawi *et al*., 2017; Tian *et al.,* 2019).

Table 2.1: **Examples, functions and sources of several polyphenols (Mohamed, 2015)**

Polyphenols	**Functions**	**Sources**
Hydroxytyrosol	Antioxidant	Olives and olive oil
Curcumin	Anti-inflammatory and modulation of gene transcription	Turmeric
Lycopene	Antioxidant, carotenoid - anti-carcinogen	Tomatoes and other fruits
Resveratrol	Anti-inflammatory, anti-thrombotic, antioxidant, anti-carcinogenic	Nuts and red wine
Anthocyanins	Antioxidant	Berries
Phlorotannin	Potent antioxidant	Seaweeds
Isoflavones and flavonoids	Antioxidant properties, favourable effects on cancer and CVDs	Soy, flaxseed oil, fruits and vegetables, whole grains
Catechins	Anti-adipogenic, antioxidant	Green tea

The different classes of polyphenols are the phenolic acids, flavonoids, stilbenes and lignans. The classification of polyphenols into these classes is dependent on the number of phenolic rings and the formed ring structure (Figure 2.1). Polyphenol structure is important as it determines bioavailability and interactions with other bioactive compounds.

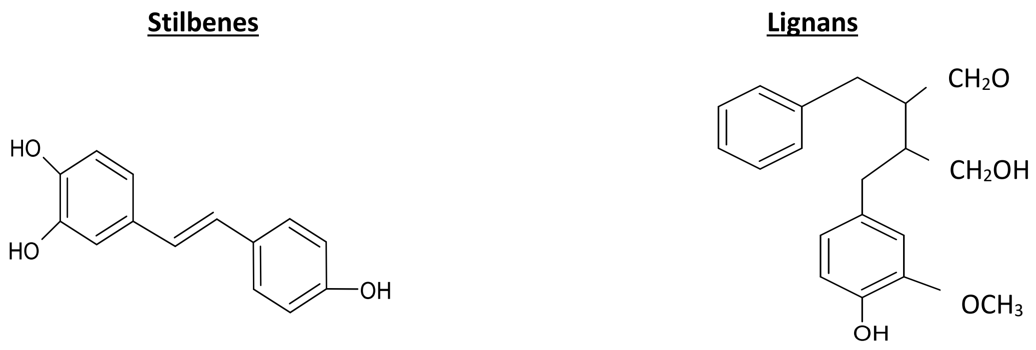
Flavonoids are the largest group, subdivided into flavanols (e.g., catechin), flavones (e.g., apigenin), flavonols (e.g., quercetin), isoflavones (e.g., genistein), anthocyanins (e.g., cyandin) and flavanones (e.g., naringenin) (Amawi *et al*., 2017). Phenolic acids are the second largest group and are predominantly found in teas and cereals and are classified as benzoic and cinnamic acid derivatives, examples include, FA, gallic acid (GA) and caffeic acid. Stilbenes are not readily found in plants; and are only produced upon pathogen invasion. Lignans are found predominantly in flaxseed products occurring either as secoisolariciresinol or matairesinol (Amawi *et al*., 2017).



Phenolic acids

(Cinnamic acids)

Flavonoids



Stilbenes

Lignans

Figure 2.1.The structure of the main classes of polyphenols (ACD/ChemSketch, 2023). These are the phenolic acids (where R1, R2 and R3 represent a carbon-based group), the flavonoids, stilbenes and the lignans.

2.3.3. Polyphenols as antioxidants

Polyphenols are the most abundant natural antioxidants found commonly in plants (Khan *et al.*, 2019). Dietary polyphenols the main sources of which are plant derived beverages, fruits, tea, legumes, chocolate, red wine etc., are potent antioxidants with the ability to scavenge free radicals and prevent oxidative cell damage (Table 2.1) (de Mello Andrade & Fasolo, 2014). Although it is understood that polyphenols and other antioxidants protect cells against oxidative damage through free radical scavenging; it is more likely that cells respond to polyphenols through direct receptor interaction or through interactions with enzymes involved in signal transduction; thus, triggering redox-dependent reactions within the cell, which in turn, results in the modification of the redox status of the cell (Scalbert *et al.,* 2005). The antioxidant activity of polyphenols is thus not limited to scavenging of ROS. Antioxidants also modulate cell signalling and gene expression as well as upregulate detoxification and antioxidant enzymes (Table 2.1) (de Mello Andrade & Fasolo, 2014).

Research on the antioxidant properties of polyphenols has provided evidence that dietary polyphenols contribute to the prevention of CVDs, neurodegenerative diseases, cancer, and other age-related disorders. This is attributed to the role of oxidative stress in the pathogenesis of these diseases. It has established that some polyphenols, whether from dietary intake or supplements, improve health status (Khan *et al.*, 2019). The is attributed to the antioxidant and pro-oxidant effects of polyphenols on cellular processes. As antioxidants, cell survival is improved while as pro-oxidants, apoptosis is induced thereby preventing the growth of tumour cells. The activities of pro-oxidants are however not fully understood; thus, more research needs to be done to elucidate their modes of action in relation/comparison to antioxidants (Khan *et al.*, 2019). Despite the considerable body of literature which supports these findings, the knowledge of dietary polyphenols in relation to their effect on specific diseases is still too limited for the formulation of recommendations to the public population (Scalbert *et al.,* 2005).

2.3.4. Anti-adipogenic effects of polyphenols

Fat cells or adipocytes are found in different regional depositories of the body and are adapted to store excess energy as triglycerides. When required, the stored energy is released as fatty acids mediated by numerous enzymes and signalling pathways, such as the endocrine and neural pathways. With starvation, this physiological system supplies the body with energy from its stored reserves for several months. Although this homeostatic physiological system is necessary to maintain balance, in the modern day of nutritional abundance and low labour lifestyles, health complications arise due to the excess adipose tissue storage, resulting in an individual being overweight and/or obese (Flier & Maratos-Flier, 2018).

Research has shown that polyphenolic compounds such as, caffeic acid, resveratrol and catechin can inhibit adipogenesis, induce adipocyte apoptosis, reduce inflammation, and increase lipolysis, leading to weight loss and improved maintenance. Consequently, the prevention and alleviation of diabetes, CVDs and other obesity related metabolic disorders is increased (Song *et al*., 2017).

In an *in vitro* study, that investigated the anti-adipogenic activity of the phenolic compounds, it was found that catechin inhibited adipocyte differentiation during early stage adipogenesis, while rutin reduced intracellular triglyceride/ adipocyte accumulation (Song *et al*., 2017). Other cell-based studies have shown that polyphenols modulate signalling pathways involved in the regulation of adipogenesis, anti-inflammatory and antioxidant responses. These include the adenosine monophosphate (AMP)-activated protein kinase, peroxisome proliferator activated receptor γ, the sterol regulatory element binding protein-1c and the peroxisome proliferator activator receptor gamma activator 1-alpha (Wang *et al.,* 2014). Adipogenesis is regulated by mainly two transcription factors, the CCAT/enhancer-binding protein α (C/EBPα) and the peroxisome proliferator activated receptor γ (PPAR γ), both of which can be modulated by dietary polyphenols (Song *et al*., 2017). Lastly, polyphenols have been shown to have protein-binding properties which inhibit lipid, protein and starch digestion in the intestinal tract leading to better glycaemic control (Cory *et al.,* 2018).

Animal studies have supported the cellular studies by strongly suggesting that dietary polyphenols have pronounced anti-adipogenic effects. This was shown by lowered body weight, lower triglycerides, increased fat oxidation and enhanced modulation of glucose haemostasis in subjects. Human based studies are few, and the information obtained is inconsistent due to differences in study designs, duration lengths, chemical forms of the polyphenols and contributing factors such as other weight-reducing compounds (Cory *et al.,* 2018).

2.4. Functional foods

Multiple foods have received scientific attention for their physiological benefits and effect on the optimization of human health. Although all foods may be considered as functional as all foods provide nutritional value, functional foods are characterised as a food or food ingredient/s which as part of a varied diet are consumed on a regular basis, which provide therapeutic benefits beyond its nutritional content (Hasler, 2002). Roberfroid (2002), defined functional foods as foods with a variety of nutrients (both classified and unclassified), which can affect a variety of bodily functions, resulting in the reduction of disease risk and an increase in well-being and health of an individual. Thus, it can be concluded that a functional food must remain as a food consumed in a diet and not in a pill or capsule and it must demonstrate beneficial effects beyond adequate nutritional effects, and in a way that is relevant in the reduction of risk of disease and improvement of health (Roberfroid, 2002).

From 2500 years ago during the time of Hippocrates, the father of medicine, food has been seen as medicine, “Let food be thy medicine and medicine be thy food”. However, with the rise in drug therapy, this philosophy has fallen into obscurity. Recently, it has been found that malnutrition-induced antioxidant deficient patients respond poorly to drug therapy or synthetic antioxidants. This has increased the search for natural non-toxic therapeutic compounds, hence the focus on functional foods. Increased intake/consumption of foods with functional attributes including high levels of antioxidants is a concept and/or strategy that is gaining importance (Hasler, 2002; Lobo *et al*., 2010; Liu *et al*., 2018).

Studies have established links between the consumption of vegetables, fruits and fibres and the lower incidence/occurrence of various degenerative diseases such as cancer, CVD, cataracts, and neural tube defects (Ortega, 2006). Examples of whole foods as functional foods, the associated benefits identified in various studies as well as the recommended intake is summarised Table 2.2 below (Lobo *et al*., 2010).

Table 2.4: Functional foods their bioactive components, health benefits, scientific evidence, and recommended consumption (Hasler, 2002)

Functional food	Bioactive component	Health benefit	Type of evidence	Recommended amount/intake frequency
Fortified margarines	Plant sterol and stanol esters	Reduce total cholesterol (TC) and low-density lipoprotein cholesterol (LDLC)	Clinical trials	1.3 g/d for sterols 1.7 g/d for stanols
Soy	Protein	Reduce TC and LDLC	Clinical trials	25 g/d
Whole oat products	β – Glucan	Reduce TC and LDLC	Clinical trials	3 g/d
Cranberry juice	Proanthrocyanidins	Reduce urinary tract infections	Small number of clinical trials	300 mL/d
Green tea	Catechins	Reduce risk of certain types of cancer	Epidemiological	Unknown
Spinach, kale, collard greens	Lutein/ zeaxnthin	Reduces risk of age-related macular degeneration	Epidemiological	6 mg/d
Fermented dairy products	Probiotics	Supports gastrointestinal health, boost immunity	*In vivo* and *in vitro* studies, limited clinical data	Daily
Maize products	Probiotics and polyphenols	Supports gastrointestinal health, boost immunity	*In vivo* and *in vitro* studies, limited clinical data	Unknown

*g/d – grams per day, mL/d – millilitres per day.

2.4.1. Superfoods

Conventional foods that have multiple unique properties and foods that are natural or medium processed and are high in nutrients and other bioactive molecules, are considered superfoods. An example is goji berries, which are a rich source of proteins, minerals, immune-stimulating polysaccharides, antioxidants; they also contain liver-cleansing betaine and anti-aging sesquiterpenes (Wolfe, 2009; Gupta & Mishra, 2021). The bioactive ingredients associated with these health benefits are probiotic micro-organisms, amino acids, antioxidants, essential minerals and vitamins, and various enzymes. The most important bioactive ingredients found in superfoods are antioxidants and include flavonoids, lycopenes, uric acid, selenium, vitamins A, C and E, β-carotene, albumin, and polyphenols such as anthocyanidins (Proestos, 2018). Several of these compounds may act synergistically, enhancing health benefits although further research is required to fully understand the associated cellular mechanisms (Wolfe, 2009, Mitra *et al*., 2023).

2.4.2. Nutraceuticals and supplements

Nutraceuticals are foods or extracted food items which have been scientifically proven to possess health benefits for the treatment and prevention of disease. Also defined as dietary supplements, nutraceuticals provide a concentrated form of a bioactive compound, in a nonfood matrix, with the aim of improving health. These range from isolated nutrients to dietary supplements, ingested in pill or capsule form, to processed dietary products such as cereals, soups, and beverages. The major nutraceutical ingredients found in plant derived products are polyphenols that act as potent antioxidants and depending on the antioxidant, may also have anti-lipogenic, anti-inflammatory, antithrombotic, anticarcinogenic, antiallergic and hepatoprotective activities (Lobo *et al*., 2010). Although some dietary supplements have been proven to be beneficial, many may contain unknown potentially toxic substances. Also, the interaction of the nutraceutical ingredients with prescription drugs can lead to side effects, with an associated risk to a patient (Halsted, 2003). Although superfoods and nutraceutical products have beneficial effects these are often expensive and availability is limited, therefore functional foods, especially those that are widely available and traditionally consumed are a cheaper option for the prevention of NCD.

2.5. Maize

Maize (*Zea mays L.)* is an ancient cereal, which is thought to be a form of two-granule wheat (*Triticum turgidum ssp. Dicoccum)*. It is the third leading crop in the world with production reaching 967 million metric tons in a year (Shah *et al.,* 2016). While not originating in Africa, for thousands of years it has been the most consumed cereal in North and East Africa. To this day, maize constitutes a large part of the average African diet and is a staple ingredient in most households. It is low in gluten, and high in amino acids such as lysine (Proestos, 2018) and one tablespoon of maize oil can satisfy the nutrient intake of dietary essential fatty acids for both healthy children and adults. In addition, maize contains an abundance of bioactive compounds such as polyphenols, carotenoids, and phytosterols. The types and concentrations of polyphenols, carotenoids, and phytosterols found in maize is listed in Table 2.3 (Shah *et al.,* 2016).

Cereals are an important source of dietary polyphenols. The phenolic content of cereals differs with maize having the highest total phenolic content (TPC) of 15.55 µg/g and the TPC of whole wheat, oats and rice being 7.99 µg/g, 6.53 µg/g and 5.56 µg/g respectively (Tian *et al.,* 2019). The most abundant phenolic acids in maize are FA and the anthocyanins (Table 2.3). Although the phenolic content of maize is high, the bioavailability of these polyphenols *in vivo* is limited (Gong *et al*., 2013); as many of the polyphenols in wheat are bound to other constituents within a solid food matrix. Nonetheless, these can be released during processing, fermentation, and digestion.

Table 2.5: The concentration of phytochemical compounds in 100 g maize (Shah *et al*., 2016)

Compounds		Concentration (mg/100 g)
Carotenoids	Carotene	2.20
	Xanthophylls	2.07
	Lutein	1.50
	Zeaxanthin	0.57
Polyphenols	Ferulic acid (FA)	**174.0**
	Anthocyanins	**141.7**
Phytosterols	Sitosterol	9.91
	Stigmasterol	1.52
	Campesterol	3.40

* Items highlighted in bold > 100 mg

2.5.1. Health benefits of maize

Maize is associated with various health benefits and therefore is often recommended as a contributory ingredient in the everyday diet. Maize contains vitamins A, C and K that act synergistically with beta-carotene and selenium to improve the immune system, by regulating the function of the thyroid gland (Shah *et al*., 2016). Vitamin B complex, is associated with improved joint motility and general health. In several Eurasian countries, maize as a functional food is also used in the treatment of urinary tract infections, fluid retention, kidney stones and jaundice. In addition to this, maize has shown to have anti-emetic effects (Shah *et al.,* 2016). The anti- Human immunodeficiency virus activity of maize is attributed to the presence of *Galanthus nivalis* agglutinin (GNA) lectin. Lectin proteins can bind to carbohydrates and carbohydrate receptors on cell membranes and consequently for some micro-organisms, such as the HIV, when the lectin protein binds to sugars or their receptors, potentially inhibiting viral activity (Shah *et al*., 2016).

2.6. Mageu

Mageu is a southern African traditional sour non-alcoholic fermented maize derived beverage. It is a popular beverage that significantly contributes to the caloric intake of a large part of the southern African population. The popularity of the beverage is reflected in the variety of indigenous names it is known as and although commonly known as Mageu, which is the shortened version of the Zulu name “amahewu”, other names include “amarhewu” (Xhosa), “machleu” (Sotho), “emahewu” (Swati), “maphulo” (Venda) and “metogo” (Pedi). This beverage may even be related to the traditional East African lactic acid fermented cereal beverage “uji” as this product contains maize as well and is described as a “yoghurt-like product” (Holzapfel & Taljaard, 2004; Nyanzi *et al.,* 2010).

Mageu has a pH of 3.5 and is prepared by boiling 8-10% of maize porridge solids into which a small amount of wheat is mixed. The wheat acts as an inoculation of lactic acid bacteria, increasing the final titratable acidity of the beverage to 0.4-0.5% (Holzapfel & Taljaard, 2004).

Mageu is commercially available and is widely consumed during the day as part of a normal working day. As it is cereal based, it has low biological value with only 7-9% proteins and lysine are deficient. One litre of Mageu provides 4000 kilojoules as maize meal carbohydrates. Thus, for normal relatively healthy labourers/ individuals, Mageu in addition to an adequate diet provides little to no nutritional value. However, for malnutritioned individuals, the consumption of Mageu may fortify and be of nutritional importance as it can supply nutrition deficient individuals with additional nutrients and probiotics. When fortified with skim milk, powder milk, soy flour or food yeast; one litre of Mageu can provide between 20-30% of an adult male's daily protein requirements (Holzapfel & Taljaard, 2004; Nyanzi *et al*., 2010).

2.6.1. Health benefits of Mageu

A study involving the randomized selection of 150 rural women in Zimbabwe with children under the age of five indicated that 94% of them were familiar with Mageu, and it was consumed by their families (Holzapfel & Taljaard, 2004). Most women also feed their infants the beverage from the age of 4 months. This study identified that Mageu is a safe and nutritious food for both young and old individuals and provided protection from different food-borne pathogens (Holzapfel & Taljaard, 2004). Another case study conducted during the cholera outbreak in Burundi and Zimbabwe, showed that only one food, Mageu, had the protective properties, which act to inactivate a variety of enteric bacterial pathogens, such as, Shigella, several strains of Salmonella, *Campylobacter jejuni* and Aeromonas (Holzapfel & Taljaard, 2004). The low pH and associated high acidity of Mageu, was identified to contribute to its bacteriostatic and bactericidal properties. In Mageu, *Bacillus cereus*, failed to grow (Byaruhanga *et al*., 1999), bacterial spores did not germinate, and the rate of growth of the bacteria responsible for spoilage was significantly reduced (Holzapfel & Taljaard, 2004; Nyanzi *et al.,* 2010).

Many nutritional benefits have been linked to cereal-based beverages and porridges. These include increased protein quantity, quality and digestibility, increased vitamin levels, improved mineral bioavailability, decreased anti-nutritional factors such as tannins, great source of probiotic microorganisms and a production of bacteriocins and prebiotics (Taylor, 2003). Likewise, Nathu *et al.,* in 2021 identified in a cell-based study additional health benefits of Mageu related to antioxidant activity, inhibition of adipocyte development and anti-inflammatory properties.

2.6.2. Phenolic acids present in Mageu

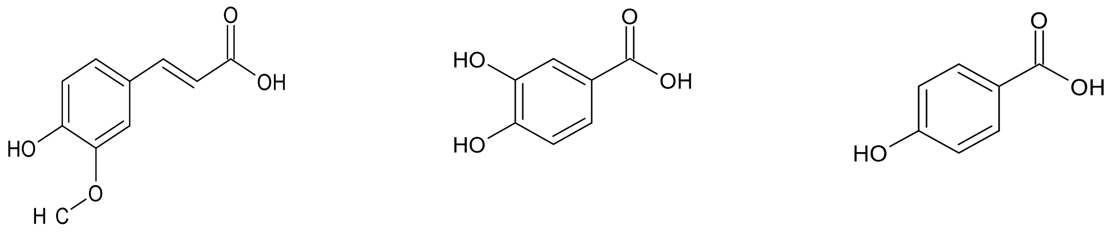
Studies have shown that the antioxidant capacities of most plant-derived products often correlate with their phenolic content (Proestos, 2018). Recently, it has been observed that the bioactivity of Mageu is in part due to the presence of the phenolic acids, such as, 3,4-DHBA, p-CA, 4-HBA, FA and CA (Nathu, 2019).

The hydroxycinnamic acid derivatives, CA, p-CA, and FA have potent antioxidant, antineoplastic and anti-inflammatory molecules (Boz, 2015; Taofiq,2017). Both, p-CA and FA have been found to decrease low density lipoprotein (LDL) peroxidation and have antimicrobial activities (Boz, 2015; Medina-Vera, 2021). In addition to this, FA has also been found to decrease the levels of inflammatory mediators such as, prostaglandin E2 (Srinivasan *et al.,* 2007; Boz, 2015). Studies have shown that CA can target and inhibit the histone demethylase oncoprotein gene, which is found and amplified in squamous cell carcinoma (Ncit, 2020).

The hydroxybenzoic acid derivative, 3,4-DHBA, is also a potent antioxidant. Studies have shown it to have great protective activities against CVD and neoplasms although the mechanism of action is unknown, although it is most likely associated with the inhibition and scavenging of ROS (Wishart *et al*., 2018).

2.7. Background to study

In a previous study the flavonoids and phenolic acid content of pre- and post-digested Mageu was determined using High-performance liquid chromatography (HPLC). No flavonoids were present according to the standards used (catechin, naringenin and chrysin), and the identified phenolic acids were 3,4 dihydrobenzoic acid (3,4-DHBA), 4-hydrobenzoic acid (4-HBA), ferulic acid (FA), p-coumaric acid (p-CA), and caffeic acid (CA) (Fig. 2.2).



3,4-DHBA **4-HBA** **FA**

Figure 2.2: The structure of phenolic acids. 3,4-DHBA, 4-HBA and FA (ACD/ChemSketch, 2023).

The study found that levels of these polyphenols were increased following *in vitro* digestion, Table 2.4 (Nathu *et al*., 2021). This translated to an increase in antioxidant activity and a 40% decrease in lipid formation in 3T3-L1 differentiated adipocytes, postdigestion. In a further study, it was necessary to confirm if these phenolic acids contribute to activity and whether synergistic interactions further contribute to observed activity. The phenolic acids, 3,4-DHBA, 4-HBA and FA, that significantly increased following digestion were the focus of this study.

Table 2.4: **Phenolic acid content (mM) the water-soluble fraction of Mageu (Nathu *et al.,* 2021).**

	3,4-DHBA	**4- HBA**	***p*-CA**	**FA**	**CA**
UD	0.78 ± 0.0233	0.51 ± 0.023	0.61 ± 0.0378	0.31 ± 0.00021	0.83 ± 0.069
GDD	1.43 ± 0.0117	2.46 ± 0.0348	0.73 ± 0.0067	0.98 ± 0.01184	0.99 ± 0.024
Fold-increase	1.83**	4.86**	1.45*	3.17**	1.2*
P-value (T-TEST)	**0.004**	**0.007**	0.042	**0.004**	0.044

Undigested (UD), gastroduodenal digestion (GDD) Significance level determined using the T-TEST at 95% confidence ($p \leq 0.05$)

2.7.1. Aim

The aim of this study was to evaluate the antioxidant, anti-adipogenic, cytotoxicity and the related polyphenol-polyphenol interactions of 3,4-DHBA, 4-HBA, and FA, the most abundant phenolic acids identified in Mageu. The aims of the study were achieved using the following objectives:

To determine the EC25 and EC50 with the physiologically relevant oxygen radical absorbent capacity (ORAC) assay.

At the EC25 and EC50 determine the reducing activity capacity of 3,4-DHBA, 4-HBA, and FA Folin-Ciocalteu (F-C) assay.

To determine the antioxidant activity of 3,4-DHBA, 4-HBA, and FA at the EC25 and EC50 with the Trolox equivalent antioxidant capacity (TEAC) assay.

To determine the antioxidant activity of 3,4-DHBA, 4-HBA and FA at the EC25 and EC50 using the ORAC assay.

At the EC25, EC50, EC(2X) and EC(5X) determine the cellular antioxidant activity of 3,4-DHBA, 4-HBA and FA at the EC25 and EC50, in the human colon adenocarcinoma (Caco-2) cell line using the dichlorofluorescein diacetate (DCFH-DA) assay.

Using the above antioxidant assays identify the type of polyphenol-polyphenol interactions between 3,4-DHBA + 4-HBA, 3,4-DHBA + FA 3,4-DHBA + FA, 3,4-DHBA + 4-HBA + FA evaluated at the EC25 and EC50.

To determine quantitatively and qualitatively the anti-adipogenic properties of 3,4-DHBA, 4-HBA, FA at the EC50 and EC25 as well as the type of polyphenol-polyphenol interactions in the mouse murine (3T3-L1) cells, using the Oil red O (ORO) and Nile red (NR) assays.

To finally, investigate the effects of the most beneficial phenolic acid or combination on cellular differentiation of 3T3-L1 adipocytes with polarisation-optical differential interference contrast (PlasDIC) microscopy

To evaluate the concentration and exposure time dependent cytotoxicity of 3,4-DHBA, 4-HBA, FA at the EC50 and EC25 and the type of polyphenol-polyphenol interactions on relative human colon adenocarcinoma (Caco-2) and 3T3-L1 cell number using the crystal violet (CV) assay.

These objectives were achieved using the experimental strategy presented in Figure 2.3.

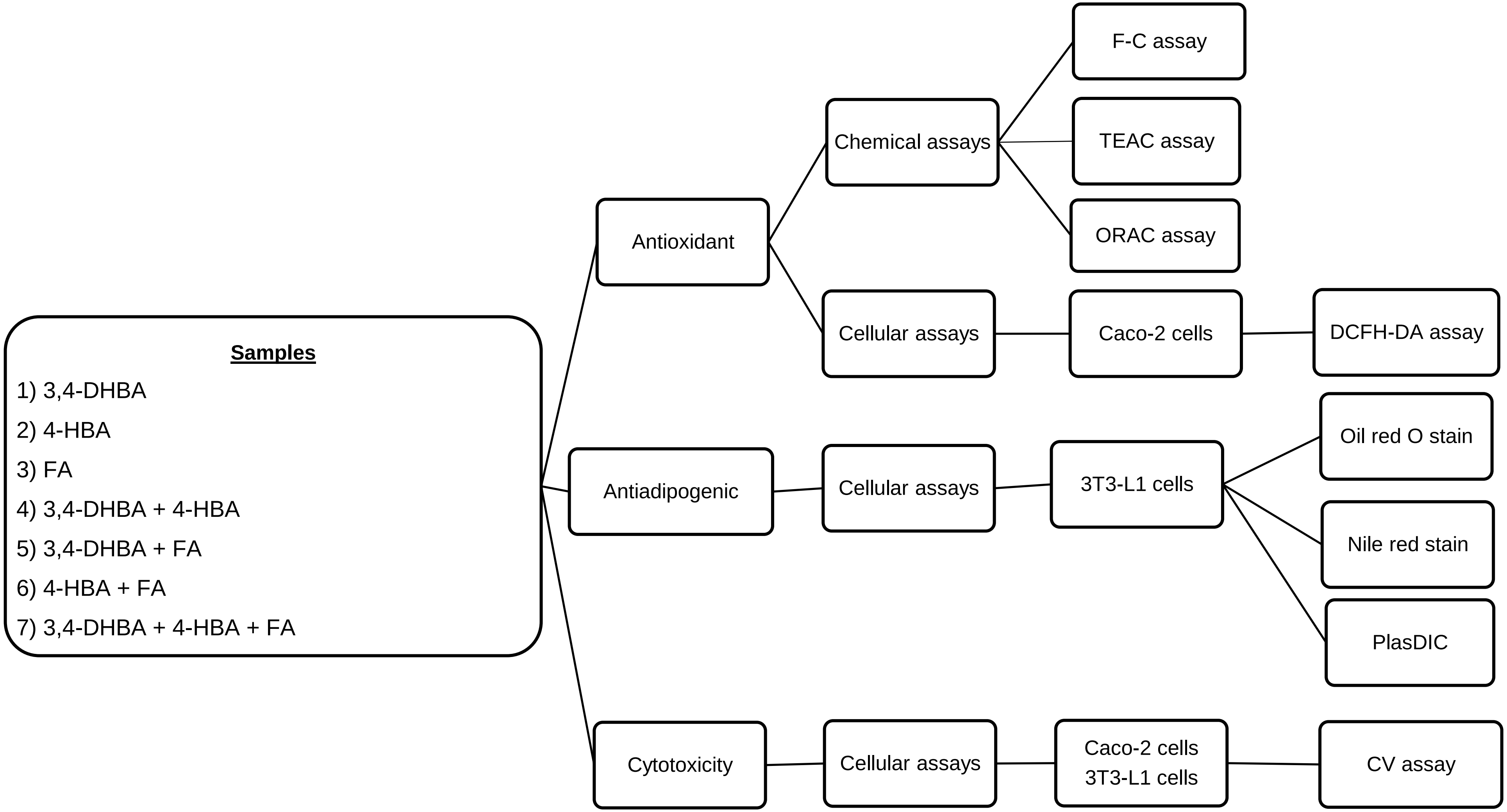


Figure 2.3:Flow diagram of experimental procedures.

Chapter 3. The antioxidant activity of the phenolic acids identified in Mageu and the associated polyphenol-polyphenol interactions

3.1. Introduction

By-products of natural biological processes are free radicals, which are molecules that contain one or more unpaired electrons (Valko *et al.,* 2007). In the maintenance of cellular and tissue homeostasis, free radicals in the form of redox reactions, destroy bacteria and regulate cell signalling pathways and as such free radicals/ ROS in moderate amounts are necessary for normal cellular functions (Sharifi-rad, 2020). In excess ROS production, due to external (pollution, radiation, toxins etc) or internal factors (intracellular metabolism), can cause cellular dysfunction and leading to the disruption of normal physiological processes, causing structural damage to cellular DNA, proteins, lipid membranes leading to cell death.

Antioxidants play a key defensive role against the harmful effects of ROS by neutralising the effect of oxidants, and repair the associated damage (Sharifi-rad, 2020). However, an excess ROS and/or low antioxidant levels can lead to oxidative stress (Young & woodside, 2001). The ability of antioxidants to break radical chain reactions is due to the heterogenous chemical structure of these molecules. Enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) either directly and/or indirectly scavenge ROS by converting already oxidized products (superoxide) to H_2O_2 and then water. Important cofactors in this process are copper, iron, manganese, and zinc (Sharifi-rad, 2020; Moussa *et al.,* 2019). Non-enzymatic antioxidants, intercept and inhibit chain free radical reactions thus preventing the formation of oxidants. Examples of non-enzymatic antioxidants include polyphenols, carotenoids, vitamin A, C and E, glutathione, and melatonin from different endogenous and exogenous sources (Moussa *et al.,* 2019).

Plant polyphenols are the most abundant exogenous antioxidants and sources include vegetables, fruits, and cereals (Rudrapal *et al.,* 2022). The most abundant polyphenols in cereals are the phenolic acids, of which FA is the most dominant component (Khan *et al.*, 2019) and accounts for 89% of the phenolic content of wheat (Khan *et al.*, 2019; Nathu *et al.,* 2021). The main mechanism of antioxidant action of these phenolic acids is attributed to their chemical structure, where the unsaturated side chains which can form resonance forms stabilised with the free radicals. In this reaction phenolic acids readily accepts a hydrogen atom and becomes a phenoxy radical (Srinivasan *et al.*, 2007; Moussa *et al.,* 2019). In addition to resonance stabilisation with free radicals, phenolic acids also can chelate with metals, thereby inhibiting lipid peroxidation (Moussa *et al.,* 2019).

The antioxidant properties of individual phenolic acids FA, 3,4-DHBA and 4-HBA are well described in literature (Chen *et al.,* 2020; Kose *et al.,*2023), little is known regarding polyphenol-polyphenol interactions with the potential for enhanced synergistic interactions leading to greater antioxidant beneficial effects (Magiera & Zaręba, 2015; Nathu *et al.,* 2021). Thus, the aim of this chapter was to determine at the EC25 and EC50 (including the EC50X2 and EC50X5 for the DCFH-DA assay) the antioxidant properties of FA, 3,4-DHBA and 4-HBA and combinations. For the double and triple combinations, determine whether synergistic, additive, or antagonistic polyphenol-polyphenol antioxidant interactions occur.

3.2. Materials

3.2.1. Cell lines

The human colon adenocarcinoma (Caco-2) cell lines were obtained from CELLONEX Separations (Johannesburg, South Africa (SA)) and is widely used in nutrition research (Glahn, 2009).

3.2.2. Reagents, equipment, glassware and disposable plasticware

Reagents and chemicals used included the Folin-Ciocalteu (F-C) reagent, hydrochloric acid (HCl), sodium chloride (NaCl) and sodium hydrogen carbonate ($NaHCO_3$) were purchased from Merck Chemicals, Modderfontein, SA. Reagents, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2'-azo-bis(2-amidinopropane) dihydrochloride (AAPH), DCFH-DA, ethanol (EtOH), Dulbecco's modified Eagle media (DMEM), fetal bovine serum (FBS), antibiotics, TrypLE Express, fluorescein, gallic acid (GA), sodium carbonate (Na_2CO_3), potassium peroxodisulfate ($K_2S_2O_8$), dimethylsulphoxide (DMSO), Trolox, 3,4-DHBA, 4-HBA and FA were obtained from Sigma-Aldrich (Johannesburg, SA).

The equipment used was the FLUOstar OPTIMA plate reader from BMG LabTech (Offenburg, Germany) and the Hermle Z300 centrifuge (Hamburg, Germany).

Glassware and plastic disposables included, 1 mL Eppendorf tubes, 96-well plates, pipette tips (10, 25, 100, 200, and 1000 µL), T-75 flasks, 50 mL, and 15 mL tubes, 0.1 - 1000 µL micro pipette tips were supplied by Greiner Bio-one obtained from LASEC (Johannesburg, SA).

3.2.3. Laboratory Facilities

All research was conducted in the research facilities of the Department of Anatomy, Faculty of Health Sciences, University of Pretoria.

3.3. Methods

3.3.1. Sample preparation

Samples and stock solutions: Stock solutions of 1 mM of 3,4-DHBA, 4-HBA and FA were prepared in ddH_2O, aliquoted and frozen at -20°C. For experiments, single mixtures (3,4-DHBA, 4-HBA, FA), double mixtures (3,4-DHBA + 4-HBA, 3,4-DHBA + FA, 4-HBA + FA) and a triple mixture (3,4-DHBA + 4-HBA + FA) were prepared each at the respective EC25 and EC50 (See Section 3.4.1).

Cell line growth and maintenance

The Caco-2 cells were maintained in DMEM with 10% FBS and 1% antibiotics (DMEM/FBS). Cells were retrieved from liquid nitrogen storage, rapidly thawed in a 37°C water bath, and subsequently transferred into a 15 mL centrifuge tube. The cell suspension was then centrifuged at 623 ×g for 2 minutes in DMEM supplemented with FBS for collection. The supernatant was discarded, and the cells re-suspended in 10mL DMEM/FBS and were then transferred to a cell culture flask and incubated at 37°C and 5% CO_2. Once confluent, cells were detached using 2 mL of TrypLE Express, for 3-5 minutes. To neutralise protease activity 5 mL DMEM/FBS was added and a 10 µL aliquot was used to determine the number of cells with a haemocytometer. The Caco-2 cells were then plated at the desired concentration and volume for each assay.

For all assays, stock solutions volumes were added to ensure that the final concentrations represent the EC25 and EC50.

3.3.2. **Determination of EC50 using the ORAC assay**

The oxygen radical absorbance is a hydrogen atom transfer assay that assesses the ability of the antioxidant to retard fluorescence quenching induced by oxidants (Prior *et al*., 2005, Litescu & Eremia, 2014). The assay was used to determine the EC50 as the ORAC assay is physiologically the most relevant antioxidant assay (Carvalho *et al.,* 2023). In addition, in the cellular DCFH-DA assay, is similar, and represents the ability of cells to scavenges AAPH generated peroxyl radicals (Nakajima *et al.,* 2013; Bardyn *et al.,* 2021).

This assay was used to determine the EC50, the antioxidant activity of the phenolic acids as well as the polyphenol-polyphenol interactions.

A modified method of Ou *et al*., (2002) was used. A 0.139 nM solution of fluorescein in phosphate-buffered saline (PBS) and 0.24 M AAPH in distilled water was prepared. In an opaque 96-well plate, to a 10 µL sample (prepared as described in Section 3.3.1), 165 µL of the fluorescein solution was added, rapidly followed by 25 µL APPH. The fluorescence was then measured every 60 seconds for 120 min, to generate 120 cycles, at excitation wavelength (Ex) of 485 nm and an emission wavelength (Em) of 520 nm, respectively. The area under the curve (AUC) was calculated, and the percentage inhibition relative to the control (APPH) only (100% oxidative damage) was calculated. The percentage reduction in oxidative damage vs concentration was plotted and the EC50 was defined as the concentration of each phenolic acid that effectively inhibited the oxidative effects of AAPH by 50% (Li *et al*., 2015). From the value for each phenolic acid the EC25 was calculated. The phenolic acid content of all solutions was 1 mM, with double mixtures, containing 0.5 mM of each phenolic acids and in the triple mixtures 0.33 mM of each phenolic acid. From this data solutions with equivalent antioxidant activity were prepared so that the final total antioxidant capacity of each solution was the same.

To investigate the phenolic acid interactions, a 1 mM stock solution of Trolox in PBS was also prepared and was used to prepare a standard curve of 0-100 µM Trolox. The antioxidant activity was then determined of the phenolic acids and the combinations with the ORAC assay as described above and the activity was expressed as µM TE.

3.3.3. The Folin- Ciocalteau assay

The Folin - Ciocalteau assay was used to determine the reducing properties of the phenolic acids and the type of polyphenol-polyphenol interactions (Prior *et al*., 2005). Activity was determined using a modified method of Amin *et al*., (2006). A 10 µL volume of the phenolic acids (prepared as described in Section 3.3.1) were added to generate final serial dilutions (0.25, 0.5 and 1X) of single, double, and triple phenolic acid solution at the EC25 and EC50. A GA standard curve of 0- 0.1 mM was prepared from a 1 mM GA stock solution. In a 96 well plate, to a 10 µL volume of sample or GA standard, 50 µL F-C reagent (diluted 15 times with ddH_2O) was added, followed by 50 µL of a 7.5% $NaCO_3$ solution. Samples were mixed well, and absorbance measured at a wavelength of 630 nm. Data was expressed as mM GA equivalents (GAE).

3.3.4. The Trolox equivalent antioxidant capacity assay

The Trolox equivalent antioxidant capacity (TEAC) assay is an electron transfer (ET) assay which is used to determine the total antioxidant capacity of a sample (Prior *et al*., 2005, Shahidi & Zhong, 2015) and in the present study was used to determine the antioxidant activity of each phenolic acid and the type of polyphenol-polyphenol interactions.

The TEAC assay was performed according to a modified procedure described by Awika *et al*., (2006). From a stock solution of ABTS (3 mM $K_2S_2O_8$; 8 mM ABTS), a 0.26 mM working solution was prepared. A standard Trolox standard curve (0.1-1 mM) was prepared from a 1 mM Trolox stock solution prepared in PBS. In a 96-well plate A10 µL volume of the phenolic acids (prepared as described in Section 3.3.1) were added to generate a final serial dilution (0.25, 0.5 and 1X) of single, double, and triple EC25 and EC50. Then 290 µL ABTS working solution was added and incubated for 15 minutes at 37˚C. The absorbance was read at 734 nm and the data was reported as µM TE.

3.3.5. The dichlorofluorescein diacetate assay in the Caco-2 cell line

In DCFH-DA assay, the probe DCFH-DA probe is used to determine intracellular radical formation. In this fluorometric assay, the non-fluorescent compound DCFH-DA is converted into a hydrophilic alcohol, Dichlorofluorescin (DCFH), which is subsequently oxidized to the fluorescent dichlorofluorescein (DCF) by ROS such as H_2O_2, lipid hydroperoxides and peroxinitrite. Consequently, DCFH is thus useful in measuring the effect of intracellular antioxidant scavenging activity which results in the inhibition of DCFH oxidation (Girard-Lalancette *et al*., 2009). In addition, as used in this study, inhibition of extracellular sources of ROS, can prevent the subsequent intracellular effects.

The cell model used in this assay is the Caco-2 cell line, derived from human colon adenocarcinoma. This cell line is typically employed to investigate the antioxidant properties of bioactive compounds and differentiated Caco-2 cells are used to simulate human intestinal tract *in vitro* (Ding *et al.,* 2021). In addition to this, Caco-2 cells are highly flexible, reliable, cost-effective and a greatly suited for the analysis of food- intestinal interactions (Angelis & Turco, 2011; Glahn, 2022).

A modified protocol used by Blasa *et al*., (2011) was used. In a 96 well plate, $2x10^4$/mL Caco-2 cells in 100 µL were plated and cultured overnight at 37°C and 5% CO_2. To the supernatant 50 µL, 75 µM DCFH-DA was added and incubated further for 1 hour. The DCFH-DA was then removed, and the attached cells were washed with 300 µL 0.1 M PBS. The Caco-2 cells were subsequently exposed to 50 µL of each sample (ensuring final concentrations of solutions prepared in 3.3.1) and 50 µL of 4 mg/mL AAPH. The fluorescence was measured at an Em of 485 nm and Ex of 520 nm for 1 hour. Negative controls were Caco-2 cells exposed to PBS only and the positive controls were cells exposed to AAPH only. Results were reported as percentage oxidative damage, with cells exposed to AAPH only representing 100% oxidative damage.

Calculation of polyphenol-polyphenol interactions

To determine if the interactions between the polyphenols were antagonistic, additive of synergistic, the differences between the experimental and expected antioxidant activities was observed. The antioxidant polyphenol-polyphenol interaction in mixtures was measured by comparing the observed experimental results to the theoretical expected results calculated by the sum of the individual polyphenols in the mixture. Additive interactions were determined to be the result of no significant difference between the two values, where the antioxidant activity is near equivalent to the sum of the individual polyphenols at a given concentration. When the observed values are significantly greater than the expected values for a mixture of polyphenols, a synergistic interaction is determined. Whereas where the observed values are significantly lower than the expected value, is indicative of an antagonistic interaction (Olszowy-Tomczyk, 2020). However, in the case of the DCFH-DA assay, a distinct criterion was applied: if the observed percentage of oxidative damage was statistically significantly lower than the expected percentage, this was indicative of a synergistic interaction. Conversely, if the observed oxidative damage was significantly higher than the expected value, it suggested antagonistic interactions within the polyphenol mixture.

3.3.6. Data management and statistics

All experiments were done at least four times in triplicate yielding 12 data points. The data was reported as the mean ± standard error of the mean (SEM). Data was then tested for normality using the D'Agostino and Pearson normality test ($p<0.05$). Data was then further analysed with one-way ANOVA with post hoc Tukey analysis for significant differences ($p<0.05$). Data is reported as mean ± SEM.

3.4. Results

3.4.1. Determination of EC25 and EC50 concentrations using the ORAC assay

The EC50 of the phenolic acids, 3,4-DHBA, 4-HBA and FA was from the ORAC dose-response curve (Figure 3.1). The graphs were linear with R^2 values of 0.992, 0,980 and 0,992 for 3,4-DHBA, 4-HBA and FA respectively. The EC50 values were 10.99 µM for 3,4-DHBA, 21.28 µM for 4-HBA, and 14.53 µM for FA. The EC25 was half the EC50 values, and the calculated values were 5.50 µM for 3,4-DHBA, 10.64 µM for 4-HBA and 7.27 µM for FA.

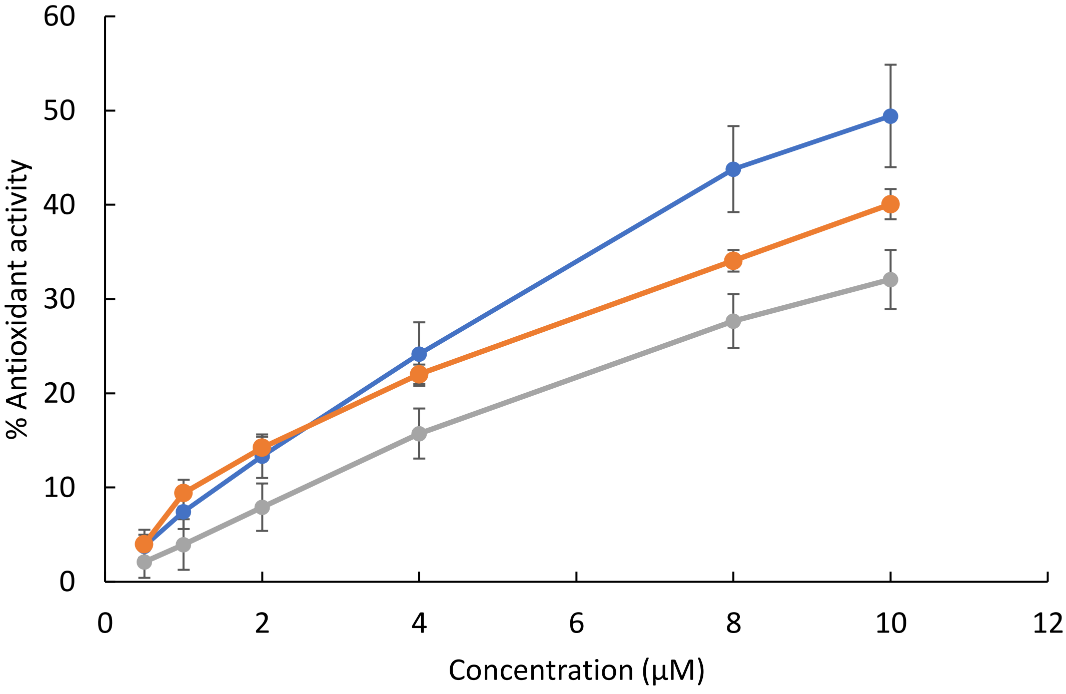


Figure 3.1. Standard curves for the determination of the EC50 of 3,4-DHBA, 4-HBA and FA determined with eth ORAC assay. The EC50 for 3,4-dihydroxybenzoic acid (3,4-DHBA) (blue), 4-hydroxybenzoic acid (4-HBA)

(grey) and ferulic acid (FA) (orange) was determined using ORAC dose-response curves. The data is represented as mean ± SEM of at least four experiments performed in triplicate.

3.4.2. The reducing activity of the phenolic acids and combinations

The reducing capacity of the samples was evaluated at the EC25 and EC50 using the F-C assay and is reported as mM GAE (Figure 3.2). The reducing capacity at the EC50 was significantly ($p < 0.05$) greater when compared with the EC25 indicating a concentration-dependent increase. At EC25, FA presented with the highest reducing capacity of 0.01 mM GAE and 3,4-DHBA presenting with the lowest 0.0038 mM GAE. The difference in reducing capacity between individual phenolic acids was not significant. The phenolic acid with the greatest reducing capacity was 3,4-DHBA and FA at 0.047 mM GAE, followed by 0.038 mM GAE for 4-HBA at the EC50. However, the difference between the individual phenolic acids was not statistically significant.

For the double combination at the EC50, 3,4-DHBA + 4-HBA, had the highest reducing capacity compared with the other double combinations. Significant differences were observed between the, 3,4-DHBA + 4-HBA and 4-HBA + FA mixture. While no significant difference was observed between the 3,4-DHBA + 4-HBA and 3,4-DHBA + FA mixtures. Similar to the effect observed for EC50, at the EC25 the 3,4-DHBA + 4-HBA mixture had the highest reducing capacity although differences between the double combinations were not statistically significant.

Polyphenol-polyphenol interactions on phenolic content were also evaluated (Table 3.1). Based on the obtained data, the polyphenol-polyphenol interactions, were expressed as a percentage of the difference (%) between the expected and observed values, showed there to be synergistic interactions between all combinations at the EC25 concentrations. At the EC50, the 3,4-DHBA + 4-HBA combination showed synergistic interaction, and the 4-HBA+FA, 3,4-DHBA + FA and triple combinations exhibited an additive effect.

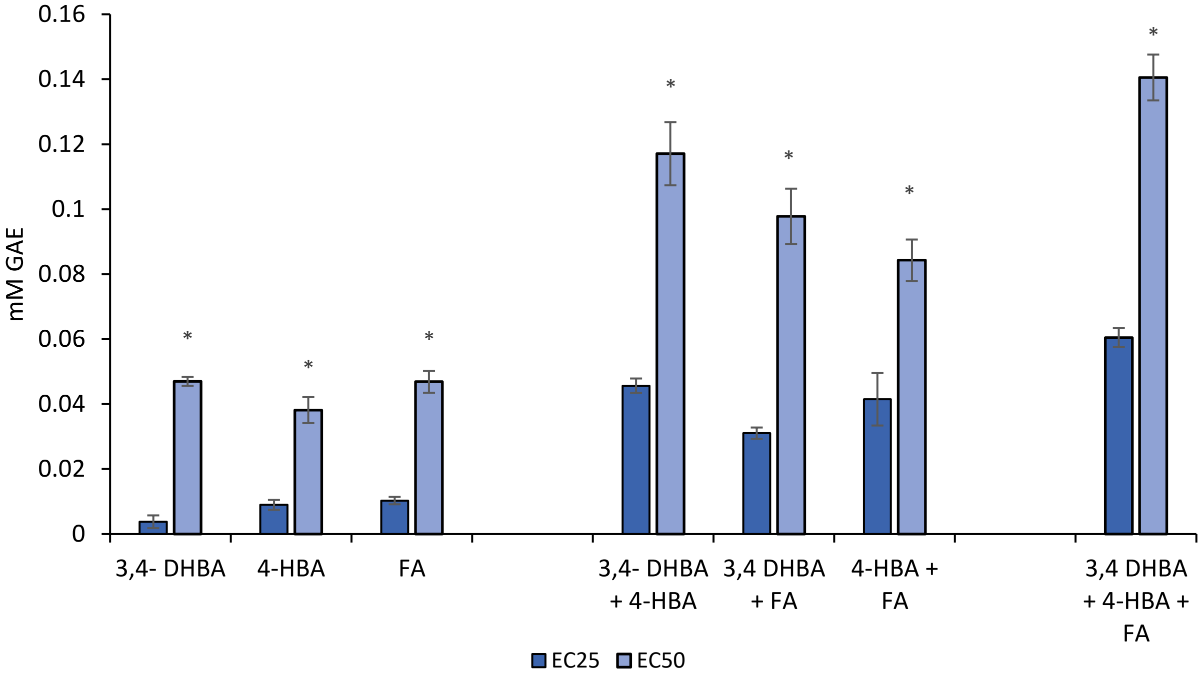


Figure 3.2. The reducing capacity of 3,4-dihydroxybenzoic acid (3,4-DHBA), 4-hydroxybenzoic acid (4-HBA) and ferulic acid (FA) and combinations at the EC25 and EC50 determined with the Folin-Ciocalteu assay. The data is represented as mean ± SEM of at least four experiments performed in triplicates and is expressed in mM gallic acid equivalence (GAE). The * denotes significant difference ($p < 0.05$) between the EC25 and EC50 values as determined using the Tukey's multiple comparisons test.

3.4.3. The antioxidant activity, TEAC assay, of the phenolic acids and combinations

The Mageu derived phenolic acids were evaluated for antioxidant activity using the TEAC assay (Figure 3.3). Only for 3,4-DHBA there was a statistically significant difference in antioxidant activity at EC25 compared with EC50. The phenolic acid with the highest antioxidant activity was FA > 3,4-DHBA > 4-HBA at both the EC25 and EC50 although differences were not statistically significant. The double combination of 3,4-DHBA and 4-HBA showed as dosage effect although differences were also not statistically significant.

The polyphenol-polyphenol interactions were the evaluated (Table 3.1). At the EC25 for the double combinations the interactions were additive and antagonistic for the triple combination. At the EC50 all interactions were antagonistic.

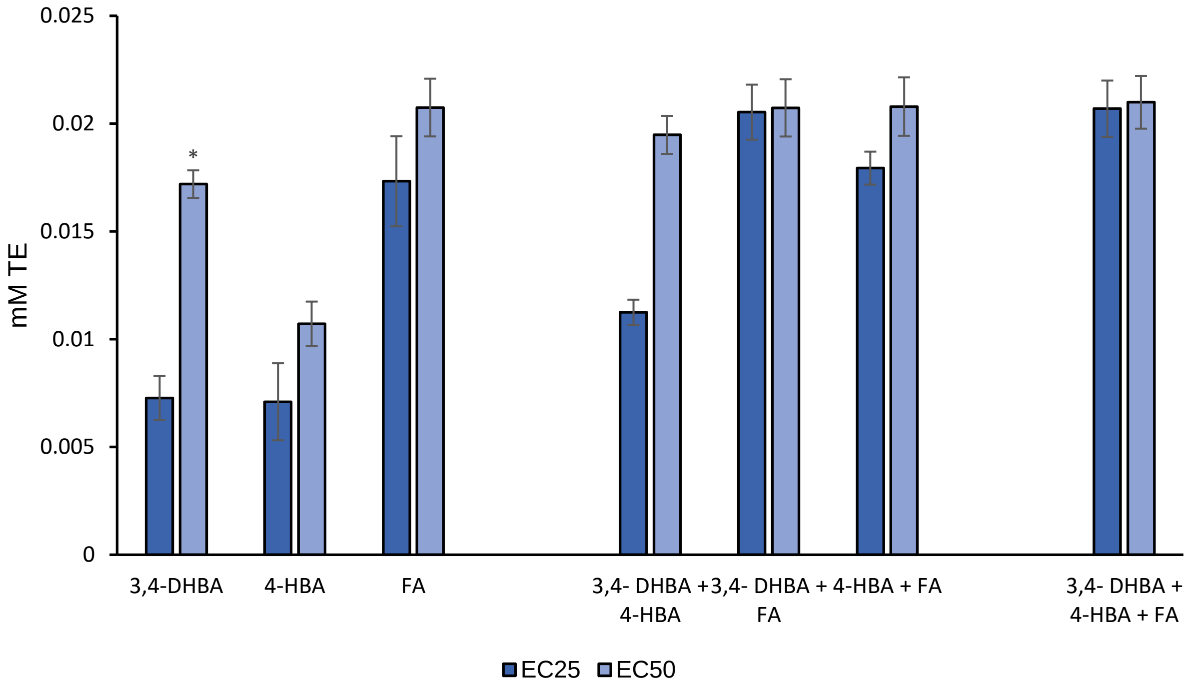


Figure 3.3. The antioxidant activity of 3,4-dihydroxybenzoic acid (3,4-DHBA), 4-hydroxybenzoic acid (4-HBA) and ferulic acid (FA) and combinations at the EC25 and EC50 determined with the TEAC assay. The data is represented as mean ± SEM of at least four experiments performed in triplicates and is expressed in µM Trolox equivalence. The * denotes significant difference ($p < 0.05$) between the EC25 and EC50 values as determined using the Tukey's multiple comparisons test. Differences between groups are not significant.

3.4.4. The antioxidant activity, ORAC assay, of the phenolic acids and combinations

The ORAC assay was used to observe the peroxyl radical scavenging capacity of the Mageu derived phenolic acids (Figure 3.4). The antioxidant activity at the EC50 was higher than the EC25 although differences each phenolic acid and combinations was not significant.

Differences between each phenolic acid at the EC25 or EC50 was also not significant. For the double combinations differences between the combinations were also not significant. The interactions between the phenolic acids using ORAC assay is presented in Table 3.1. For the double combinations, the interactions at the EC25 were additive for 3,4-DHBA + 4-HBA as well as 3,4-DHBA + FA, while for 4-HBA + FA was antagonistic. At the EC50 for all the double combinations effects were antagonistic. For the triple combination at EC25 the interactions were however synergistic and at the EC50 effects were additive. Like the TEAC values, there is no significant difference in the antioxidant activity of the different phenolic acids; single and combined EC25 and EC50 compounds.

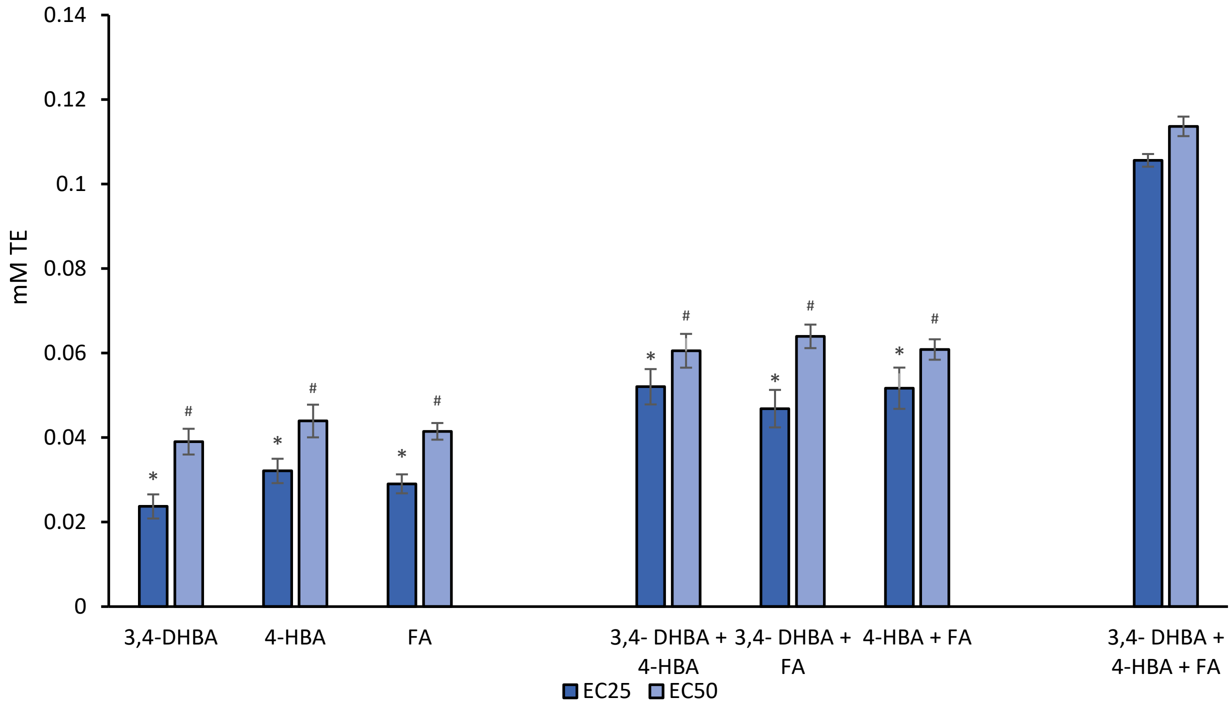


Figure 3.4. The antioxidant activity of 3,4-dihydroxybenzoic acid (3,4-DHBA), 4-hydroxybenzoic acid (4-HBA) and ferulic acid (FA) and combinations at the EC25 and EC50 determined with the ORAC assay. The data is represented as mean ± SEM of at least four experiments performed in triplicates and is expressed in µM Trolox equivalence (TE). * and $^{\#}$ indicates significant difference ($p < 0.05$) between the single or double combinations compared to the triple combinations at EC25 and EC50 respectively determined using the Tukey's multiple comparisons test.

Table 3.1: Polyphenol-polyphenol interactions on phenolic content and antioxidant activity.

A. F-C (µM GAE)	**3,4-DHBA/4-HBA**	**3,4-DHBA/FA**	**4-HBA/FA**	**3,4-DHBA/4-HBA/FA**
		EC25		
Expected	12.75 ± 2.24	14.08 ± 2.23	19.27 ± 2.59	23.05 ± 3.02
Observed	45.68 ± 2.21*	31.06 ± 1.75*	41.50 ± 8.08*	60.45 ± 2.92*
p value	**0.001**	**0.001**	0.030	**0.002**
Interaction	**Synergistic**	**Synergistic**	**Synergistic**	**Synergistic**
		EC50		
Expected	85.17 ± 5.70	93.92 ± 3.65	85.02 ± 5.8	132.05 ± 6.51
Observed	117.08 ± 8.18*	97.81 ± 8.91	84.28 ± 6.99	140.54 ± 7.00
p value	**0.007**	0.729	0.941	0.178
Interaction	**Synergistic**	Additive	Additive	Additive
B. TEAC (µM TE)	**3,4-DHBA/4-HBA**	**3,4-DHBA/FA**	**4-HBA/FA**	**3,4-DHBA/4-HBA/FA**
		EC25		
Expected	11.82 ± 2.73	21.52 ± 3.11	20.85 ± 3.82	27.10 ± 4.80
Observed	11.25 ± 0.58	20.53 ± 1.28	17.93 ± 0.76	20.69 ± 1.31
p value	0.814	0.676	0.414	0.181
Interaction	Additive	Additive	Additive	Additive
		EC50		
Expected	27.90 ± 1.06	37.94 ± 1.26	31.45 ± 2.37	48.65 ± 2.25
Observed	19.48 ± 0.88*	20.73 ± 1.33*	20.79 ± 1.36*	20.99 ± 1.22*
p value	< 0.001	< 0.001	0.002	< 0.001
Interaction	Antagonistic	Antagonistic	Antagonistic	Antagonistic
C. ORAC (µM TE)	**3,4-DHBA/4-HBA**	**3,4-DHBA/FA**	**4-HBA/FA**	**3,4-DHBA/4-HBA/FA**
		EC25		
Expected	55.81 ± 4.37	52.76 ± 3.11	61.16 ± 4.70	84.86 ± 5.42
Observed	52.03 ± 4.00*	46.85 ± 2.79	51.68 ± 2.42*	105.61± 2.31*
p value	**0.038**	0.077	0.044	**0.031**
Interaction	**Antagonistic**	Additive	Antagonistic	**Synergistic**
		EC50		
Expected	82.96 ± 6.25	80.51 ± 4.65	85.40 ± 5.37	124.43 ± 7.84
Observed	60.54 ± 4.18*	63.95 ± 4.44 *	60.85 ± 4.88*	113.65 ± 1.50
p value	0.002	0.005	< 0.001	0.222
Interaction	Antagonistic	Antagonistic	Antagonistic	Additive

The data is represented as mean ± SEM of at least four experiments performed in triplicates. 3,4-DHBA: 3,4-dihydroxybenzoic acid, 4-HBA: 4-hydroxybenzoic acid, FA: ferulic acid, F-C: Folin-Ciocalteu assay, mM GAE: millimolar gallic acid equivalents, TEAC: Trolox equivalent antioxidant capacity, µM TE: micromolar Trolox equivalents, ORAC: oxygen radical absorbance capacity. The * denotes significant difference (*p* < 0.05) between the expected and observed values as determined using the student's paired t-test.

3.4.5. The Caco-2, cellular antioxidant activity, DCFH-DA assay of the phenolic acids and combinations

The DCFH-DA assay was used to evaluate the %CAA (cellular antioxidant activity) of Mageu derived samples and their combinations at the EC25, EC50, twice EC50X2 (21.98 µM 3,4-DHBA, 42.56 µM 4-HBA and 29.06 µM FA), and five times the EC50, EC50X5 (54.95 µM 3,4-DHBA, 106.4 µM 4-HBA and 72.65 µM FA) (Figure 3.5) in the Caco-2 cell line. At these concentrations, except for the triple combination, no dosage effect was observed. At the EC25 only 4-HBA, caused a significant decrease in AAPH induced oxidative damage. At EC50 4-HBA and FA as well as the triple combination protected the Caco-2 cells against oxidative damage. At EC50X2, a pro-oxidant effect was observed for 3,4-DHBA and 3,4-DHBA+ FA. Following exposure to EC50X5 a significant cellular antioxidant effect was observed for 3,4-DHBA , 3,4-DHBA +FA , 4-HBA+ FA and the triple combination.

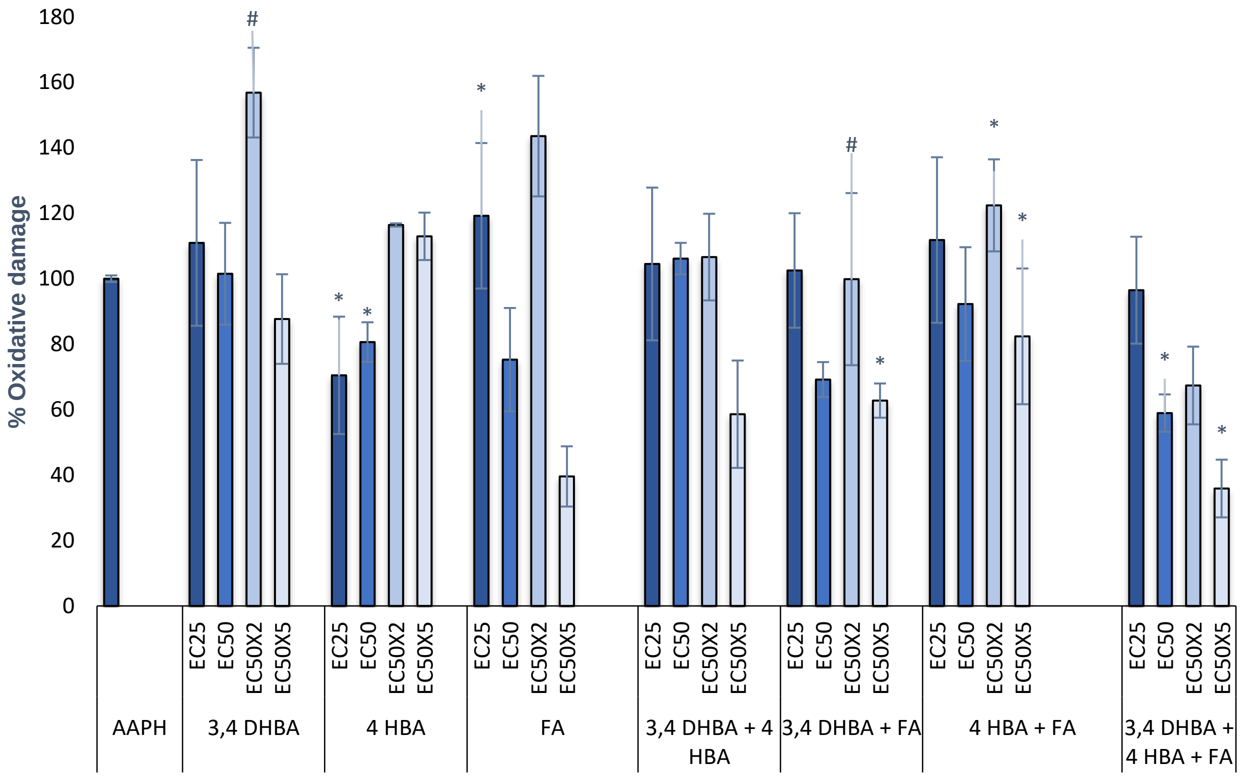


Figure 3.5. The cellular antioxidant activity in the Caco-2 cell line of 3,4-dihydroxybenzoic acid (3,4-DHBA), 4-hydroxybenzoic acid (4-HBA) and ferulic acid (FA) and the combinations at the EC25, EC50, EC50X2 and EC50X5 determined with the DCFH-DA assay. Data is expressed as % oxidative damage relative to the positive control AAPH. The data is represented as mean ± SEM of at least three experiments performed in triplicates. Differences between control and groups are not significant. * Indicates significant difference ($p < 0.05$) between the single or double combinations compared to the EC50X2, of 4 HBA. #Indicates significant difference ($p < 0.05$) between the single or double combinations and EC50X5 3,4-DHBA+4HBA+FA. Significant differences were determined using the Tukey's multiple comparisons test.

The study examined the interactive effects of the polyphenols 3,4-DHBA, 4-HBA, and FA, on cellular antioxidant activity, quantified by DCFH-DA oxidative damage assay (Table 3.1). At the EC25 level, all polyphenol combinations exhibited additive effects, with observed oxidative damage closely aligning with expected values, evidenced by non-significant p-values (p > 0.05). However, at the EC50 level, significant synergistic interactions are detected for 3,4-DHBA/4-HBA (p = 0.045), 4-HBA/FA (p = 0.033), and the triple mixture 3,4-DHBA/4-HBA/FA (p = 0.031), indicating enhanced antioxidant efficacy beyond additive expectations. Notably, the synergistic interactions were also observed at EC50(X2) and EC50(X5) for the triple mixture (p = 0.015 and p = 0.047, respectively) and for 3,4-DHBA/4-HBA at EC50(X5) (p = 0.023). These findings highlighted that specific combinations potentiate cellular antioxidant defences through synergistic mechanisms, particularly at higher concentrations.

Table 3.1: Polyphenol-polyphenol interactions on cellular antioxidant activity

DCFH-DA (% Oxidative damage)	**3,4-DHBA/4-HBA**	**3,4-DHBA/FA**	**4-HBA/FA**	**3,4-DHBA/4-HBA/FA**
		EC25		
Expected	90.71 ± 21.94	115.08 ± 26.99	94.85 ± 18.46	100.21 ± 21.38
Observed	104.5 ± 23.33	102.52 ± 17.49	111.82 ± 25.30	96.48 ± 16.34
p value	0.314	0.351	0.180	0.427
Interaction	additive	additive	additive	additive
		EC50		
Expected	91.07 ± 9.95	88.38 ± 14.19	77.95 ± 13.50	85.80 ± 11.45
Observed	106.14 ± 4.4	69.17 ± 5.34	92.26 ± 17. 37	58.93 ± 5.71
p value	**0.045** *	0.137	**0.033** *	**0.031** *
Interaction	**Antagonistic**	Additive	**Antagonistic**	**Synergistic**
		EC50(X2)		
Expected	136.64 ±43.60	150.21 ± 14.53	129.99 ± 42.32	138.95 ± 32.78
Observed	106.61 ±13.23	99.86 ± 46.27	122.41 ± 14.06	67.37 ± 11.87
p value	0.050	0.286	0.115	**0.015** *
Interaction	Additive	Additive	Additive	**Synergistic**
		EC50(X5)		
Expected	100.31 ± 9.22	63.62 ± 47.49	76.26 ± 42.74	80.06 ± 31.25
Observed	58.59 ± 16.40	62.75 ± 5.23	82.38 ± 20.74	35.88 ± 8.81
p value	**0.023** *	0.120	0.262	**0.047** *
Interaction	**Synergistic**	Additive	Additive	**Synergistic**

The data is represented as mean ± SEM of at least four experiments performed in triplicates. 3,4-DHBA: 3,4-dihydroxybenzoic acid, 4-HBA: 4-hydroxybenzoic acid, FA: ferulic acid, DCFH-DA: dichloro-dihydrofluorescein diacetate. The * denotes significant difference (*p* < 0.05) between the expected and observed values as determined using the student's paired t-test.

3.5. Discussion

The phenolic acids in wheat or cereals, are either benzoic acid or cinnamic acid derivatives. Benzoic derived phenolic acids found in wheat include 4-HBA, protocatechuic acid (3,4-DHBA) and vanillic acids, whereas the cinnamic acid derivatives and includes FA as well as CA and p-CA. These phenolic acids are known to largely exist in bound form, and thus not readily bioavailable (Horvat *et al.*, 2020). The most abundant phenolic acid found in maize is FA that accounts for 82-92% of the TPC (Méndez-Lagunas *et al.*, 2020; Shahidi *et al.*, 2022) and accounts for an estimated 74% of the bound phenolic content in major cereals including maize. The rest of the phenolic acids, 4-HBA, p-CA, protocatechuic acid, CA, vanillic acid is present in smaller quantities (Horvat *et al*., 2020).

Both fermentation and digestion, can release these phenolic acids and consequently, the antioxidant activity is increased. The antioxidant properties of these phenolic acids can potentially prevent oxidative stress through multiple mechanisms including free radical scavenging and/or electron or proton transfer (Leváková, 2017; Gao *et al*., 2022).

Studies claim that the antioxidant capacity of a product cannot be explained by the total phenolic content of phenolic acids alone, it is more plausible that the combined effect of all the phenolic acids is responsible for the observed bioactivity (Gallardo, 2006). The investigation of the interactions of polyphenols found in maize is important in further uncovering the actual antioxidant activity of the phenolic acids in solution. These polyphenol-polyphenol interactions are used to explain the bioactive properties of phenolic rich foods and or extracts where the dominant individual bioactive molecules and or phenolic acids are not identified as the sole contributors of the total antioxidant acidity (Skroza *et al*., 2022)

In a previous study the release of 3,4-DHBA, 4-HBA and FA was increased following the simulated digestion of Mageu a fermented maize product (Nathu *et al*., 2021). Therefore, the aim of the research presented in the is chapter was to evaluate the antioxidant, and the associated polyphenol-polyphenol interactions of 3,4-DHBA, 4-HBA, and FA, the three most abundant phenolic acids identified in Mageu following simulated digestion. The reducing capacity of the Mageu derived phenolic acids was evaluated with the F-C assay and the antioxidant activity with the TEAC and the ORAC assays.

To determine these effects, it was necessary to determine a working concentration that could be used for all assays. As the ORAC assay is physiologically relevant and as a fluorescence-based assay is the most sensitive, this assay was selected to determine these concentrations as the EC50, from which the EC25 was calculated.

A linear correlation between antioxidant content and the reducing activity determined with the F-C assay has been reported (Aryal *et al.*, 2019). Significant differences in reducing activity were observed between each phenolic acid at EC25 and EC50. For the combinations effects were synergistic for all combinations evaluated at the EC25. Indicating at a low concentration the reducing activity is increased. In contrast at the higher concentration at the EC50 these effects are additive.

Studies indicate that the carboxylic group adjacent to a C-C double bond provides additional attack sites for free radicals thereby providing additional protection against ROS (Laddomada *et al*., 2015). Also, the number of OH groups a phenolic compound is directly linked to an increase in TEAC value and subsequently antioxidant activity (Villaño *et al*., 2005). The authors reported that for GA, protocatechuic acid and vanillic acid with three, two and one hydroxyl group, activity was 1.98 ± 0.01, 0.32 ± 0.01 mM TE and inactive respectively. For the phenolic acids, 3,4-DHBA has two OH groups compared with 4-HBA, with a single OH group (Figure 2.1), the activity of 4-HBA at the EC50 measured with the TEAC assay was less than the activity measured for 3,4-DHBA, although not statistically significant. When comparing cinnamic acids to benzoic acids however, the effect of side chains in addition to OH groups was observed to enhance the ABTS scavenging property of phenolic acids, correlates with antioxidant capacity (Villaño *et al.,* 2005; Zhou *et al*., 2006). Thus, the increased antioxidant capacity of FA in comparison with the other two phenolic acids can be attributed to the methoxy (CH_3O) side chain in addition to the OH group.

Differences in antioxidant activity can be attributed to the number of -OH groups, but also the position of the groups. Hydroxyl groups at position 3 and 5 significantly influences the hydrogen transfer mechanism of action; whereas, when the -OH group is at the ortho position it results in reduction of bond dissociation energy, consequently leading to decreased/ less antioxidant activity (Skroza *et al*., 2022). Thus 3,4-DHBA which has -OH groups on position two and three will exhibit higher antioxidant activity than 4-HBA which has one -OH group at position two (Figure 2.1).

Interactions between the phenolic acids at the EC25 were additive for the double combinations and antagonistic for the triple combination. At the higher EC50 all interactions were antagonistic. Differences in effects observed at EC25 and EC50 potentially is related to ratio of phenolic acids and the amount of ATBS.

In the ORAC assay, the chemistry is different and in this assay the ability of an antioxidant to scavenge peroxyl radicals is determined. The antioxidant activity between the EC25 and EC50 of 3,4-DHBA, 3-HBA and FA was significant, indicating a dosage effect. A similar effect was observed for the double combinations but not for the triple phenolic acid combination.

With the ORAC assay, there was no significant differences in antioxidant activity between the two hydroxybenzoic acids. Skroza *et al.* (2022) reported an ORAC value of 30 ± 0.9 µM TE and 57 ± 2 µM TE for 2.5 and 5 µM 3,4-DHBA. In the present study the ORAC values were 23.7 ± 2.86 µM TE and 39.0 ± 3.05 µM TE for 5.5 and 10.99 µM respectively. Although the relative activity is lower in the present study, activity is also in the micromolar range. Differences are possibly related to various reasons including measuring methods, discrepancy of results or differences in procedures (Gallardo *et al.,* 2006).

In general, the most prevalent phenolic acids found in Mageu following simulated GIT digestion, were at the EC25, synergistic when measured with the F-C and ORAC assay, and antagonistic when evaluated with the TEAC assay. In contrast at the EC50 the interactions were mostly additive, except for the 3,4-DHBA/4-HBA mixture which was synergistic when measured with the F-C; and antagonistic when evaluated with the TEAC and ORAC assay. Similarly, Skroza *et al.,* (2022) investigated the interaction of benzoic derived phenolic acids in double and triple combinations, the triple combination containing 3,4-DHBA showed the greatest synergistic effect at the lowest tested concentration.

This study confirms that Mageu derived phenolic acids have radical scavenging capacity, thus functioning as reducing agents with the ability to quench oxygen free radicals as identified in a previous study (Nathu *et al.,* 2021). The capacity of these phenolic acids to scavenge free radicals is attributed to the ability of these compounds to transfer electrons and activate certain endogenous mechanisms, such as the Nrf2 pathway which is an antioxidant pathway. Consequently, endogenous antioxidant enzyme levels are increased, thereby decreasing oxidative stress (Laddomada *et al*., 2015).

The < 3 KDa fraction of the Mageu digests showed significant CAA evaluated with the DCFH-DA assay (Nathu *et al.,* 2021). The contribution of the identified phenolic acids to the cellular antioxidant was determined. Concentration effects were highly variable, no dosage effects were observed and generally no CAA was observed although a few combinations at a specific concentration showed either beneficial cellular antioxidant or pro-oxidant effects.

Under neutral conditions, phenolic acids act as antioxidants, however, studies have shown that in specific circumstances, antioxidants can become pro-oxidants. Depending on the environment, whether acidic or basic, or on the concentration of the antioxidant in the food matrix, antioxidants may promote oxidative reactions (Sotler *et al*., 2019; Tian *et al*., 2022). Under aerobic conditions for example, phenolic acids generate superoxide radicals which react with reduced metal ions to form ROS (Sotler *et al*., 2019). Phenolic acids can also chelate transition metals in a manner that promotes their catalytic activity, or even reduce metal ions, resulting in the formation of more free radicals (Tian *et al*., 2022)

Among the antioxidants that have been reported to exhibit pro-oxidant activity is the phenolic acids FA and 4-HBA and these phenolic acids promote lipid peroxidation only after reaching a certain concentration limit. Using the ß-carotene bleaching assay, Gao *et al*., 2022, reported that this limit was 25 µg/mL. This current study observes pro-oxidant activity at the EC25 and some EC50 concentrations and antioxidant activity at EC50(X5).

Studies explain that the interaction that phenolic compounds have with other phenolic compounds may be synergistic or antagonistic depending on the food matrix. Interactions with other polyphenols, macro- and micro-compounds may affect bioactivity, under certain conditions (e.g., concentration of phenolic acids in the matrix) either inducing synergistic or antagonistic effects (Erskine *et al*., 2022). In the present study polyphenol-polyphenol interactions on CAA were evaluated. The results showed that combinations of all three of these polyphenolic compounds result in synergistic interactions evident at higher concentrations (EC50, EC50(X2), EC50(X5)) for 3,4-DHBA/4-HBA, 3,4-DHBA/FA and the 3,4-DHBA/4-HBA/FA combinations. Some combinations such as 3,4-DHBA/FA and 4-HBA/FA show additive effects. This study thus suggests that the combination of these polyphenolic compounds can potentiate their antioxidant effects against oxidative damage, especially at higher concentrations, demonstrating persistent synergistic interactions in triple combinations.

Smaller unidentified <3 kDa molecules in Mageu (Nathu *et al.,* 2021), such as peptides may also contribute to activity and the identification of these molecules should be the focus of future studies.

3.6. Conclusion

Mageu derived polyphenols exhibit antioxidant activity consistent with literature, with FA showing the highest antioxidant activity followed by 3,4-DHBA and 4-HBA. The observed differences in antioxidant activity can be attributed to differences in structure, regarding the number of -OH groups attached to the phenolic ring, as well as the arrangement of the groups and sidechains. In addition to the structure, concentration plays a role in the antioxidant capacity of the phenolic acids. It is observed that synergistic interactions occur at low concentrations and continue to do so until a certain concentration threshold limit is reached as observed for the TEAC and ORAC assays. However, no dosage effects were observed using the DCFH-DA assay and generally no CAA was observed although a few combinations at a specific concentration showed either beneficial cellular antioxidant or pro-oxidant effects at varying concentrations.

Chapter 4. The anti-adipogenic of the phenolic acids identified in Mageu and the associated polyphenol-polyphenol interactions

---

4.1. Introduction

Currently obesity is the leading metabolic disease in the world, affecting both the developed and developing world and in South Africa the prevalence of obesity is high. By 2025, it is predicted that the prevalence of obesity will increase to 47.7% in females and 23.3% in males (Manafe, 2022). Obesity is characterised by the cellular increase in the number and size of adipocytes differentiated from preadipocytes in adipose tissue. The prevalence of this condition has led to an increase in research aimed at elucidating influences surrounding adipose tissue mass (Hsu *et al.,* 2007). A significant area of study has focused on the association of plant derived food and their effects on adiposity (Luna-Vital *et al.,* 2019).

The inclusion of whole grains such as maize in the diet reduces obesity and promotes weight loss (Lee *et al.*, 2022). Mageu, which is a fermented maize product, has been identified as source of bioactive molecules, that if regularly consumed can potentially the development of metabolic disorders. Nathu *et al.,* in 2021 demonstrated that the bioactive molecules that is potentially responsible for the cellular antiadipogenic effects of Mageu are phenolic acids, with 3,4-DHBA, 4-HBA and FA being bioavailable. In this study, Mageu was found to prevent adipocyte differentiation and lipid accumulation rather than the direct reduction of accumulated lipid droplets in differentiated adipocytes (Nathu *et al.,* 2021). This effect is attributed to the ability of phenolic acids to interact with adipose tissue directly and/or indirectly; through adipocyte oxidation, suppression of adipogenesis, lipogenesis, gluconeogenesis and other energy storing mechanisms (Wang *et al.,* 2014, Luna-Vital *et al.*, 2019).

Synergistic, additive or antagonistic effects among other factors impact polyphenol bioavailability, stability and metabolic pattern which results in complex mixtures with variable bioactivities (Zhang *et al;* 2019). The phenolic acid, FA has been shown to stimulate adiponectin secretion in 3T3-L1 adipocytes and therefore inhibit adipocyte differentiation and lipid accumulation in cell culture models (Ilavenil *et al.,* 2017). Protocatechuic acid, 3,4-DHBA elicits anti-obesity effects through inhibition of lipid mediator generating enzymes and by indirectly affecting glucose homeostasis thereby improving glucose tolerance and insulin sensitivity in mice (Rivera-Piza *et al.,* 2017). In contrast, 4-HBA has no effect on adipocyte differentiation in 3T3-L1 cells (Hu *et al.,* 2013). This indicates that individually theses phenolic acids have varied effects on adipocyte differentiation and lipid accumulation; however, there exists a need to understand the polyphenol-polyphenol interactions exhibited by these Mageu associated phenolic acids as part of a food matrix (Nathu *et al.,* 2021). Thus, of the research presented in this chapter was to determine the anti-adipogenic properties of 3,4-DHBA, 4-HBA, FA at the EC50 and EC25 as well as the type of polyphenol-polyphenol interactions in the mouse murine (3T3-L1) cells, using the ORO and NR assays. Then to investigate the effects of the most beneficial phenolic acid or combination on cellular differentiation of 3T3-L1 adipocytes with PlasDIC microscopy.

4.2. Materials

3.6.1. Cell lines

Mouse fibroblast pre-adipocytes (3T3-L1) were obtained from CELLONEX Separations (Johannesburg, SA) and used between passage numbers p46 – p49.

3.6.2. Reagents, equipment, glassware and disposable plasticware

Mageu derived phenolic acids and combinations were prepared as described in Chapter 3. The dyes, ORO catalogue number 102419, and NR catalogue number 7385-67-3; as well as formalin, isopropanol, dexamethasone (DEX), insulin, 3-isobutyl-1-methylxanthine (IBMX), rosiglitazone, were purchased from Merck Chemicals, Modderfontein, SA. All equipment and glassware were the same as listed in Sections 3.2.2.

The equipment used was the Olympus IX71 inverted phase contrast microscope (Tokyo, Japan).

3.6.3. Laboratory facilities and equipment

The laboratory facilities and the equipment used in this study was the same as described in Sections 3.2.3.

3.6.4. Samples

All samples were prepared as described in Section 3.3.1.

4.3. Methods

3.6.5. Culturing 3T3-L1 cells

The 3T3-L1 cell line was maintained in a humidified incubator at 37°C, in growth media, consisting of DMEM/FCS, in a 5% CO_2 atmosphere and 90% humidity in cell culture flasks until confluent. To establish cultures of the 3T3-L1 cells, cryopreserved cells were removed from liquid nitrogen and thawed in 37°C water bath. The cells were then transferred into a 15 mL centrifuge tube. To the cells, DMEM/FCS was then the cells were collected with centrifugation at 623 xg for 2 minutes. The supernatant was then discarded, and the cells were re-suspended in DMEM/FCS medium, following before transfer into a cell culture flask and cultivation at 37°C and 5% CO_2. The cells were passaged at 3-to-4-day intervals. This was achieved by first removing the media and then rinsing the monolayer with 0.1 M PBS (0.062 M $Na_2HPO_4.2H_2O$, 0.038 M $NaH_2PO_4.H_2O$, 0.15 M NaCl, pH 7.4) following which, 1 mL of TrypLE Express was added to the cells. The cells were then incubated at 37°C for 2 – 5 minutes, until the cells detached. Then 4 mL DMEM/FCS was added to the culture flask to suspend the cells before the suspension was transferred into a 15 mL centrifuge tube. The cells were collected by centrifugation at 623 xg for two minutes. The cells were then finally re-suspended in 10 mL DMEM/FCS and then plated and cultured at 37°C. The media was replaced at 2–3-day intervals.

3.6.6. Differentiation of 3T3-L1 cells

For differentiation the pre-adipocyte cells were seeded at a concentration of 1×10^3 cells per 100 µL in the wells of a 96 well plate and grown to confluence for three to four days in DMEM/FCS. After confluence, differentiation was induced by replacing the DMEM/FCS with differentiation medium (DM1, Table 4.1) that contains insulin, IBMX and DEX and rosiglitazone. The media is replaced with fresh, DM1 on day 4, 7 and 10. On day 10 medium was switched to differentiation medium 2 (DM2, Table 4.1) for a further 3 days.

Table 4.1: Differentiation medium

Medium	Content
Growth medium	DMEM +10% FBS + 1% antibiotics (DMEM/FCS)
DM1 - Differentiation medium 1	DMEM/FCS + IBMX (500 mM) + insulin (10 µg/mL) + dexamethasone (5 mM) +rosiglitazone (10 mM)
DM2 - Differentiation medium 2	DMEM/FCS + insulin (10 µg/mL)

3.6.7. Prevention and reduction of lipid droplet formation in adipocyte 3T3-L1 cells

To investigate the ability of the phenolic acid samples to inhibit lipid accumulation (Table 4.2). To test for prevention of lipid formation, 10 µL of each sample was added with DM1 on days 4 and 7; as well as with DM2 and DMEM on days 10 and 13 respectively (Table 4.2).

Table 4.2: Differentiation of 3T3-1 cells

Day	Prevention of lipid droplet accumulation
1	Plated 3T3-L1 cells: $1x10^3$ cells/100 µL DMEM/FCS
4	Change medium: 90 µL DM1 + 10 µL phenolic acid samples
7	Change medium: 90 µL DM1 + 10 µL phenolic acid samples
10	Change medium: 90 µL DM2 + 10 µL phenolic acid samples

13	Change medium: 90 µL DMEM/FCS + 10 µL phenolic acid samples
14	Differentiation complete: Stain with Oil Red O (ORO) (Qualitative: morphology Quantitative: ORO solubilisation) Stain with Nile Red (NR) (Qualitative: morphology Quantitative: fluorescence)

3.6.8. **Lipid accumulation, Oil Red O staining**

The dye ORO is a lipid soluble dye used to discern adipocytes from other cells, as it can be used to detect neutral lipids and cholesterol esters. The ORO stain is hydrophobic, and therefore it can associate with lipids within cells or tissues, thus making it an ideal stain for the quantification and visualisation of lipids (Costa *et al.,* 2018; Histalim, 2019).

For staining, a 5% w/v solution of ORO was prepared in 60% isopropanol and before use was further diluted 3:2 v/v with distilled water. The 3T3-L1 adipocytes were washed with PBS and then fixed with 2% formaldehyde for 30 minutes at a temperature of 37°C. The fixative was then removed and 100 µL working ORO solution was added to the wells. After 1 hour staining at room temperature, the excess dye was removed using distilled water. For quantification, the stain in the cells was solubilised with 60% isopropanol and measured at an absorbance of 405 nm. The data was expressed relative to the untreated control.

Prior to solubilisation, phase contrast images were taken with an Olympus IX71 inverted phase contrast light microscope (Tokyo, Japan) to visualize the effect of Mageu derived phenolic acids on 3T3-L1 adipocytes.

3.6.9. **Lipid accumulation, Nile Red staining**

The dye, NR is a (9-diethylamino-5H-benzo[a]phenoxazine-5-one) is a meta-chromic fluorescent dye intracellular fluorescent stain, which can stain intracellular lipid droplets of living cells (Greenspan *et al.,* 1985; Alemán-Nava *et al*., 2016). Fluorescent dyes such as NR when compared with ORO offer a more accurate quantitative measurement as NR specifically binds to the intended compounds and are not prone to staining unspecific, non-fat cellular structures (Escorcia *et al.*, 2018). Although it is a more accurate assay, caution needs to be taken regarding its sensitivity as fluorescence fades. Thus, when correlating the lipid content of exposed cells to fluorescence, consideration of the fluorescence extinction needs to be taken (Rumin *et al.,* 2015). In addition, the method is more rapid as the fixation of the cells is required.

A stock solution of NR in DMSO (1 mg/mL) was prepared and stored at 4°C in the dark. To the differentiated adipocytes 198 µL PBS, 2 µL NR was added to generate a 1:100 dilution (final concentration 10 µg/mL). After 30 mins the excess dye was gently rinsed off with PBS and then 100 µL of PBS added to the wells. The amount of staining was measured at an Ex of 450nm and Em of 528 nm using the plate reader, and the data expressed as a percentage relative to the control.

4.3.1.1. PlasDIC microscopy

PlasDIC is a microscopy technique that uses polarisation-optical transmitted light differential interference contrast when visualising live cells to provide a pseudo-3-dimensional shadow case contrasted image (Arnaout *et al.,* 2016). Exposed 3T3-L1 cells were visualised on day 7 and 13 of the differentiation protocol and were images of cells where the phenolic acids and combinations caused a significant reduction in lipid accumulation.

3.6.10. Calculation of polyphenol-polyphenol interactions

To classify the interactions between the polyphenols as antagonistic, additive, or synergistic, we evaluated the differences between the experimental and expected antioxidant activities. The anti-adipogenic effects of polyphenol mixtures were assessed by comparing the observed experimental results to the theoretical expected results, which were calculated based on the sum of the individual polyphenols in the mixture. Synergistic interaction occurs when the observed value is statistically lower than the expected value, indicating that the polyphenols collectively reduce lipid accumulation more effectively than predicted.Additive interaction is observed when the observed value closely matches the expected value, signifying that the combination’s effect is equivalent to the sum of the individual effects. Antagonistic interaction is defined as when the observed value is statistically higher than the expected value, suggesting that the combination results in less reduction in lipid accumulation than anticipated, despite still being lower than the control.

3.6.11. Data management and statistics

All experiments were done at least four times in triplicate yielding 12 data points. Data was then tested for normality using the D'Agostino and Pearson normality test ($p<0.05$). Data was then further analysed with one-way ANOVA with post hoc Tukey analysis for significant differences ($p<0.05$). Data is reported as mean ± SEM.

4.4. Results

3.6.12. The antiadipogenic effects (Oil red O assay) of the phenolic acids and combinations

The anti-adipogenic activity of the Mageu derived samples were evaluated at the EC25, EC50, EC50(X2) and EC50(X5) and reported as percentage lipid formation relative to the untreated control. The data illustrated in Figure 4.1 shows the ability of these Mageu derived phenolic acids and combinations to prevent lipid accumulation in 3T3-L1 adipocytes. For all phenolic acids there was a dosage effect with reduced lipid accumulation at EC50(X2) and EC50(X5) compared with EC25 and EC50.

The reduction in percentage lipid accumulation of the individual phenolic acids at the EC25 and EC50 concentrations was non-significant and ranged from 87.42 ± 5.87% to 96.55 ± 4.60% and 86.24 ± 3.28% to 93.85 ± 4.10% respectively. No significant ($p > 0.05$) difference was observed when comparing the individual EC25 and EC50 samples with their respective combinations at the same concentrations. The EC25 and EC50 double combinations presented with percentage lipid accumulation ranging from 94.22 ± 5.98% to 100.96 ± 5.03%, and 89.66 ± 8.50% to 98.45 ± 9.35%, respectively (Figure 4.1). The EC25 and EC50 triple combinations, 3,4-DHBA + 4-HBA + FA, presented with lipid accumulation of 85.28 ± 2.21% and 93.11 ± 3.57% respectively. These effects were additive interaction for the double and synergistic for the triple combination at the EC25 and EC50 (Table 4.3).

At the EC50(X2) concentrations, significant anti-adipogenic activity ($p < 0.05$) compared to the untreated control was observed for the individual phenolic acids, 4-HBA (54.38 ± 3.86%) > FA (57.31 ±.3.53%) > 3,4-DHBA (67.66 ± 5.66%) as well as for the combination 4-HBA + FA (68.65 ± 4.09%), and for the triple combination 3,4-DBHA+4-HBA+FA (60.56 ± 7.89%). At the EC50(X2) and the EC50(X5) compared with the control, the percentage lipid accumulation was significantly reduced at the EC50(X2) for 4-HBA and at EC50(X2) and the EC50(X5) for FA. For the EC50(X2) and EC50(X5) double combinations, the percentage lipid accumulation ranged from 58.10 ± 8.92% to 68.66 ± 4.09%, and 69.41 ± 4.49% to 73.08 ± 6.52%, respectively and was significantly reduced ($p > 0.05$) compared with the untreated control. For these double combinations all effects were additive (Table 4.3).

The EC50(X2) and EC50(X5) triple combinations, 3,4-DHBA + 4-HBA + FA, presented with percentage lipid accumulation of 60.56 ± 7.89% and 58.55 ± 3.17% respectively. Both EC50(X2) and EC50(X5) triple combinations showed significant decrease in percentage lipid accumulation when compared with the control. For the double polyphenol-polyphenol interactions, effects were additive and for the triple combination was additive and synergistic for EC50(X2) and EC50(X5) respectively (Table 4.3).

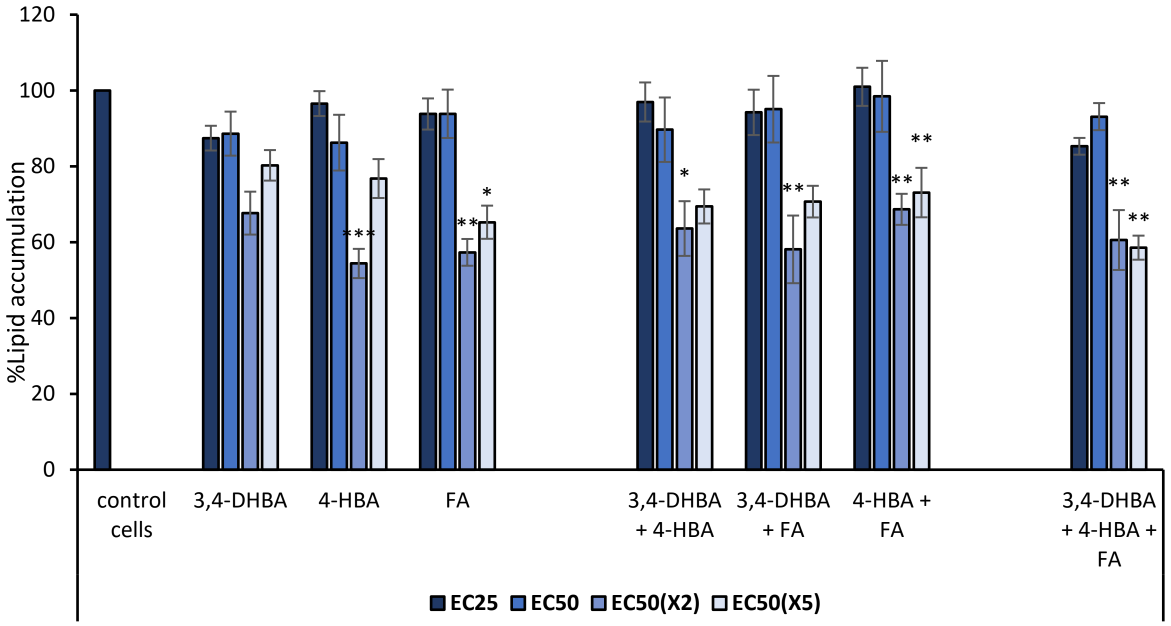


Figure 4.1: Inhibition of lipid droplet formation in differentiating 3T3-L1 cells by of 3,4-dihydroxybenzoic acid (3,4-DHBA), 4-hydroxybenzoic acid (4-HBA) and ferulic acid (FA) and combinations at the EC25, EC50, 2X EC25 and 2X EC50 quantified with the Oil red O assay. The data is represented as mean ± SEM of at least three experiments performed in duplicates. * denotes significant difference ($p < 0.05$) from the control. Differences between groups are not significant.

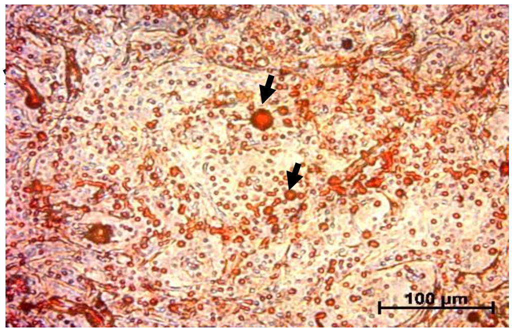
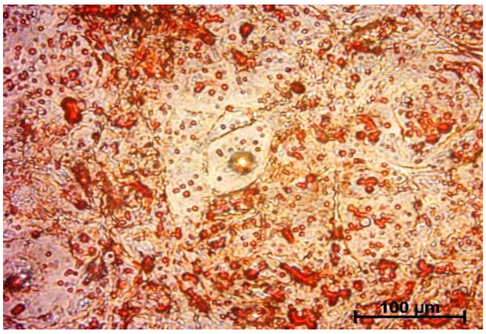
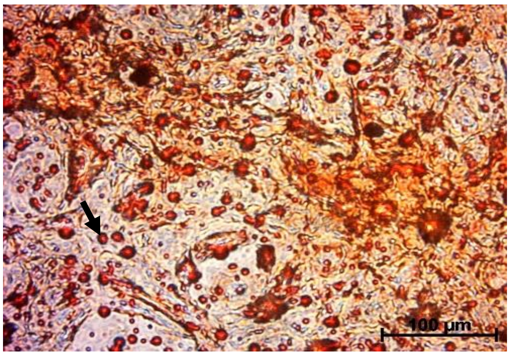
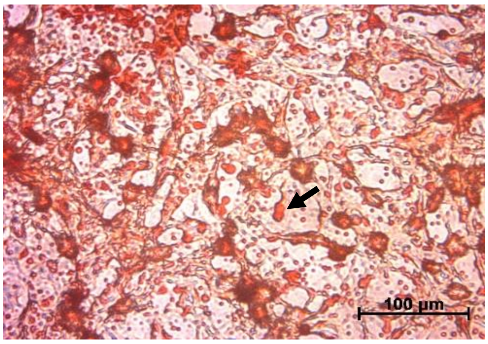
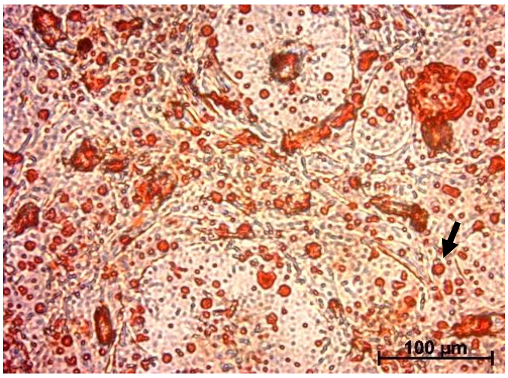
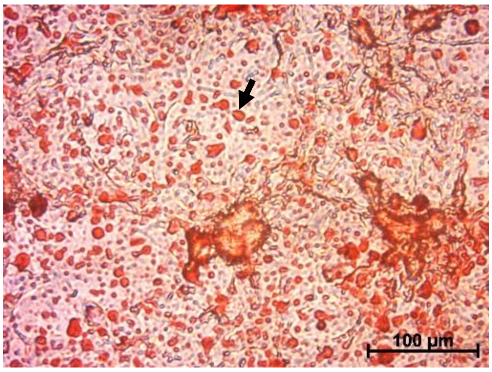
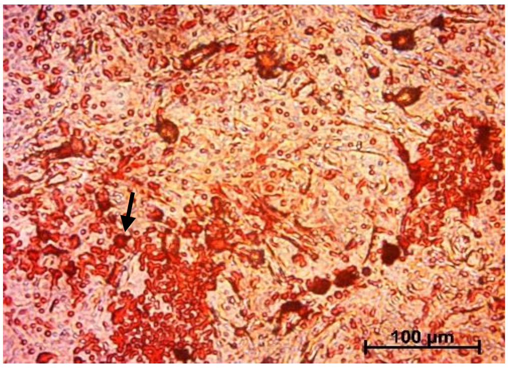
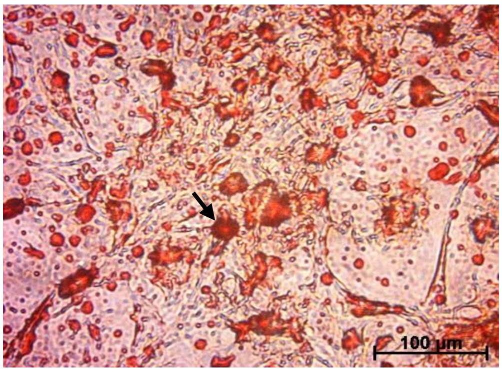
Table 4.3. Polyphenol-polyphenol interactions on lipid accumulation (ORO staining) in 3T3-L1 adipocytes.

Oil red O (%)	3,4-DHBA/4-HBA	3,4-DHBA/FA	4-HBA/FA	3,4-DHBA/4-HBA/FA
		EC25		
Expected	91.98 ± 6.77	90.62 ± 7.80	95.17 ± 6.80	92.59 ± 6.83
Observed	96.93 ± 5.77	94.23 ± 6.47	100.96 ± 5.45	85.28± 6.83*
p value	0.702	0.721	0.841	**0.008**
Interaction	Additive	Additive	Additive	**Synergistic**
		EC50		
Expected	87.43 ± 3.68	91.23 ± 4.69	90.05 ± 4.19	89.57 ± 3.76
Observed	89.66 ± 8.5	95.06 ± 8.78	98.45 ± 9.36	93.11 ± 3.57
p value	0.556	0.760	0.168	0.580
Interaction	Additive	Additive	Additive	Additive
		EC50(X2)		
Expected	61.02 ± 8.20	62.48 ± 6.18	55.85 ± 5.25	59.78 ± 6.07
Observed	63.59 ± 13.25	58.10 ± 16.39	68.66 ± 7.52*	60.56 ± 14.50
p value	0.648	0.570	0.004	0.913
Interaction	Additive	Additive	Antagonistic	Additive
		EC50(X5)		

Expected	78.52 ± 11.60	72.76 ± 11.08	71.02 ± 6.71	74.10 ± 8.97
Observed	69.42 ± 15.49	70.69 ± 16.41	73.08 ± 11.49	58.55 ± 8.94*
p value	0.219	0.813	0.678	**0.021**
Interaction	Additive	Additive	Additive	**Synergistic**

The ORO data is represented as mean ± SEM of at least four experiments performed in triplicates. 3,4-DHBA: 3,4-dihydroxybenzoic acid, 4-HBA: 4-hydroxybenzoic acid, FA: ferulic acid, ORO: Oil red O. * denotes significant difference ($p < 0.05$) between the expected and observed values as determined using the student's paired t-test.

The inhibition of lipid droplet formation by the EC25 and EC50 samples was further studied using light microscopy to visualize the lipid droplets. Images of the ORO-stained cells showing the distribution of the lipids is presented in Figure 4.2. The untreated differentiated adipocytes presented with a uniform monolayer, with areas of intense staining, representing lipid droplets and other regions with lighter staining, probably membrane associated lipids. Compared with the control adipocytes, exposure to 3,4-DHBA and FA at the EC50 resulted in a monolayer similar to the control, with small lipid droplets in the cytoplasm. Minimal reduction in red staining was observed in the cells exposed to the EC25 and EC50 samples compared to the control cells confirming quantitative data (Figure 4.1). For the double combinations and triple combination, a similar distribution and intensity of staining was observed confirming the lack of toxicity and inhibition of lipid accumulation at these concentrations.

	Control	
		
	Individual phenolic acids	
	EC25	EC50
3,4-DHBA		
4-HBA		
FA		
	Double combinations	

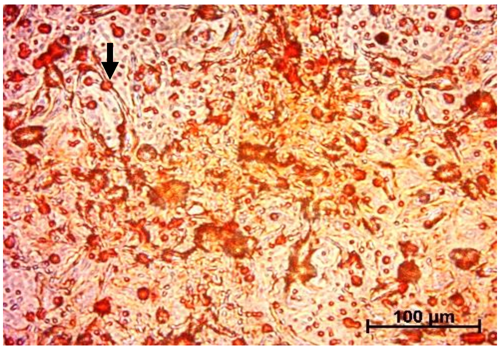
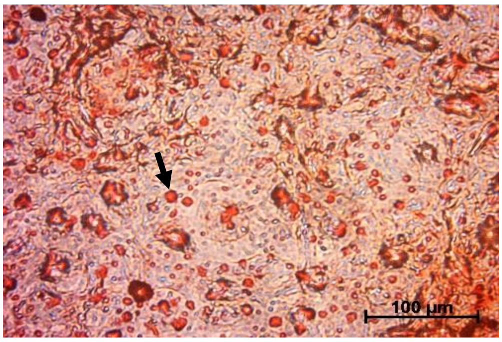
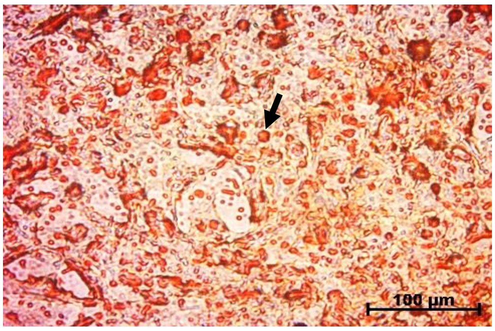
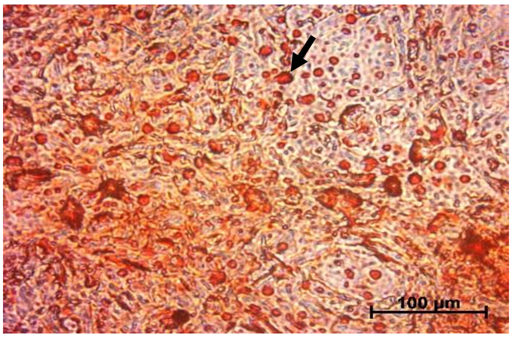
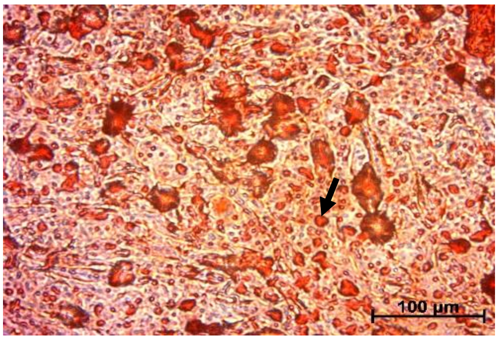
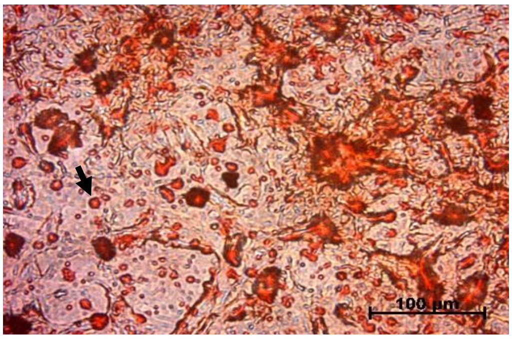
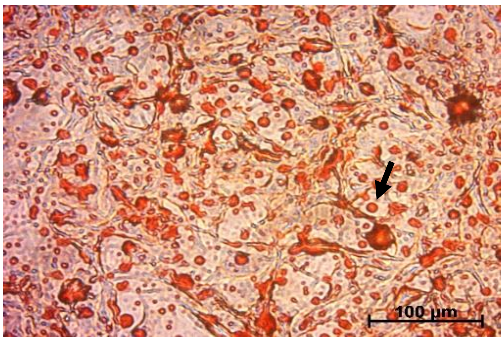
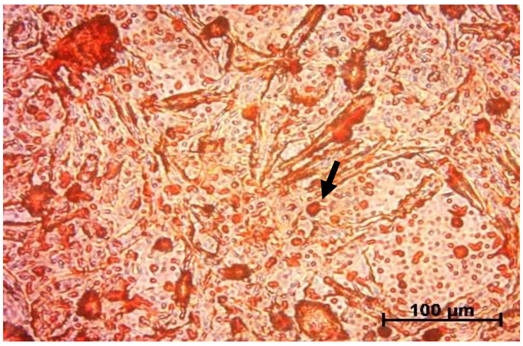
3,4-DHBA + 4-HBA		
3,4-DHBA + FA		
4-HBA + FA		
	Triple combination	
		

Figure 4.2. Light micrographs of differentiated 3T3-L1 cells exposed to 3,4-dihydroxybenzoic acid (3,4-DHBA), 4-hydroxybenzoic acid (4-HBA) and ferulic acid (FA) and combinations at the EC25, and EC50 and stained with Oil Red O stain. Scale bar is 100 µm. Arrows indicate stained lipid droplets.

3.6.13. The anti-adipogenic effect (Nile Red assay) of the phenolic acids and combinations

In the current study, anti-adipogenic activity of the Mageu derived samples was evaluated using the NR stain at the EC25, EC50, EC50(X2) and EC50(X5). The individual phenolic acids at EC25 and EC50 presented with lipid accumulation ranging from 82.24 ± 5.82% to 99.04 ± 2.19%, and from 72.65 ± 11.18% to 88.88 ± 3.99%, respectively (Figure 4.3) with 3,4-DHBA having the least anti-adipogenic activity at both concentrations when compared with the other individual phenolic acids. No significant ($p > 0.05$) inhibition on lipid accumulation was observed when the individual EC25 and EC50 phenolic acids were compared with the control. For the EC50(X2) and EC50(X5) individual phenolic acids, activity ranged from 85.40 ± 2.57 – 91.96 ± 2.49% and 84.13 ± 4.07 - 91.95 ± 4.13% lipid accumulation, respectively: with 3,4-DHBA again having the least anti-adipogenic activity at both concentrations. There were no significant ($p > 0.05$) differences in lipid accumulation inhibition for the individual EC50(X2) and EC50(X5) phenolic acids compared with the control.

For the double combinations a significant reduction ($p < 0.05$) in lipid droplet formation at higher concentrations than observed with the ORO assay (Figure 4.1). The double combinations at EC50(X2) and EC50(X5) presented with lipid accumulation ranging from 65.77 ± 3.32% to 85.17 ± 2.31%, and from 65.46 ± 6.04% to 82.98 ± 4.46%, respectively. Although all double combinations significantly ($p < 0.05$) inhibited the accumulation of lipid droplets at EC50(X2) and EC50(X5) when compared with the sum of the effects of the individual phenolic acids, the double combinations containing FA exhibited greater inhibitory effect (Table 4.4). At both the EC50(x2) and the EC50(X5) these interactions were additive for both 3,4-DHBA and 3-HBA as well as 3,4-DHBA and FA. In contrast the interaction between 3-HBA and FA was synergistic at both concentrations.

The 3,4-DHBA+4-HBA+FA triple combination at EC50(X2) and EC50(X5) concentrations also presented with significant ($p < 0.05$) reduction in the percentage lipid accumulation compared with the control of 71.57% and 76.32%, respectively and at both concentrations these effects were synergistic (Table 4.4). For the combinations, synergistic effects were observed for 3,4-DHBA+FA and 3,4-DHBA+4-HBA+FA following NR staining (Table 4.4). With ORO staining for 3,4-DHBA+FA these effects were additive and for 3,4-DHBA+4-HBA+FA was only synergistic at EC50(X5).

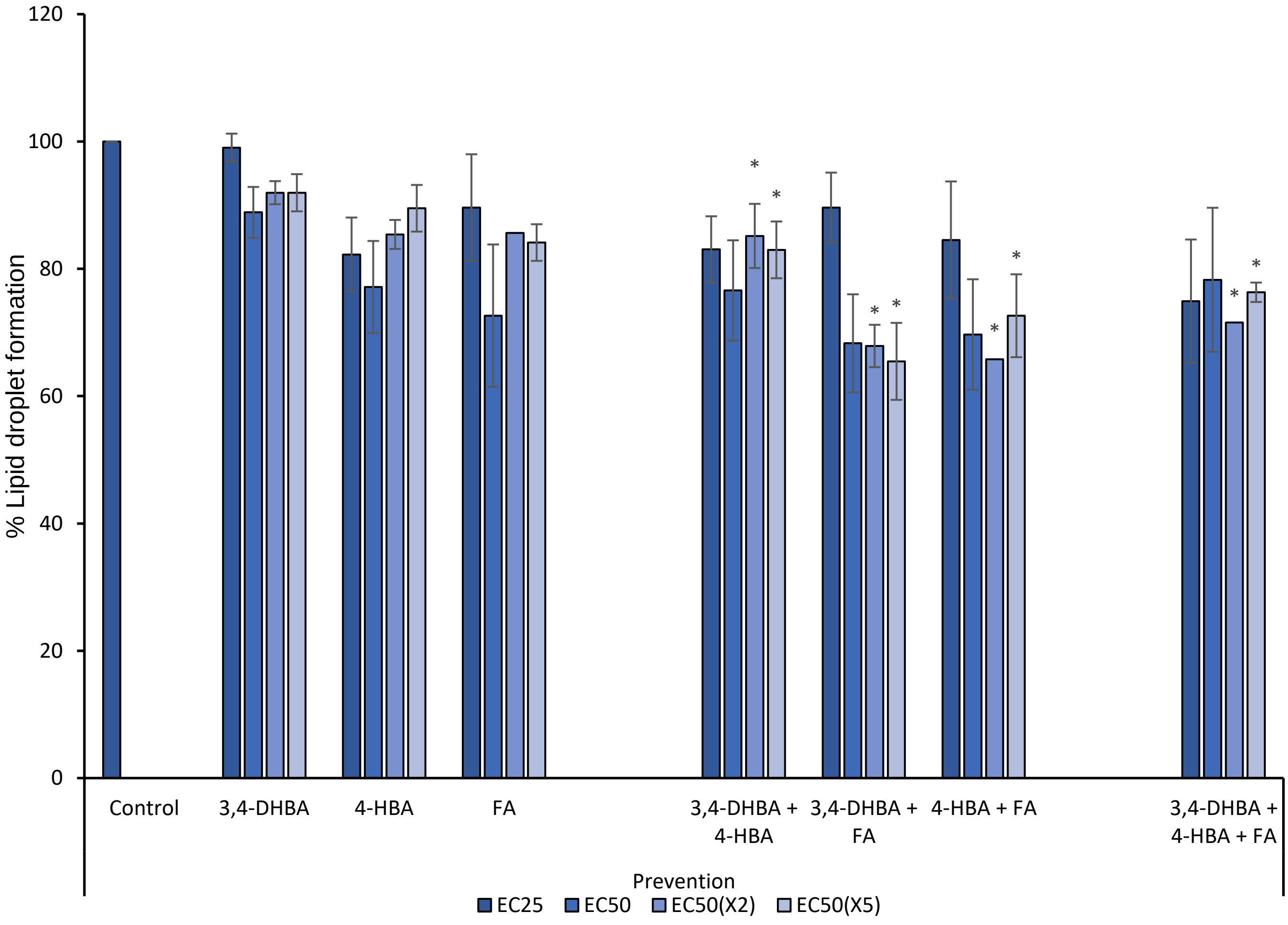


Figure 4.3. Inhibition of lipid droplet formation in differentiating 3T3-L1 cells by of 3,4-dihydroxybenzoic acid (3,4-DHBA), 4-hydroxybenzoic acid (4-HBA) and ferulic acid (FA) and combinations at the EC25, EC50, 2X EC50 and 5X EC50 quantified with the Nile red assay. The data is represented as mean ± SEM of at least three experiments performed in duplicates. * denotes significant difference ($p < 0.05$) from the control. Differences between groups are not significant.

Table 4.4: Polyphenol-polyphenol interactions on lipid accumulation (NR staining) in 3T3-L1 adipocytes.

Nile red (%)	3,4-DHBA/4-HBA	3,4-DHBA/FA	4-HBA/FA	3,4-DHBA/4-HBA/FA
		EC25		
Expected	90.64 ± 5.25	94.33 ± 6.77	85.93 ± 9.70	90.30 ± 7.09
Observed	83.04 ± 7.37	89.63 ± 7.75	84.53 ± 13.00	74.90 ± 13.72*
p value	0.347	0.506	0.226	**0.008**
Interaction	Additive	Additive	Additive	**Synergistic**
		EC50		
Expected	83.01 ± 7.11	80.76 ± 9.52	74.90 ± 12.30	79.56 ± 9.34
Observed	76.60 ± 11.15	68.30 ± 10.89	69.69 ± 12.27	78.28 ± 16.00
p value	0.355	0.665	0.529	0.333
Interaction	Additive	Additive	Additive	Additive
		EC50(X2)		
Expected	88.68 ± 1.61	88.79 ± 2.21	85.51 ± 1.51	87.66 ± 1.33
Observed	85.17 ± 3.28	67.88 ± 7.12*	65.77 ± 4.70*	71.57 ± 5.43*
p value	0.294	**0.046**	**0.024**	**0.0029**
Interaction	Additive	**Synergistic**	**Synergistic**	**Synergistic**
		EC50(X5)		
Expected	90.73 ± 4.58	88.04 ± 3.45	86.82 ± 4.22	88.53 ± 3.98
Observed	82.98 ± 6.31	65.46 ± 8.55*	72.63 ± 9.21	76.32 ± 2.15*
p value	0.070	**0.048**	0.120	**0.031**
Interaction	Additive	**Synergistic**	Additive	**Synergistic**

The Nile red data is represented as mean ± SEM of at least three experiments performed in duplicates. 3,4-DHBA: 3,4-dihydroxy-benzoic acid, 4-HBA: 4-hydroxybenzoic acid, FA: ferulic acid, NR: Nile red. The * denotes significant difference (*p* < 0.05) between the expected and observed values as determined using the student's paired t-test.

3.6.14. The anti-adipogenic effect of the phenolic acids and combinations on 3T3-L1 adipocytes morphology evaluated with PlasDIC microscopy.

During differentiation, the 3T3-L1 cells in the confluent monolayer changes from fibroblastic to the rounder morphology associated with adipocytes (Figure 4.4). Inhibition of this process would result in fewer differentiated adipocytes (Figure 4.5). Therefore, using PlasDIC microscopy the effects of individual phenolic acids and the triple combination at the EC50(X2) and at day 7 and 13 of differentiation was evaluated.

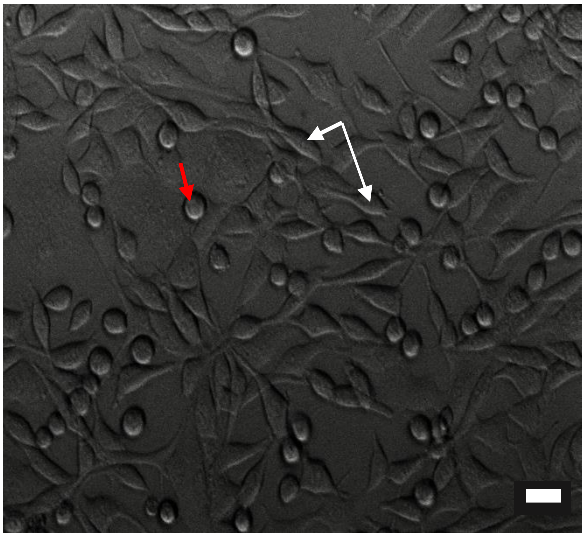
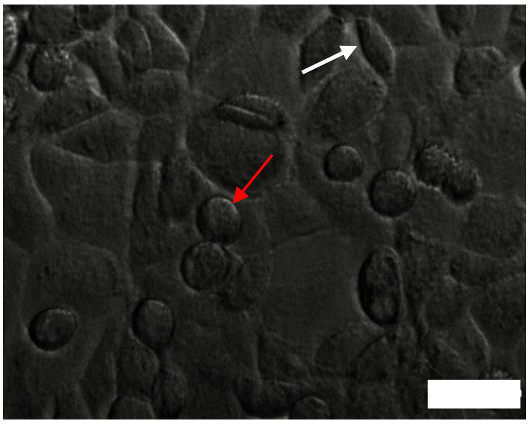
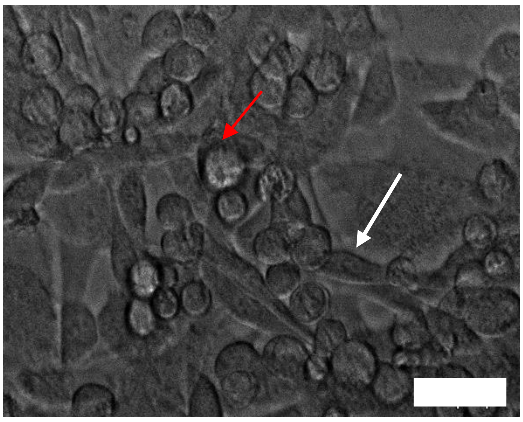
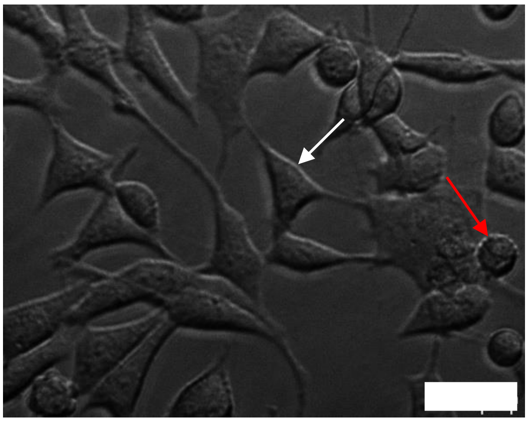
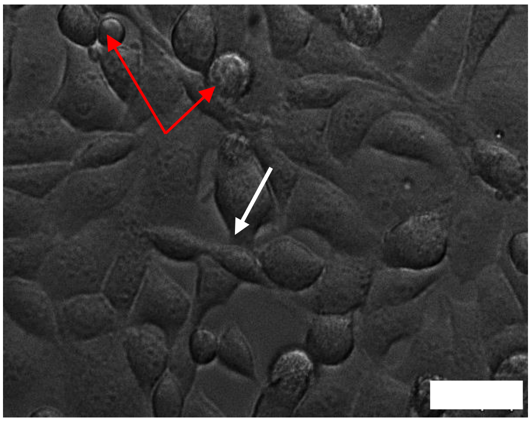
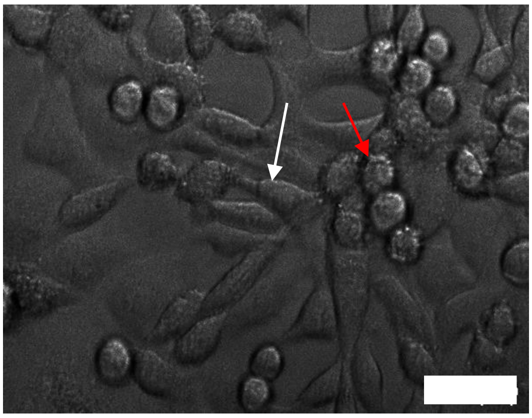
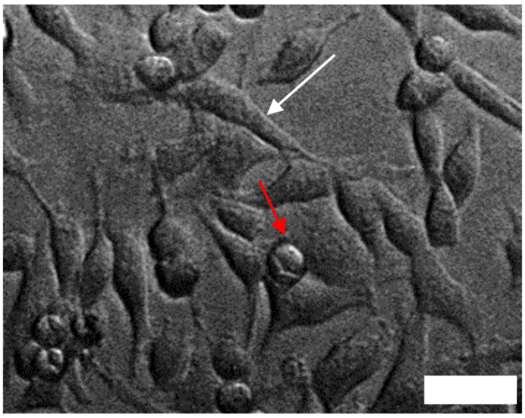


Figure 4.4. Morphology of undifferentiated 3T3-L1 fibroblasts as visualised with PlasDIC microscopy at 10x magnification. The scale bar represents 100 µm. White arrows indicate fibroblasts, and the red arrow shows rounded cells associated with adipocytes.

The micrographs also show the difference in cell differentiation between cells after day 7 in differentiation medium 1 (DM 1) and after day 13 in DMEM/FCS. In the control, the number of adipocytes at day 13 are more than observed on day 7. For exposure at 3,4-DHBA and 4-HBA at day 7 and 13 the number of adipocyte-like cells are less when compared with the respective controls. Although following exposure to 4-HBA, at day 7 the number of adipocytes is increased, at day 13, the number appears reduced. For FA, at day 13 most cells have a typical fibroblastic morphology. The effects of the double combination on cell morphology were not determined, however for the triple combination, in contrast to the effects of the single phenolic acids, the observed effect was an increased number of rounded, adipocyte-like cells.

	DM 1 (Day 7)	DM2 (Day 13)
Control		
3,4-DHBA		
4-HBA		

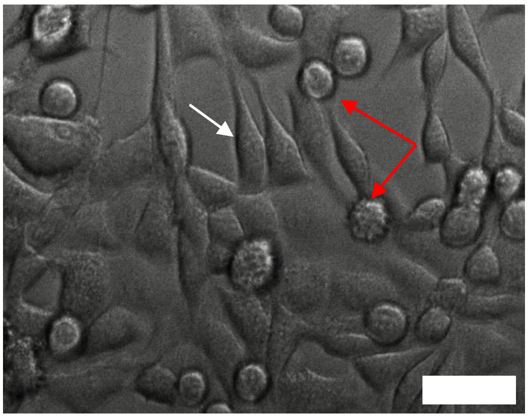
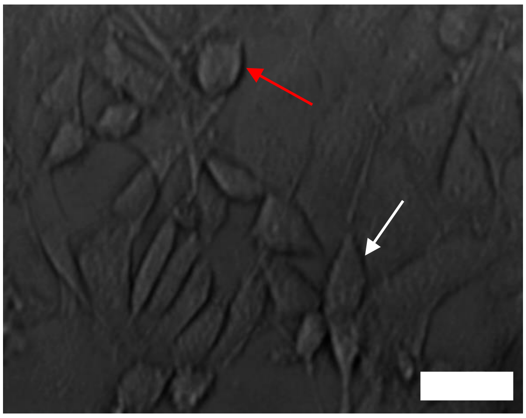
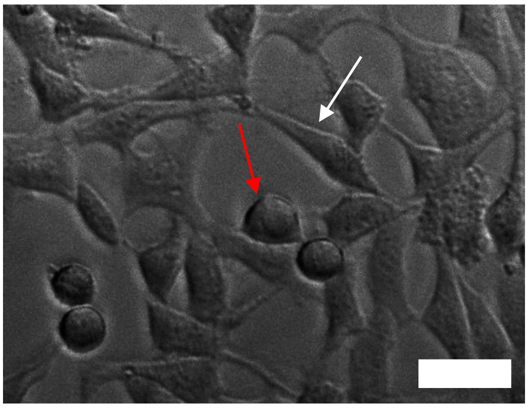
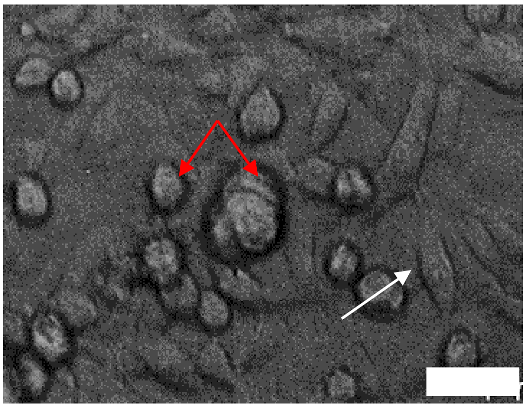
FA		
3,4-DHBA+4-HBA +FA		

Figure 4.5. Morphology of undifferentiated 3T3-L1 fibroblasts as visualised with PlasDIC microscopy after exposure to the EC50(X2) of 3,4-dihydrobenzoic acid (3,4-DHBA), 4-hydrobenzoic acid (4-HBA), ferulic acid (FA) and the combination 3,4-DHBA+4-HBA + FA at day 7 and 13 of differentiation/exposure. Red arrows show round adipocytes. White arrows show fibroblast cells.The scale bars represent 100µm at a 40× objective.

4.5. Discussion

In vitro studies (Seo *et al.,* 2015, Hithamani & Ganesan, 2022) and random controlled clinical trials (Most *et al.,* 2018, Delage *et al.,* 2023) suggest that polyphenolic compounds promote the inhibition of adipocyte differentiation and reduction in proliferation of adipocytes in addition to their antioxidant and anti-inflammatory effects (Cory *et al.,* 2018). Dietary phenolic acids may exert these beneficial effects through synergistic interactions (Herranz-López *et al*., 2012). Thus, there exists a need to determine the composition-bioactivity relationship or more specifically polyphenol-polyphenol effect of phenolic compounds on adipogenesis (Zhang *et al.,* 2019).

While studies assume additive or synergistic polyphenol-polyphenol interactions on the inhibition of lipid droplet formation in differentiating 3T3-L1 cells as quantified with the ORO assay, studies that have investigated these interactions on mixtures of pure polyphenols are limited (Zebisch *et al.,* 2012; Nathu *et al.,* 2021). This current study evaluated the effects of Mageu derived phenolic acid mixtures on lipid droplet formation using the ORO staining. Except for 3,4-DHBA a decrease in lipid accumulation was observed at EC50(2X) and EC50(5X), for 3-HBA and FA and the combinations.

A similar dose-dependent inhibitory effect was observed by Podsędek *et al.,* (2020) where phenolic extracts of *Viburnum opulus* elicited a dose dependent inhibitory effect on the formation of lipid droplets in differentiated 3T3-L1. Similarly, Seo *et al.,* (2015) reported dose-a dependent inhibitory effect for a mixture of coumaric and FA. Ilavenil *et al.,* (2017) observed that with increasing concentrations of FA, lipid accumulation in 3T3L1 adipocytes were reduced. In addition to this FA, in an *in vivo* study, significantly reduced the body weight and therefore adipose tissues mass, of high fat diet induced obese mice (Ilavenil *et al.,*2017). The mechanism of action perhaps relates to the structure of FA in the interaction with the other polyphenols in exerting the anti-adipogenic activity. Mosqueda-Solís, *et al.,* (2017) demonstrated that chemical structure and concentration of polyphenols are important for the exertion of inhibitory activity on lipid droplet formation in 3T3-L1 cells.

The NR assay, which uses a hydrophobic fluorescent dye to stain intracellular neutral lipids, is often use in adipogenicity studies because of its accuracy compared with ORO staining as NR specifically binds to the intended cellular structures (Escorcia *et al*., 2018). This explains the differences in the quantitative results observed with regards to the inhibitory effects of the phenolic acid mixtures on adipogenesis of 3T3-L1 cells quantified with ORO compared with NR assay.

With the NR assay, a decrease in lipid accumulation was only observed for the double and triple phenolic acid combinations. No effect was observed for 3,4-DHBA, 3-HBA and FA. Aranaz, *et al.,* (2019) also reported a lack of significant inhibition of lipid droplet formation as quantified with the NR assay in differentiated 3T3-L1 cells by various polyphenols including phenolic acids such as p-coumaric acid, ellagic acid, FA, GA, and vanillic acid. It was concluded that effects depended on the concentration and stage of the 3T3-L1 cell differentiation. Similarly, for a semi-purified phenolic extract of the *Viburnum opulus* flowering plant, a dose dependent inhibitory effects on the formation of lipid droplets in differentiated 3T3-L1 evaluated with NR staining was observed, where at low concentrations no inhibition was observed (Podsędek *et al.,* 2020).

The phenol-phenol interactions were generally additive. Synergistic polyphenol-polyphenol anti-adipogenic interactions were observed in the triple combinations at the EC25 and

EC50(X5). With NR staining, at EC50(X2) a synergistic effect was observed for all combinations except for 3,4-DHBA and 3-HBA. For either method, synergism was a common interaction observed for the triple combination. Contributing to synergism are that firstly, different polyphenols may exert their anti-adipogenic effects through distinct mechanisms, such as inhibition of adipocyte differentiation, suppression of lipid accumulation, or modulation of adipogenic gene expression. When combined, these polyphenols may act synergistically by targeting multiple pathways involved in adipogenesis, leading to enhanced anti-adipogenic activity (González-Castejón *et al.,* 2011). Secondly, certain polyphenols may enhance the bioavailability of others by influencing their absorption, distribution, metabolism, and excretion (ADME). By improving the bioavailability of co-administered polyphenols, synergistic effects on antiadipogenic activity may be achieved (Manach *et al.,* 2004). Lastly, polyphenols can modulate various signaling pathways involved in adipogenesis and lipid metabolism, including AMP-activated protein kinase (AMPK), peroxisome proliferator-activated receptor gamma (PPARγ), and CCAAT/enhancer binding protein α (C/EBPα) pathways (Ahmed, 2014.). Triple combinations of polyphenols may exert synergistic effects by concurrently targeting multiple signaling pathways, leading to enhanced inhibition of adipogenesis and adipocyte hypertrophy (Herranz-López *et al.,* 2012).

During the differentiation process, 3T3L1 cells differentiate into adipocytes and the associated change in cell morphology is from fibroblastic to the typical rounded morphology of adipocytes. The PlasDIC micrographs, show that the controls following differentiation presented with many mixed round and spindle shaped cells, with the round cells being hypertrophied. This is characteristic of adipogenesis/ adipogenic differentiation as obesity is marked by the differentiation of preadipocytes with subsequent morphological transition from fibroblast to round, triglyceride filled lipid droplets (Park *et al*., 2019, Singh *et al.,* 2022). The 3T3-L1 cells which, when correctly stimulated with a differentiation cocktail inclusive of essential adipogenic agents, such as insulin, DEX and IBMX and rosiglitazone, elevate cyclic adenosine monophosphate (cAMP) concentrations in the FBS containing media and stimulates the differentiation of 3T3-L1 from fibroblasts to adipocyte cells, usually within 10 to 12 days (Zebisch*, et al*., 2012, Ruiz-Ojeda, *et al.,* 2016). The transition from spindle to round shape accompanied by lipid accumulation observed in the differentiated control cells is consistent with the results of Rao *et al.,* (2015) and Jasaszwili, *et al., (*2019).

The phenolic acids 3,4-DHBA, 4-HBA and FA all showed cells that mostly maintained their fibroblast morphology. Phytochemicals like the β-indoloquinazoline alkaloid, bouchardatine, have been shown to inhibit the differentiation of 3T3-L1 cells through cell cycle arrest, thus inhibiting proliferation (Rao *et al.,* 2015). Similarly, Mageu rich in phenolic acids investigated in the current study have been reported to directly hinder the differentiation of 3T3-L1 fibroblasts into adipocytes (Nathu *et al*., 2021). However, in the 3,4-DHBA/4-HBA/FA triple combination, although fibroblast cells are seen, the spherical cells look to be hypertrophied and are comparable to the control cells. Hypertrophic adipocytes are known to promote the recruitment of preadipocytes and their differentiation into mature adipocytes by secreting specific paracrine factors (Longo *et al.,* 2019).

Upon the initiation of differentiation in preadipocytes, there is a subsequent rapid elevation in the transcription factors C/EBPβ and C/EBPσ, which are responsible for the induction of PPARγ and C/EBPα. These transcription factors remain elevated throughout the duration of the differentiation period regulating triglyceride uptake, fatty acid metabolism, and lipid storage through controlling the expression of adipocyte-specific genes i.e. Fatty acid synthase (Fasn), Lipoprotein lipase (Lpl) or stearoyl-CoA desaturase 1 (Scd1), that play key role in fat metabolism (Aranaz, *et al*., 2019). It has however been postulated that most phenolic acids strongly inhibit the early stages of differentiation, thereby inhibiting the action of these transcription factors to bring about their anti-adipogenic effect. Other studies however suggest that there are some phenolic acids that have their anti-adipogenic activity restricted to the initial stages of differentiation and not maintained throughout differentiation, this could the reason for the microscopic observation seen in the triple combination (Aranaz, *et al*., 2019).

4.6. Conclusion

Mageu derived phenolic acids exhibit dose-dependent anti-adipogenic effects as significant anti-adipogenic activity is observed at higher concentrations when compared to control cells in both the ORO and NR assay. Polyphenol-polyphenol interactions in the EC25, EC50 and EC50(X2) are predominantly additive. Synergistic interactions between phenolic acids are observed consistently in triple combinations, and at the higher concentrations. The phenolic acids also synergistically inhibit differentiation of preadipocytes, and thereby maintaining their fibroblast morphology. Considering all the presented quantitative and qualitative data, the observed additive and synergistic polyphenol-polyphenol interactions may be responsible for the previously reported anti-adipogenic activity of Mageu rich in 3,4-DHBA, 4-HBA, and FA.

Chapter 5. The cytotoxicity of the phenolic acids identified in Mageu and the associated polyphenol-polyphenol interactions

5.1. Introduction

Studies have shown that consumption of phenolic rich beverages such as Mageu, results in a vast range of physiological effects including antioxidant, anti-inflammatory, antimicrobial and other bioactive benefits (Mu, 2022). *In vitro* and *in vivo* studies have validated these benefits and their potential application in the prevention and treatment of ROS induced chronic degenerative diseases. As a result, great interest has been focused in extracting these polyphenols and producing supplements and/or nutraceuticals with higher concentrations in comparison to their original source. However, side effects have been reported when isolated phenolic acids were consumed in high doses outside of their original food source. These side effects are thought to be linked to the difficulties of the phenolic acids in mimicking the *in vivo* conditions observed when part of a food matrix. Thus, performing cytotoxicity assays is essential in the determination of the maximum beneficial dosage in which isolated phenolic acids can be consumed (Lopez-Corona *et al.,* 2022).

Cytotoxicity is a vital indicator for the evaluation of bioactive compounds in *in vitro* studies. Exogenous sources of molecules such as polyphenols can affect cellular metabolism potentially leading to multiple cytotoxic mechanisms including, prevention of protein synthesis, the destruction of cell membranes, and activation of certain pathways and/or receptors. To determine the potential damage and subsequent cell death, accurate, reliable, and reproducible protocols are required. These cytotoxicity and cell viability assays are used as screening methods to determine the cytotoxic effects of chemicals on cell viability or proliferation (Adan *et al.,* 2016; Aslantürk, 2018).

The results are often dependent on the type of molecule evaluated, the assay used and the sensitivity of the method. The MTT assay is widely used and although highly sensitive, as an assay measuring the reducing capacity of a cell, the evaluation of polyphenols with reducing capacity has been identified to be problematic (Śliwka *et al.,* 2016; Karakas *et al.,* 2017). In contrast a staining method such as the CV assay does not measure the effects on cell metabolism but rather the total protein and DNA content and therefore is metabolism independent (Śliwka *et al.,* 2016; Aslantürk, 2018)

Various studies have shown the 3,4-DHBA, 4-HBA and FA show greater cytotoxicity in cancer cells when compared with normal cells (Nunes *et al.,* 2021; Mu, 2022). However, the synergistic, additive and/or antagonistic effects of the phenolic acids on cell viability have yet to be determined.

The aim of the research presented in this chapter is to determine the cytotoxicity of EC25 and EC50 of 3,4-DHBA, 4-HBA, FA and combinations and the type of polyphenol-polyphenol interactions on the Caco-2 cell number using the CV assay after 24-, 48- and 72-hours exposure. Then to determine the cytotoxicity of EC25, EC50, EC50(2X) and EC50(5X) of 3,4-DHBA, 4-HBA, FA and combinations at the on the relative cell number of 3T3-L1 cells using the CV assay after 24-, 48- and 72 hours exposure.

5.2. Materials

5.2.1. Cell lines

The human colon adenocarcinoma (Caco-2) and murine fibroblast (3T3-L1) cell lines were established, cultured and maintained as described in Sections 3.2.1 and 4.2.1.

5.2.2. Reagents, equipment, glassware and disposable plasticware

Mageu derived phenolic acids, 3-4DHBA, 4-HBA and FA, were prepared as in Chapter 3. Additional reagents were CV, formic acid and acetic acid purchased from Merck Chemicals, Modderfontein, SA.

All equipment and glassware were the same as indicated in Sections 3.2.2

5.2.3. Laboratory facilities

All research was conducted in the research facilities of the Department of Anatomy of the Faculty of Health Sciences, University of Pretoria.

5.3. Methods

5.3.1. Crystal violet assay

Crystal violet is a dye that stains the DNA and proteins of attached/ viable cells. It is based on the principle that adherent cells which are undergoing cell death detach from the cell culture plate and thus are lost (Aslanturk, 2017). In addition, with increased toxicity the permeability of the plasma membrane is compromised and leakage of the cell content including ions, metabolites, proteins, and RNA also occurs. Consequently, cells can still be attached but CV staining is reduced. Therefore, the CV assay provides an indication of the degree of staining relative to the control and is expressed as the % cell number as this assay does not measure if attached cells are dead or alive.

The cell line cultures were established and cultured as described in Sections 3.3.1 for the Caco-2 cell line and Sections 4.3.1 for the 3T3L1 cell line.

For the evaluation of cytotoxicity, the Caco-2 and 3T3-L1 cells were plated at 11.1x10^4/mL, in 90 µL (final concentrations 10x10^4 cells/mL and cultured left overnight at 37°C and 5% CO_2.

Cells were then exposed to 10 µL of sample (ensuring final EC25 and EC5 prepared in Section 3.3.1.) for 24, 48 and 72 hours. After exposure, 11 µL of 20% formaldehyde (final concentration 2%) was added to fix the cultured cells. After the 30 minutes, the fixative and spent medium was removed and the plate left to dry. This was followed by the addition of 100 µL of 0.1% (w/v) CV staining solution prepared in 0.75% formic acid. After 30 minutes, the dye was removed, and the plate gently washed with distilled water. The dye was then extracted in 100 µL of 10% v/v acetic acid and the absorbance was measured at 630 nm. Vehicle controls included cells exposed to 10 µL of double distilled water.

5.4. Results

5.4.1. The Caco-2 cell cytotoxicity of the phenolic acids and combinations

To determine the cytotoxic effects of the Mageu derived phenolic acid mixtures, the CV assay, was used to quantify the cell number as a percentage relative to the negative control. The cytotoxic effects of 3,4-DHBA, 4-HBA and FA, at single, double, and triple combinations were evaluated at the EC25 and EC50 concentrations after 24-, 48- and 72-hours exposure (Figure 5.1). Relative to the negative control, 3,4-DHBA, 4-HBA and FA after 24, 48 and 72 hrs caused no significant reduction in the % cell number. For the double combinations, at the EC25 significant a decrease in the % cell number ($p < 0.01$) was observed for the combination of 4-HBA + FA (83.7 ± 1.88 %) at 24 hours but not at 48 and 72 hours. Likewise, 3,4-DHBA, 4-HBA + FA caused a decrease in the % cell number to 81.23± 4.69% after 24 hours but not after 48 and 72 hours.

At the EC50, 3,4-DHBA, 4-HBA and FA were also not cytotoxic, while after 24 hours exposure 3,4-DHBA+ 4-HBA (80.78 ± 1.91%) ($p < 0.001$), 3,4-DHBA + FA (89.17 ± 3.75%) ($p< 0.05$) and 4-HBA +FA (85.32 ± 1.50%) caused a decrease in the % cell number, which after 48 and 72 hours exposure was similar to the control. Between the groups, EC25 and EC50, differences were not statistically significant.

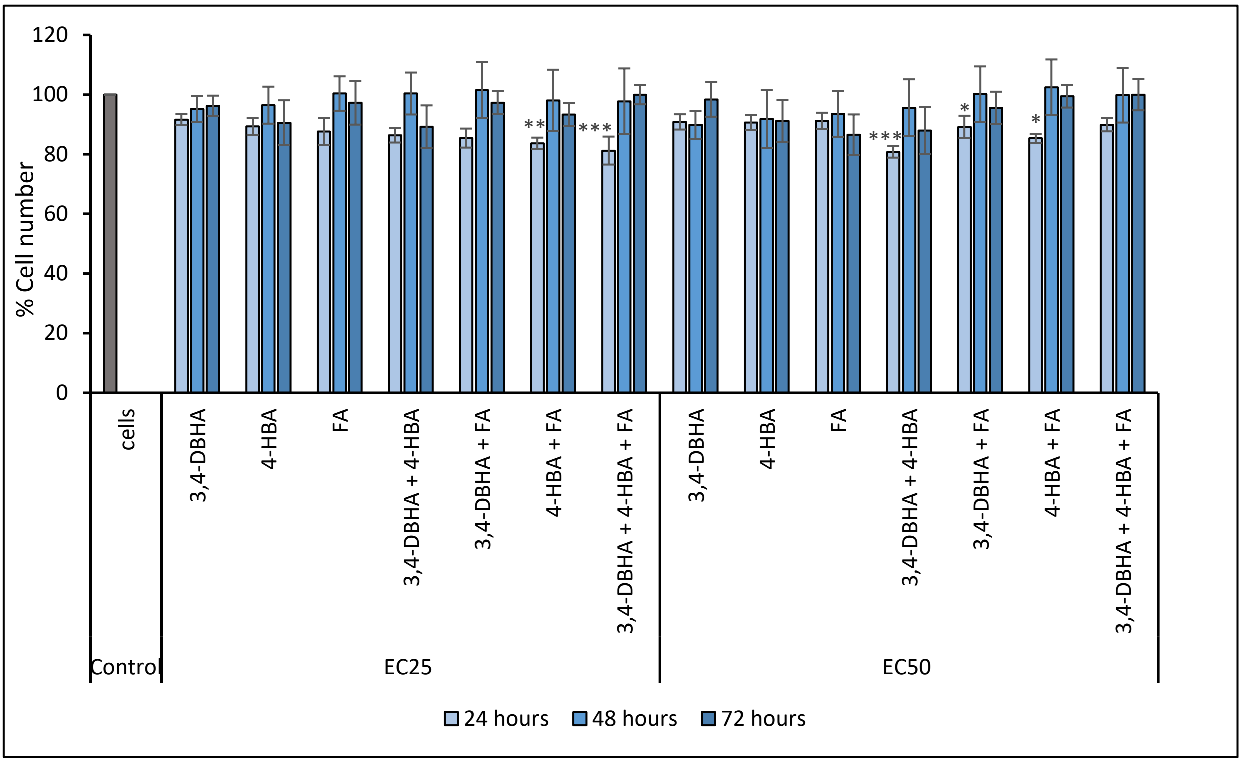


Figure 5.1. Cytotoxicity in Caco-2 cells of 3,4-dihydroxybenzoic acid (3,4-DHBA), 4-hydroxybenzoic acid (4-HBA) and ferulic acid (FA) and combinations at the EC25 and EC50 evaluated after 24, 48 and 72 hours quantified with the Crystal Violet assay. The data is represented as mean ± SEM of at least four experiments performed in triplicate. Significant difference from the control is illustrated as $p<0.05$ = *, $p<0.01$ = **, $p<0.001$ = ***, $p<0.0001$ = ****. Between EC25 and EC50, no statical differences were found.

5.4.2. The 3T3-L1 cell cytotoxicity of the phenolic acids and combinations

The cytotoxicity of the Mageu derived phenolic acids and mixtures at the EC25 and EC50 were assessed in the 3T3-L1 cells after 24-, 48-, and 72-hours exposure. In addition, cytotoxicity was also evaluated at the EC50(2X) and EC50(5X) to determine whether the observed anti-adipogenic effects was not due to increased cytotoxicity. At the EC25, after 72 hours (Figure 5.2) the % cell number was significantly reduced for all phenolic acids and combinations. The range of % cell number after 72 hrs exposure to the EC25 and EC50 was 78.6 - 90,6% and was statistically significantly reduced compared with the control.

No time dependent decrease in the % cell number was observed although after 72 hours, the % cell number was reduced. Compared with the Caco-2 cell line, the 3T3-L1 cell line is more sensitive to the toxicity of the phenolic acids and the combinations.

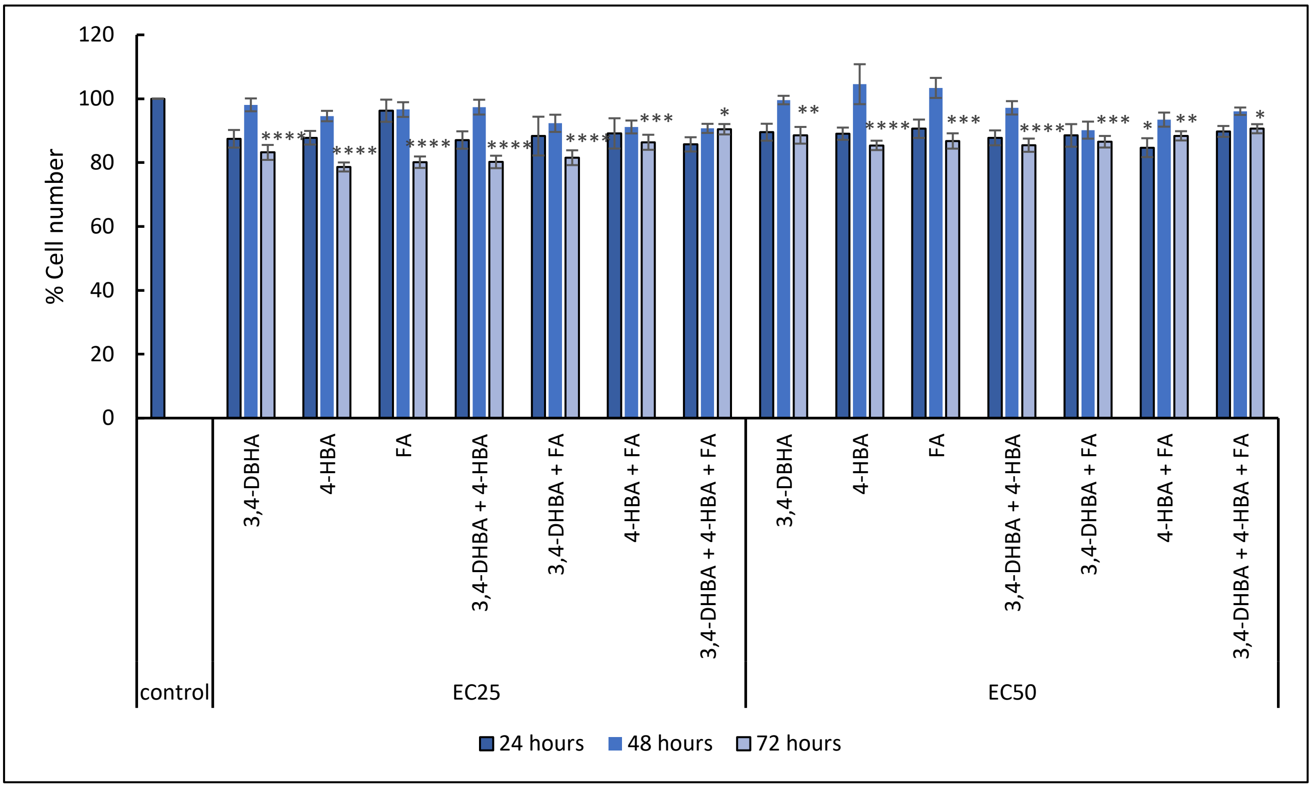


Figure 5.2. Cytotoxicity in 3T3-L1 cells of 3,4-dihydroxybenzoic acid (3,4-DHBA), 4-hydroxybenzoic acid (4-HBA) and ferulic acid (FA) and combinations at the EC25 and EC50, quantified after 24, 48 and 72 hours. quantified with the Crystal Violet assay. The data is represented as mean ± SEM of at least four experiments performed in triplicate. Significant difference from the control is illustrated as $p<0.05$ = *, $p<0.01$ = **, $p<0.001$ = ***, $p<0.0001$ = ****. Differences between groups are not significant.

In Chapter 4, Table 4.3 beneficial synergistic effects were observed for 3,4-DHBA/4-HBA/FA combination at EC50(5X) with the ORO and NR stains. Reduced lipid formation potentially can be the result of increased cytotoxicity and therefore toxicity was re-evaluated at these concentrations with 72 hours representing the intervals of administration during differentiation. Figure 5.3 shows the results obtained after the exposure of 3T3-L1 cells with the phenolic acids alone and in combinations at the concentrations EC50(X2) and EC50(X5) for 72 hours.

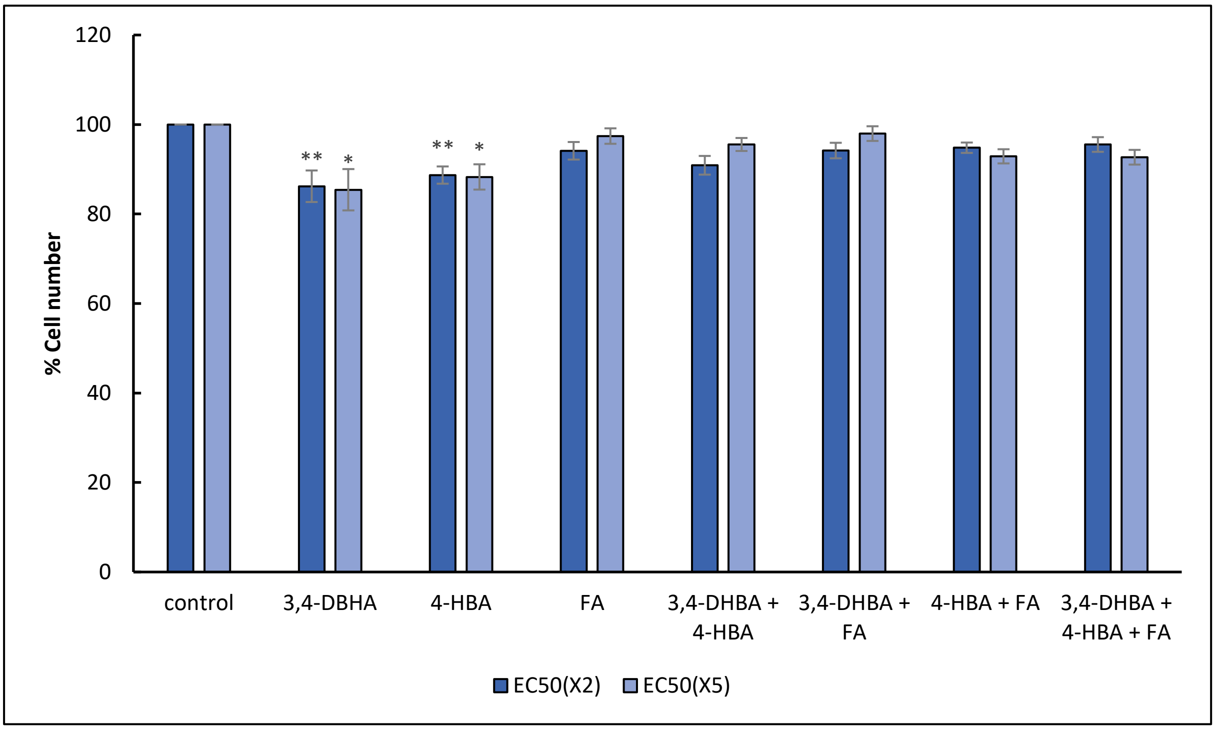


Figure 5.3. Cytotoxicity in 3T3-L1 cells of 3,4-dihydroxybenzoic acid (3,4-DHBA), 4-hydroxybenzoic acid (4-HBA) and ferulic acid (FA) and combinations at the EC25(X2) and EC50(X5), quantified after 72 hours. quantified with the Crystal Violet assay. The data is represented as mean ± SEM of at least four experiments performed in triplicate. Significant difference from the control is indicated as $p<0.05$ = *, $p<0.01$ = **, $p<0.001$ = ***, $p<0.0001$ = ****. Differences between groups are not significant.

Significant difference between the control and 3,4-DHBA and 4-HBA in both EC50(X2) and EC50(X5) concentrations were observed, with a % cell number of 86.20 ± 3.52% ($p<0.05$) and 88.70 ± 1.93% ($p<0.005$) at EC50(X2) concentration and 85.42 ± 4.61% ($p<0.05$) and 88.28 ± 2.83% ($p<0.005$) at EC50(X5) concentration, respectively. For all other phenolic acids at EC50(X2) and EC50(X5) no significant cytotoxicity was seen. This shows that 3,4-D4HBA and 4-HBA consistently exhibit cytotoxic effects on 3T3-L1 cells after 72 hours of exposure for all tested concentrations; namely, EC25, EC50, EC50(X2) and EC50(X5) concentrations. For the double and triple phenolic acid mixtures which showed no significant difference when compared with the control cells after 72 hours for both EC50(X2) and EC50(X5) concentrations. This confirms that reduction in the % lipid accumulation is not due to increased cytotoxicity but rather the inhibition of the biochemical pathways associated with lipid accumulation.

5.5. Discussion

Various research studies have shown that phenolic acids have antioxidant, anti-inflammatory, and anti-adipogenic properties. To determine their non-cytotoxic doses as individual compounds and mixtures, cell viability tests were utilized to observe the potential cytotoxic effect of 3,4-DHBA, 4-HBA, and FA on intestinal Caco-2 and preadipocyte 3T3-L1 cells.

For the individual Mageu phenolic acid derivatives, the samples at EC25 and EC50 in both cell lines revealed an initial decline in % cell number after 24 hours, followed by cell proliferation and improved cell viability after 48 hours. The observed decrease in % cell number after 24 hours is attributed to changes in cell culture parameters such as temperature, pH, sample addition, and/or shock, as cell growth is impacted by environmental factors including alterations in cell culture parameters, exposure to pharmacological agents and growth factors, and in response to numerous disease states. Thus, the subsequent increase in cell number after 48 hours is indicative of the cell recovery period (Aslanturk, 2017; Xu *et al.,* 2022). Similarly, Mavhungu (2012) observed an effect of phenolic acid containing extract on the cell viability of Caco-2 cells was associated with an initial decrease in cell viability followed by cell proliferation at 72 hours. Thus, likewise individual, and mixtures of phenolic acids can promote cell growth and viability (Mavhungu, 2012).

After 72 hours, the % cell number was unaltered compared with the control, indicating no cytotoxicity to Caco-2 cells. However, all phenolic acid samples, including mixtures at EC25 and EC50 concentrations, were significantly ($p < 0.05$) cytotoxic to murine 3T3-L1 preadipocyte, indicating a time-dependent cytotoxic effect.

Kampa *et al.* (2004) reported that phenolic acids such as CA, sinapic acid, syringic acid, FA, and 3,4-DHBA exhibit a time- and dose-dependent antiproliferative activity, with FA and 3,4-DHBA having nearly identical cytotoxic potency. Furthermore, both polyphenols, at a concentration of conentration10^{-7} M, reduced cell proliferation by 40% over 6 days at a (Kampa *et al.,* 2004). This is consistent with the observed 20% drop in cell viability over three days in this study.

Studies have indicated that mixtures that exhibit similar/complementary cytoprotective/anti-cancer mechanisms would allow for enhanced complementary/synergistic interactions between the compounds (Niedzwieki *et al.,* 2016). Thus, the observed protective effects of containing mixtures could be attributed to enhanced interactions secondary to complementary cytoprotective mechanisms.

Studies on the phenolic acid, 3,4-DHBA indicate that it plays a significant role in the reduction of cell proliferation and has demonstrated significant pro-apoptotic properties on various cancer cell lines (Kakkar & Bais, 2014; Acquaviva *et al.,* 2021). In a study by Yıldız *et al.,* (2023), investigating the cytotoxic effects of 3,4-DHBA alone and in combination with chemotherapeutic drugs on Caco-2 cells, the results showed that the phenolic acid alone and in combination significantly suppressed the cell viability of the Caco-2 cells. The cytotoxic effects were observed to increase with dose and time, with a significant reduction in cell viability notable at 48 hours post exposure, starting from a concentration of 50 µM (Yıldız *et al.,* 2023). The pro-apoptotic effect of 3,4-DHBA is postulated to be mediated by the modulation of the redox balance and heme oxygenase -1 system which leads to the activation of the p21 (Acquaviva *et al*., 2021). However, the mechanism of action is still not clearly elucidated, and requires further investigation.

The phenolic acid, 4-HBA has antiproliferative activity against cancerous cell lines with 4-HBA shown to decrease the cell viability of doxorubicin- sensitive K562 and doxorubicin-resistant K562/Dox leukemia cells in a dose and time-dependent manner in a study by Zambonin *et al* (2012). The study confirmed that *in vitro*, phenolic acids suppress the development of cancer cells, indicating an additional preventive action against hormone-dependent breast cancers (Zambonin *et al.,* 2012). According to Kampka *et al.,* (2004), FA, showed a bimodal/biphasic cytotoxic effect. This effect refers to the ability of FA to exert both cytotoxic and cytoprotective effects at different concentrations and or times, consisting of a short time acting and a long-time acting cytotoxic or cytoprotective effect (Kampka *et al.,* 2004).

While the cytotoxic properties of the individual phenolic acids and mixtures containing these phenolic acids are well studied (Quideau *et al*., 2011; Li *et al.,* 2014); Studies on the combinations investigated in the present study have not been undertaken and therefore requires further elucidation.

The toxicity of the Mageu phenolic acids at all investigated concentrations (EC25, EC50, EC50(X2), and EC50(X5)) was consistently less than 20% when compared to the control after 72 hours, and cytotoxicity was time dependent. The samples did not, however, show a dose-dependent effect on cell viability since FA and phenolic acid combinations had no cytotoxic effects on 3T3-L1 cell viability after 72 hours at the highest concentrations evaluated. Similarly, the *Antidesma bunius* L. MM extract, which contains phenolics and anthocyanins such as GA, catechin, and protocatechuic acid, had a low cytotoxic effect on pre-adipocytes at all tested concentrations. Cell viability was high (86.3 ± 2.5-106 ± 2%), thus exhibiting toxicity levels of less than less than 20% as well. At the highest tested concentration of 2 mg/mL, of the MM extract a significant reduction in cell viability compared to the control was observed (Krongyut & Sutthanut, 2019). This indicates that like the phenolic acid mixtures, plant extracts that also contain mixtures of polyphenols generally require high concentrations to mediate a significant level of cytotoxicity. Therefore, the long-lasting cytoprotective effect on cell viability can be explained by the multifaceted mechanism of action of phenolic acids that includes antioxidant and anti-inflammatory activity as well as through the modulation of cell signaling pathways involved in cell survival and proliferation, such as the phosphoinositide 3-kinase (PI3K/Akt) and mitogen-activated protein kinase (MAPK pathways).

In Chapter 3 the phenolic acids and combinations did not cause oxidative damage and in addition, no dosage- and time dependent toxic effect was observed, indicating at the concentrations used to determine antioxidant effects no toxicity was observed. In Chapter 4, the effect on the differentiation of 3T3L1 cells into adipocytes was evaluated. Although a statistical reduction in the % cell number was observed for 3,4-DHBA and 4-HBA, in general, the anti-lipogenic effects observed for the phenolic acid combinations are not due to toxicity. Yang *et al.,* (2008) and later Aranaz *et al.,* (2019) demonstrated that polyphenols such as resveratrol and quercetin or luteolin and kaempferol at 100 and 1 µM can decrease 3T3-L1 cell viability by up to 20 and 25%, respectively and concluded that the observed anti-adipogenic action was not related to the cytotoxic effects of the samples.

5.6. Conclusion

This present study suggests that Mageu derived phenolic acids although not cytotoxic to adenocarcinoma (Caco-2) cells, exert a mild time-dependent cytotoxic effect on preadipocyte mouse fibroblast (3T3-L1) cells, however, the anti-adipogenic effects of the phenolic acids and their mixtures were not linked to their cytotoxic activity.

Chapter 6. Concluding discussion

Medicinal foods, categorized as functional foods, represent a class of consumables hypothesized to provide distinct health benefits beyond their primary nutritional components (Dixit *et al*.,2023). Dietary polyphenols have been reported to be abundant in cereals. Cereals have varied amounts of phenolic content, of which, maize has the highest polyphenol content. Mageu, which is a traditional food is a sour non-alcoholic beverage derived from maize. Identified cellular health benefits are antioxidant, anti-adipogenic and anti-inflammatory (Nathu, 2021). The contributing polyphenols were not the flavonoids but the phenolic acids, 3,4-DHBA, 4-HBA, FA, p-CA, and CA. With simulated *in vitro* digestion, the levels of these polyphenols were increased. Thus, the purpose of this study aimed to ascertain whether the identified phenolic acids in Mageu are responsible for the observed antioxidant and anti-adipogenic effects of Mageu and whether synergic polyphenol-polyphenol interactions mediated these effects. The three phenolic acids, 3,4-DHBA, 4-HBA and FA that were identified to be significantly increased following digestion were the focus of this study.

6.1. Summary of results

The antioxidative potential of the phenolic acids extracted from Mageu was assessed using F-C assay, the TEAC and the ORAC assays. Subsequently the antioxidant effects were evaluated in the Caco-2 cells with the DCFH-DA assay at their EC25 and EC50.

To calculate the EC50, a modified method by Ou *et al.,* (2002) was used, involving a 0.139 nM fluorescein solution in PBS and a 0.24 M AAPH solution in distilled water. Fluorescence was measured every 60 seconds for 120 minutes at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The area under the curve (AUC) was calculated for each sample, and the percentage inhibition relative to the control (AAPH only) was determined. The EC50 was identified as the concentration of phenolic acid that reduced oxidative damage by 50%, and from this value, the EC25 was derived, representing 25% inhibition.

Mageu derived phenolic acids were tested for reducing capacity at the EC25 and EC50 concentrations, using the F-C assay. The reducing capacity of the individual samples at the EC25 was 0.047 mM, 0.047 mM and 0.038 mM for FA, 3,4-DHBA and 4-HBA respectively. At the EC50, the reducing capacity was 0.01 mM, 0.009 mM and 0.0038 mM for FA, 4-HBA and 3,4-DHBA respectively. The phenolic acid, FA was shown to have the highest reducing capacity at both concentrations. In the case of the EC25 combinations, the reducing capacity was 0.05 mM (3,4-DHBA + 4-HBA) > 0.04 mM (4-HBA+FA) > 0.03 mM (3,4-DHBA + FA), with 3,4-DHBA + 4-HBA having the highest reducing capacity. No significant difference between the double and single samples was observed. Neither was there any significant difference between the double and the triple combination/s. The EC50 double combinations with the highest reducing capacity of 0.12 mM for 3,4-DHBA + 4-HBA, 0.1 mM 3,4-DHBA + 4-HBA and 0.08 mM for 4-HBA + FA. The EC50 triple combination (3,4-DHBA + 4-HBA + FA) presented with the highest reducing capacity of 0.14 mM, significantly higher than the single phenolic acids and their double combinations. The EC50 samples showed to have significantly ($p < 0.05$) higher reducing capacity when compared with the EC25 counterparts, thus indicating a concentration-dependent increase in reducing capacity. For the polyphenol-polyphenol interactions, showed synergisms for all combinations at the EC25. At the EC50, the triple and 3,4-DHBA + 4-HBA combinations showed synergistic interactions, whereas the 4-HBA+FA and 3,4-DHBA + FA combinations exhibited an additive effect.

The antioxidant capacity of the individual phenolic acids measured with the TEAC assay followed the order: FA > 3,4-DHBA > 4-HBA, for both EC25 and EC50 concentrations with FA exhibiting the highest antioxidant activity of 0.02 mM. The higher antioxidant activity of FA compared with 3,4-DHBA and 4HBA was attributed to its OH group and CH_3O side chain. For the individual phenolic acids at the EC25 compared with EC50, only 3,4-DHBA presented with a dosage dependent increase in antioxidant activity. The double and triple combinations showed no significant difference in antioxidant activity when compared with the individual phenolic acids and the combinations at both the EC25 and EC50. At the EC25, the polyphenol-polyphenol interactions were mostly additive, while at the EC50, an antagonistic effect was observed. Thus, indicating that when in combinations, other factors, such as assay conditions, can affect polyphenol interactions (Gallardo *et al.,* 2006).

Antioxidant activity was further evaluated with the ORAC assay. At the EC25 the activity for FA, 3,4-DHBA and 4-HBA was 0.0173 mM, 0.0073 mM, 0.0071mM respectively. At the EC50, antioxidant activity was 0.021 mM, 0.017 mM and 0.011 mM for FA, 3,4-DHBA and 4-HBA respectively. Similar to the TEAC results, no significant difference was observed between the antioxidant activity or dosage dependent increase in activity was observed between of the individual phenolic acids and the combinations at both concentrations. However, unlike the TEAC results, the triple combinations at the EC25 and EC50 exhibited high radical scavenging capacity of 0.02 mM and 0.021 mM respectively; showing a significant difference in antioxidant activity when compared with the single phenolic acids as well as the double combinations. For the triple combinations at the EC25 and EC50 synergistic and additive interactions respectively were observed. For all other phenolic combinations, the interactions were antagonistic.

The DCFH-DA assay was used to determine antioxidant activity in a cellular model. At the EC25 concentration, only 4-HBA significantly decreased AAPH-induced oxidative damage in the Caco-2 cells. At the EC50 level, both 4-HBA and FA, as well as their triple combination with 3,4-DHBA, effectively protected the cells from oxidative damage. Conversely, at EC50X2, a pro-oxidant effect was noted for 3,4-DHBA alone and in combination with FA. However, at the higher concentration of EC50X5, 3,4-DHBA and the triple combination exhibited a strong cellular antioxidant effect. These results indicate that the protective effects of polyphenols on oxidative damage are concentration-dependent, with varying interactions observed at different concentration levels.

The evaluation of polyphenol-polyphenol interactions revealed differential interactions contingent on concentration levels. At the EC25, all combinations exhibited additive effects with no significant differences from expected values ($p > 0.05$). At the EC50 level, synergistic interactions were significant for 3,4-DHBA/4-HBA ($p = 0.045$), 4-HBA/FA ($p = 0.033$), and the triple combination ($p = 0.031$), indicating enhanced antioxidant efficacy. Synergistic interactions persisted at EC50(X2) and EC50(X5) for the triple mixture ($p = 0.015$ and $p = 0.047$) and for 3,4-DHBA/4-HBA at EC50(X5) ($p = 0.023$). These findings thus highlighted the concentration-dependent nature of polyphenol interactions in enhancing cellular antioxidant defences.

The anti-adipogenic activity as inhibition of 3T3-L1 adipocytes differentiation, was evaluated with the ORO and NR assays, at the EC25, EC50, EC50(X2) and EC50(X5). From the ORO data, a dose-dependent increase in anti-adipogenic activity was observed. Polyphenol-polyphenol interactions of double combinations at the EC25, EC50, EC50(X2) and EC50(X5) concentrations were additive while the triple combination was additive at EC50(X2) and synergistic at EC50(X5). Similar to the ORO assay, NR results showed a dose dependent increase in anti-adipogenic activity of Mageu derived phenolic acids. No significant ($p > 0.05$) inhibition on lipid accumulation was observed for the individual phenolic acids compared with the control. However, with the double combinations a significant reduction ($p < 0.05$) in lipid droplet formation was observed at higher concentrations than was observed after ORO staining. Synergistic polyphenol-polyphenol interactions were observed in FA containing double and the triple combinations at the EC25, EC50(X2) and EC50(X5). All the combinations that were additive with both the ORO and the NR assays. Light microscopy, of ORO-stained cultures exposed to EC25 and EC50 compared with the control cells, showed reduced staining. Furthermore, with PlasDIC microscopy the effect of the single phenolic acids and the triple combination on adipocyte differentiation was evaluated. Fewer adipocytes relative to undifferentiated fibroblastic cells were observed for 3,4-DHBA and 4-HBA but not the triple combination.

The toxicity of the phenolic acids and mixtures was evaluated with the CV assay in the Caco-2 and 3T3-L1 cells. The individual phenolic acid exhibited no Caco-2 cytotoxicity following exposure to EC25 and EC50 for 24, 48 and 72 hrs. In general, the 3T3-L1 cells were more sensitive with increased cytotoxicity observed for EC25 and EC50 after 72 hrs exposure. Notably at EC25 and EC50 concentrations in both cell lines an initial decline in cell viability after 24 hours was observed, followed by cell proliferation, and improved cell viability after 48 hours. The observed decrease after 24 hours is possibly attributed to stress factors with a recovery at 48 and 72 hrs. Thus, its inferred that the phenolic acids and their combinations are not cytotoxic to Caco-2 cells and potentially exert a mild time-dependent cytotoxic effect on preadipocyte 3T3-L1 cells. At the concentrations and exposure times used for the determination of CAA and the effects on lipid accumulation in 3T3L1 cells the observed effects were no due to cytotoxicity.

In conclusion Mageu derived phenolic acids possess antioxidant and anti-adipogenic properties that are either additive or synergistic. Mageu, when consumed at the concentrations tested, could offer significant antioxidant and anti-adipogenic benefits. The concentration-dependent polyphenol interactions, showing both synergistic and additive effects, imply that regular ingestion of Mageu could support cellular health by reducing oxidative stress and inhibiting adipogenesis. These findings reinforce Mageu's potential role as a functional food, particularly due to its rich phenolic acid profile, enhancing both its traditional value and its application in modern dietary interventions aimed at mitigating oxidative damage and supporting metabolic health.

6.2. Limitations and recommendations

This study focused on Mageu derived phenolic acids and their interactions at specific concentrations, in future studies a concentration dosage effect can be evaluated. From this data the change in antioxidant activity can be determined. This will eliminant possible problems associated with depletion of the substrate in the assays as effects in the linear region of the assay will be evaluated. This will also provide more robust data for the analysis of phenolic-phenolic interactions. The data generated from the CAA assay, was highly variable and a dosage effect will better identify concentrations that have an antioxidant vs a pro-oxidant effect.

In this study PlasDIC was utilised to visualise the morphology of sample exposed 3T3-L1 cells. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) may be utilised to study structures on a nanoscale level- elucidating the effects of Mageu derived phenolic acids on cellular interactions, subcellular components, and potential ultrastructural changes such as the effect on lipid droplet formation. Western blotting analysis of specific adipocyte associated markers such as Uncoupling protein 1 (UCP1) expression, adipose triglyceride lipase (ATGL) and activated protein kinase (AMPK) will provide an opportunity to quantify these effects. Pacifici *et al.*, (2023) assessed the effect of a mixture of polyphenols called A5$^+$ using Western blot analysis of adipose associated markers (UCP1, ATGL and AMPK) on differentiated 3T3-L1 cells. All tested markers were observed to have significantly increased in A5+-treated cells when compared to control cells ($p < 0.05$).

Moreover, a methodological limitation of this study was the use of Oil Red O (ORO) staining for qualitative observations of lipid accumulation. Although ORO is commonly employed, it lacks the specificity of more advanced techniques. In future research, the use of fluorescence microscopy with Nile Red (NR) staining should be considered, as NR binds more selectively to lipid droplets, providing a more accurate and specific qualitative observation of lipid accumulation. Unfortunately, due to time constraints, fluorescence microscopy could not be integrated into the current study. Moving forward, incorporating NR staining would greatly enhance the precision of qualitative lipid accumulation observations and provide a more robust assessment of the anti-adipogenic effects of Mageu-derived phenolic acids.

The most significant finding of this study is the effect on the adipocytes. Various transcription factors that play key roles in fat metabolism. The effect of these phenolic acids on the fatty acid synthase (Fasn), lipoprotein lipase (Lpl) and stearoyl-CoA desaturase 1 (Scd1) should be investigated in either cell or animal models to determine the mechanism by which these transcription factors are modulated. Knowledge of these mechanisms in addition to the polyphenol- polyphenol and polyphenol-protein interactions can lead to the development of targeted therapeutic formulations.

Lastly, maize contains other bioactive molecules (Table 2.3), and the effects of these are matrix associated effects will be important parameters that can also be evaluated in future studies and will provide key information on how functional foods such as Mageu has beneficial health promoting properties.

Chapter 7. References

1. ACD/ChemSketch. (2023). Version 2023.2. Advanced Chemistry Development, Inc. Toronto, ON, Canada.
2. Adefegha, S.A., 2018. Functional foods and nutraceuticals as dietary intervention in chronic diseases; novel perspectives for health promotion and disease prevention. *Journal of dietary supplements*, *15*(6), pp.977-1009.
3. Acquaviva, R., Tomasello, B., Di Giacomo, C., Santangelo, R., La Mantia, A., Naletova, I., Sarpietro, M.G., Castelli, F. and Malfa, G.A., 2021. Protocatechuic acid, a simple plant secondary metabolite, induced apoptosis by promoting oxidative stress through HO-1 downregulation and p21 upregulation in colon cancer cells. *Biomolecules*, *11*(10), p.1485-1497.
4. Adan, A., Kiraz, Y. and Baran, Y. 2016. Cell proliferation and cytotoxicity assays. *Current Pharmaceutical Biotechnology*, 17(14), pp.1213-1221.
5. Alemán-Nava, G., Cuellar-Bermudez, S., Cuaresma, M., Bosma, R., Muylaert, K., Ritmann, B. and Parra, R. 2016. How to use Nile Red, a selective fluorescent stain for the microalgal neutral lipids. *The Journal of Microbiological Methods*, 128, pp.74-79.
6. Amin, I., Norazaidah, Y. and Hainida, K. 2006. Antioxidant activity and phenolic content of raw and blanched Amaranthus species. *Food Chemistry*, 94 (1), pp.47-52.
7. Angelis, I.D. and Turco, L. 2011. Caco-2 cells as a model for intestinal absorption. Current protocols in toxicology, 47(1), pp.6-20.
8. Aranaz, P., Navarro-Herrera, D., Zabala, M., Miguéliz, I., Romo-Hualde, A., López-Yoldi, M., Martínez, J.A., Vizmanos, J.L., Milagro, F.I. and González-Navarro, C.J. 2019. Phenolic compounds inhibit 3T3-L1 adipogenesis depending on the stage of differentiation and their binding affinity to PPARγ. *Molecules* 2019, *24*, doi:10.3390/molecules24061045.
9. Aryal S., Baniya M.K., Danekhu K., Kunwar P., Gurung R. and Koirala N. 2019.Total phenolic content, flavonoid content and antioxidant potential of wild vegetables from Western Nepal. *Plants* (Basel). 2019 Apr 11;8(4), p 96. doi: 10.3390/plants8040096. PMID: 30978964; PMCID: PMC6524357.
10. Aslantürk, Ö. 2018. In vitro cytotoxicity and cell viability assays: Principles, advantages, and disadvantages. [Online]. Available at: https://www.intechopen.com/books/genotoxicity-a-predictable-risk-to-our-actual-world/in-vitro-cytotoxicity-and-cell-viability-assays-principles-advantages-and-disad-vantages [Accessed 11 February. 2020].
11. Awika, J., Rooney, L., Wu, X., Prior, R. and Cisneros-Zevallos, L. 2006. Screening methods to measure antioxidant activity of sorghum (*Sorghum bicolor*) and sorghum products. *Journal of Agricultural and Food Chemistry*, 51 (23), pp. 6657−6662.
12. Bardyn, M., Allard, J., Crettaz, D., Rappaz, B., Turcatti, G., Tissot, J.D. and Prudent, M. 2021. Image-and fluorescence-based test shows oxidant-dependent damages in red blood cells and enables screening of potential protective molecules. *International Journal of Molecular Sciences*, *22*(8), p.4293-4307.
13. Blasa, M., Angelino, D., Gennari, L. and Ninfali, P. 2011. The cellular antioxidant activity in red blood cells (CAA-RBC): a new approach to bioavailability and synergy of phytochemicals and botanical extracts. *Food Chemistry*, *125* (2), pp.685-691.
14. Boz, H. 2015. p-Coumaric acid in cereals: presence, antioxidant and antimicrobial effects. *International Journal of Food Science & Technology*, 50 (11), pp. 2323-2328.
15. Byaruhanga, Y.B., Bester, B.H. and Watson, T.G., 1999. Growth and survival of Bacillus cereus in mageu, a sour maize beverage. World Journal of Microbiology and Biotechnology, 15, pp.329-333.
16. Carvalho, J.R., Meireles, A.N., Marques, S.S., Gregório, B.J., Ramos, I.I., Silva, E.M., Barreiros, L. and Segundo, M.A., 2023. Exploiting kinetic features of ORAC assay for evaluation of radical scavenging capacity. *Antioxidants*, *12*(2), pp.505 - 519.
17. Cory, H., Passarelli, S., Szeto, J., Tamez, M. & Mattei, J. 2018. The role of polyphenols in human health and food systems: A mini-review. *Frontiers in Nutrition*, 5 (87): doi: 10.3389/fnut.2018.00087
18. Costa, F., Alves, S., Diaz, C. and Pessolani, M. 2018. Procedures for the staining of lipid droplets with Oil Red O. *PLoS Pathogens,* 14 (7). [Online] Available at: https://doi.org/10.1371/journal.ppat.1007151 [Ac-cessed 10 Feb. 2020]
19. Chen, J., Yang, J., Ma, L., Li, J., Shahzad, N. and Kim, C.K., 2020. Structure-antioxidant activity relationship of methoxy, phenolic hydroxyl, and carboxylic acid groups of phenolic acids. *Scientific reports*, *10*(1), p.2611.

20. de Mello Andrade, J. and Fasolo, D. 2014. Polyphenol Antioxidants from natural sources and contribution to health promotion. In: Watson, R., Preedy, V. & Zibadi, S. (eds.) *Polyphenols in Human Health and Disease.* Amsterdam: Elsevier, pp. 253-265. 25
21. Delage, P., Ségrestin, B., Seyssel, K., Chanon, S., Vieille-Marchiset, A., Durand, A., Nemeth, A., Métairon, S., Charpagne, A., Descombes, P. and Hager, J. 2023. Adipose tissue angiogenesis genes are down-regulated by grape polyphenols supplementation during a human overfeeding trial. *The Journal of Nutritional Biochemistry*, *117*, p.109334- 109343.
22. Ding, X., Hu, X., Chen, Y., Xie, J., Ying, M., Wang, Y. and Yu, Q. 2021. Differentiated Caco-2 cell models in food-intestine interaction study: Current applications and future trends. *Trends in Food Science & Technology*, 107, pp.455-465.
23. Dixit, V., Joseph Kamal, S.W., Bajrang Chole, P., Dayal, D., Chaubey, K.K., Pal, A.K., Xavier, J., Manjunath, B.T. and Bachheti, R.K. 2023. Functional foods: exploring the health benefits of bioactive compounds from plant and animal sources. *Journal of Food Quality*, *2023*(1), pp.5546753 - 5546775.
24. Erskine, E., Gültekin Subaşı, B., Vahapoglu, B. and Capanoglu, E. 2022. Coffee phenolics and their interaction with other food phenolics: Antagonistic and synergistic effects. *ACS Omega*, *7*(2), pp.1595-1601.
25. Flier, J. S. and Maratos-Flier, E. 2018. Pathobiology of Obesity. In: Jameson, J. L., Fauci, A. S., Kasper, D. L., Hauser, S. L., Longo, D. L. & Loscalzo, J. (eds*.) Harrison's Principles of Internal Medicine*, 20e. New York: McGraw-Hill Education. [Online] Available at: http://accessmedicine.mhmedical.com/Con-tent.aspx?bookid=2129&sectionid=192288213 [Accessed 04 Feb. 2020].
26. Gallardo, C., Jimenez, L. and Garcia-Conesa, M.T. 2006. Hydroxycinnamic acid composition and *in vitro* antioxidant activity of selected grain fractions. *Food Chemistry*, *99*(3), pp.455-463.
27. Gao, Q., Li, Y., Li, Y., Zhang, Z. and Liang, Y. 2022. Antioxidant and prooxidant activities of phenolic acids commonly existed in vegetables and their relationship with structures. *Food Science and Technology*, *42*, p.e07622- 07631.
28. Glahn, R.P .2009. The use of Caco-2 cells in defining nutrient bioavailability: application to iron bioavailability of foods. In: McClements, D. and Decker, E. (eds.) *Designing Functional Foods*. Boca Raton: CRC Press, pp.340-361.
29. Glahn, R.P. 2022. The caco-2 cell bioassay for measurement of food iron bioavailability. Journal of Visualized Experiments, (182), p.e63859.
30. Girard-Lalancette, K., Pichette, A. and Legault, J. 2009. Sensitive cell-based assay using DCFH oxidation for the determination of pro-and antioxidant properties of compounds and mixtures: Analysis of fruit and vegetable juices. *Food Chemistry*, *115*(2), pp.720-726.
31. Gong, L., Jin, C., Wu, X. and Zhang. 2013. Relationship between total antioxidant capacities of cereals measured before and after *in vitro* digestion. *International Journal of Food and Nutrition,* 64 (7), pp. 850–856.
32. González-Castejón, M. and Rodriguez-Casado, A. 2011. Dietary phytochemicals and their potential effects on obesity: a review. Pharmacological research, 64(5), pp.438-455.
33. Greenspan, P. & Fowler, S.D. 1985. Spectrofluorometric studies of the lipid probe, Nile Red. *Journal of Lipid Res*earch, 26, pp. 781-789.
34. Greenspan, P., Mayer, E.P. and Fowler, S.D.1985. Nile Red: A selective fluorescent stain for intracellular lipid droplets. *The Journal of Cell Biology*. 100, pp. 965 – 973.
35. Gupta, E. and Mishra, P. 2021. Functional food with some health benefits, so called superfood: a review. *Current Nutrition & Food Science*, *17*(2), pp.144-166.
36. Halsted, C. 2003. Dietary supplements and functional foods: 2 sides of a coin? *The American Journal of Clinical Nutrition*, 77 (4), pp.1001S-1007S.
37. Hasler, C. 2002. Functional Foods: benefits, concerns and challenges—a position paper from the American council on science and health. *The Journal of Nutrition*, 132 (12): 3772-3781.
38. Histalim. 2019. Oil red O (staining on mouse liver) - Histalim histology services. [Online] Available at: https://www.histalim.com/home/activities/our-services/histology/oil-red-o/ [Accessed 10 Feb. 2020].
39. Hithamani, G. and Ganesan, P. 2022. Polyphenols from Indian cereal grains inhibit 3T3-L1 adipogenesis through modulating early and late phase adipogenic markers. *Food Bioscience*, *50*, p.102075 - 102085.
40. Holzapfel, W.H.& Taljaard, J.L. 2004. Industrialization of Mageu fermentation in South Africa. In: Stein-kraus, K.H. (eds). *Industrialisation of indigenous fermented foods*. New York: Marcel Deker, pp.363-407.

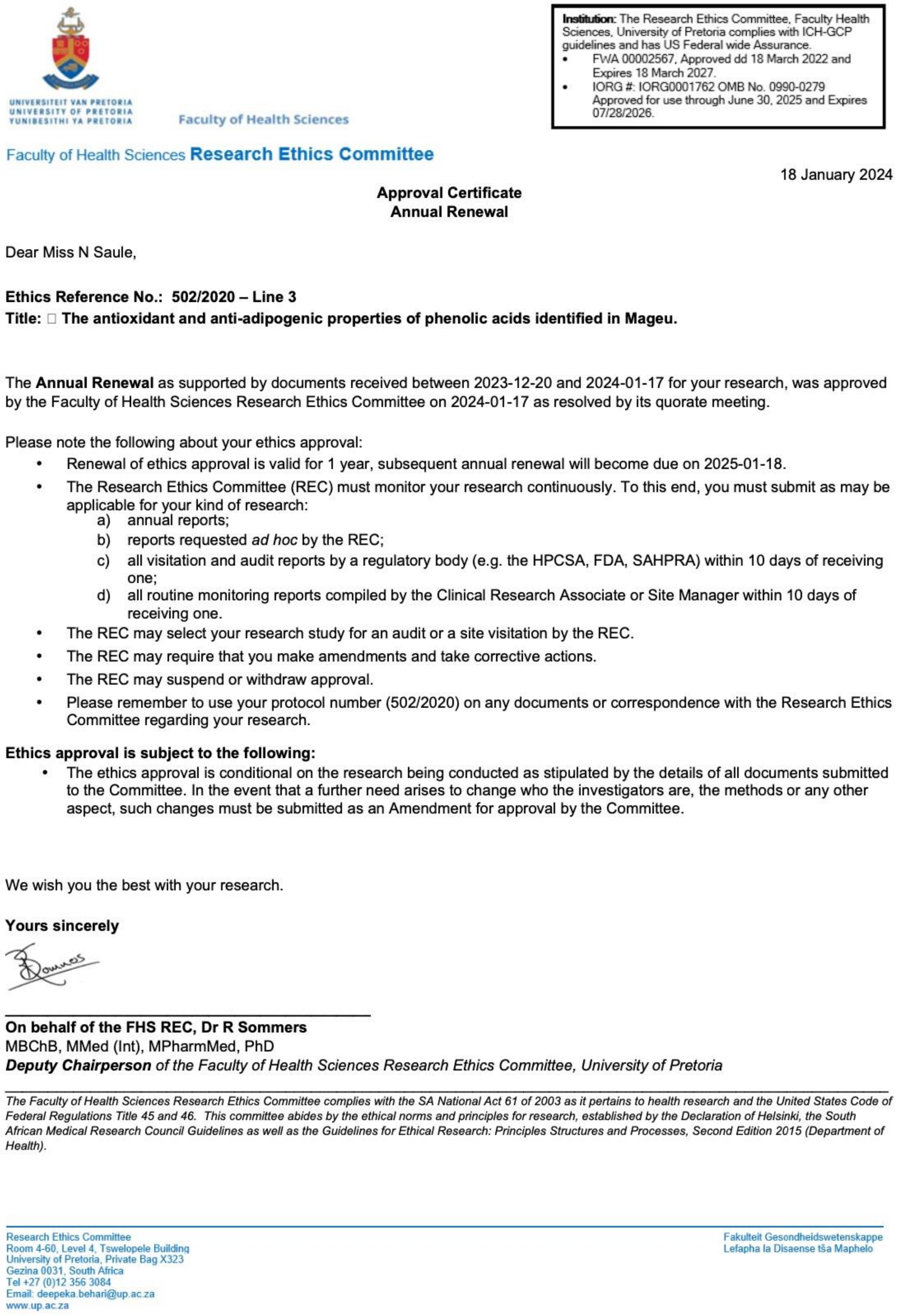
41. Horvat, D., Šimić, G., Drezner, G., Lalić, A., Ledenčan, T., Tucak, M., Plavšić, H., Andrić, L. and Zdunić, Z. 2020. Phenolic acid profiles and antioxidant activity of major cereal crops. *Antioxidants*, *9*(6), pp.527- 539.
42. Ilavenil, S., Kim, D.H., Srigopalram, S., Kuppusamy, P., Arasu, M.V., Lee, K.D., Lee, J.C., Song, Y.H., Jeong, Y.I. and Choi, K.C. 2017. Ferulic acid in *Lolium multiflorum* inhibits adipogenesis in 3T3-L1 cells and reduced high-fat-diet-induced obesity in Swiss albino mice via regulating p38MAPK and p44/42 signal pathways. *Journal of Functional Foods*, *37*, pp.293-302.
43. Kakkar, S. and Bais, S. 2014. A review on protocatechuic acid and its pharmacological potential. *International Scholarly Research Notices*, *2014*(1), p.952943- 952952.
44. Kampa, M., Alexaki, V.I., Notas, G., Nifli, A.P., Nistikaki, A., Hatzoglou, A., Bakogeorgou, E., Kouimtzoglou, E., Blekas, G., Boskou, D. and Gravanis, A. 2004. Antiproliferative and apoptotic effects of selective phenolic acids on T47D human breast cancer cells: potential mechanisms of action. *Breast Cancer Research*, 6, pp.1-12.
45. Karakaş, D., Ari, F. and Ulukaya, E. 2017. The MTT viability assay yields strikingly false-positive viabilities although the cells are killed by some plant extracts. *Turkish Journal of Biology*, *41*(6), pp.919-925.
46. Khan, M.T., Paniwnyk, L. and Hassan, S. 2019. Polyphenols as Natural Antioxidants: Sources, Extraction and Applications in Food, Cosmetics and Drugs. In: Li, Y & Chemat, F. (eds.) *Green Chemistry and Sustainable Technology*. Singapore: Springer Nature, p.197-235.
47. Knez, E., Kadac-Czapska, K. and Grembecka, M. 2023. Effect of fermentation on the nutritional quality of the selected vegetables and legumes and their health effects. *Life*, *13*(3), p.655-669.
48. Kose, T., Sharp, P.A. and Latunde-Dada, G.O. 2023. Phenolic acids rescue iron-induced damage in murine pancreatic cells and tissues. Molecules, 28(10), p.4084-4098.
49. Krongyut, O. and Sutthanut, K. 2019. Phenolic profile, antioxidant activity, and anti-obesogenic bioactivity of Mao Luang fruits (*Antidesma bunius L.). Molecules*, 24(22), pp.4109 - 4124.
50. Kruger, H., Puoane, T., Senekal, M. and van der Merwe, M. 2005. Obesity in South Africa: challenges for government and health professionals. *Public Health Nutrition*, 8 (5), pp.491- 500. 26
51. Laddomada, B., Caretto, S. and Mita, G. 2015. Wheat bran phenolic acids: Bioavailability and stability in whole wheat-based foods. *Molecules*, *20*(9), pp.15666-15685.
52. Leváková, Ľ. and Lacko-Bartošová, M. 2017. Phenolic acids and antioxidant activity of wheat species: A review. *Agriculture (Pol'nohospodárstvo)*, *63*(3), pp.92-101.
53. Li, M., Chou, J., King, K.W., Jing, J., Wei, D. and Yang, L. 2015. ICECAP: an integrated, general-purpose, automation assisted IC50/EC50 assay platform. *Journal of Laboratory Automation*, *20*(1), pp.32-45.
54. Litescu, S., Eremia, S., Tache, A., Vasilescu, I. and Radu, G.2014. The use of oxygen radical absorbance capacity (ORAC) and Trolox equivalent antioxidant capacity (TEAC) assays in the assessment of beverages' antioxidant properties. In: Preedy, V. (eds.) *Processing and Impact on Antioxidants in Beverages*. Oxford: Academic Press, p.245-251.
55. Liu, Z., Ren, Z., Zhang, J., Chuang, C., Kandaswamy, E., Zhou, T. and Zuo, L. 2018. Role of ROS and nutritional antioxidants in human diseases. *Frontiers in Physiology*, 9: 477 - 491.
56. Lobo, V., Patil, A., Phatak, A. and Chandra, N. 2010. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy Reviews*, 4 (8), pp. 118 – 126.
57. Lopez-Corona, A.V., Valencia-Espinosa, I., González-Sánchez, F.A., Sánchez-López, A.L., Garcia-Amezquita, L.E. and Garcia-Varela, R., 2022. Antioxidant, anti-inflammatory and cytotoxic activity of phenolic compound family extracted from raspberries (*Rubus idaeus*): A general review. *Antioxidants*, 11(6), pp.1192- 1207 doi:10.3390/antiox11061192.
58. Maleta K. 2006. Undernutrition. *Malawi Medical Journal: The Journal of Medical Association of Malawi*, *18* (4), pp.189–205.
59. Manach, C., Scalbert, A., Morand, C., Rémésy, C., and Jime´nez, L. 2004. Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition,* 79, pp. 727-747.
60. Mathur, P., and Pillai, R. 2019. Overnutrition: Current scenario and combat strategies. *The Indian Journal of Medical Research*, *149* (6), pp. 695–705.
61. Magiera, S. and Zaręba, M. 2015. Chromatographic determination of phenolic acids and flavonoids in Lycium barbarum L. and evaluation of antioxidant activity. *Food Analytical Methods*, *8*, pp.2665-2674.
62. Masisi, B., Beta, T. and Moghadasian, M.H. 2016. Antioxidant properties of diverse cereal grains: A review on *in vitro* and *in vivo* studies. *Food Chemistry* 196, pp.90–97.
63. Mavhungu, N.P.2012. Antioxidant properties and cellular protective effects of selected African green leafy vegetables (Doctoral dissertation, University of Pretoria).

64. Mbogori, T. and Mucherah, W. 2019. Westernization of traditional African diets and the development of chronic diseases in Africa. [Online] Available at: https://www.researchgate.net/publication/336653227 [Accessed 04 Feb 2020].
65. Méndez-Lagunas, L.L., Cruz-Gracida, M., Barriada-Bernal, L.G. and Rodríguez-Méndez, L.I. 2020. Profile of phenolic acids, antioxidant activity and total phenolic compounds during blue corn tortilla processing and its bioaccessibility. *Journal of Food Science and Technology*, *57*, pp. 4688-4696.
66. Medina-Vera, I., Gómez-de-Regil, L., Gutiérrez-Solis, A.L., Lugo, R., Guevara-Cruz, M., Pedraza-Chaverri, J. and Avila-Nava, A. 2021. Dietary strategies by foods with antioxidant effect on nutritional management of dyslipidemias: a systematic review. Antioxidants, 10(2), p.225-244.
67. Minatel, I., Borges, C., Ferreira, M., Gomez, H., Chen, C. and Lima, G. 2017. Phenolic compounds: functional properties, impact of processing and bioavailability. [Online] Available at: https://www.intechopen.com/books/phenolic-compounds-biological-activity/phenolic-compounds-func-tional-properties-impact-of-processing-and-bioavailability [Accessed 4 Feb 2020].
68. Mitra, S., Tareq, A.M., Das, R., Emran, T.B., Nainu, F., Chakraborty, A.J., Ahmad, I., Tallei, T.E., Idris, A.M. and Simal-Gandara, J. 2023. Polyphenols: A first evidence in the synergism and bioactivities. *Food Reviews International*, *39*(7), pp.4419-4441.
69. Mohamed, S. 2015. Antioxidants as functional foods in health and diseases. *Austin Journal of Nutrition and Food Science*, 3 (3), pp. 1067-1075.
70. Morrison, S. and McGee, S.L., 2015. 3T3-L1 adipocytes display phenotypic characteristics of multiple adipocyte lineages. *Adipocyte*, *4*(4), pp.295-302.
71. Mosqueda-Solís, A., Lasa, A., Gómez-Zorita, S., Eseberri, I., Picó, C. and Portillo, M.P. 2017. Screening of potential anti-adipogenic effects of phenolic compounds showing different chemical structure in 3T3-L1 preadipocytes. *Food and Function Journal*, 8, pp. 3576-3586, doi:10.1039/c7fo00679a.
72. Most, J., Warnke, I., Boekschoten, M.V., Jocken, J.W., de Groot, P., Friedel, A., Bendik, I., Goossens, G.H. and Blaak, E.E. 2018. The effects of polyphenol supplementation on adipose tissue morphology and gene expression in overweight and obese humans. *Adipocyte*, *7*(3), pp.190-196.
73. Moussa, Z., Judeh, Z.M. and Ahmed, S.A. 2019. Nonenzymatic exogenous and endogenous antioxidants. *Free Radical Medicine and Biology*, pp.1-22.
74. Nakajima, A., Masuda, Y., Matsuda, E., Tajima, K., Sameshima, H. and Ikenoue, T. 2013. Abilities of antioxidants to eliminate the peroxyl radical derived from 2, 2′-azobis (2-amidinopropane) dihydrochloride (AAPH). *Applied Magnetic Resonance*, *44*, pp. 997-1005.
75. Nathu, H. 2019. An exploratory investigation on the bioactivity of *in vitro* digested, commercially fer-mented maize product (Mageu) [dissertation]. South Africa: University of Pretoria.
76. Nathu, H., Mbuyama, K.R., Adarkwah-Yiadom, M., Serem, J.C., Ibrahim, M.A., Duodu, K.G., Gaspar, A.R. and Bester, M.J. 2021. Antioxidant properties and inhibition of lipid formation in 3T3-L1 adipocytes of *in vitro* digested Mageu, a commercial sample. *Journal of Food Biochemistry*, 45(10), p.e13929 doi: 10.1111/jfbc.13929
77. Ncit. 2020. *NCI Thesaurus*. [Online] Available at: https://ncit.nci.nih.gov/ncitbrowser/ConceptReport.jsp?dictionary=NCI_Thesaurus&ns=NCI_Thesaurus&code=C68540 [Accessed 04 Feb. 2020].
78. Niedzwiecki A, Roomi MW, Kalinovsky T, and Rath M. Anticancer efficacy of polyphenols and their combinations. *Nutrients*. 2016 Sep 9;8(9):552. doi: 10.3390/nu8090552. PMID: 27618095; PMCID: PMC5037537.
79. Nyanzi, R., Jooste, P., Abu, J. and Beukes, E. 2010. Consumer acceptability of a synbiotic version of the maize beverage Mageu. *Development Southern Africa*, 27 (3):447-463.
80. Olszowy-Tomczyk, M., 2020. Synergistic, antagonistic and additive antioxidant effects in the binary mixtures. Phytochemistry Reviews, 19, pp.63-103.
81. Ou, B., Huang, D., Hampsch-Woodill, M., Flanagan, J.A. and Deemer, E.K. 2002. Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study. *Journal of Agricultural and Food Chemistry*, 50 (11), pp. 3122-3128.
82. Roberfroid, M. 2002. Global view on functional foods: European perspectives. *British Journal of Nutrition,* 88 (S2), pp. S133-S138.
83. Pandey, K. and Rizvi, S. 2009. Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity*, 2 (5), pp.270-278.

84. Pant, S. and Vaidya, A. 2018. Over-nutrition in children and influence of parental attributes. *Journal of Kathmandu Medical College*, *7*(3), pp.102-109.
85. Prior, R., Wu, X. and Schaich, K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, 53 (10), pp.4290-4302.
86. Proestos, C. 2018*.* Superfoods: Recent data on their role in the prevention of diseases*. Current Research in Nutrition and Food Science Journal.* 06 (3), pp.576-593.
87. Ramezani-Jolfaie, N. and Mohammadi, M. 2023. Functional Foods for the Prevention and Treatment of Cardiovascular Diseases Including Hypertension. In Applications of Functional Foods and Nutraceuticals for Chronic Diseases, pp. 197-230. CRC Press.
88. Rudrapal, M., Khairnar, S.J., Khan, J., Dukhyil, A.B., Ansari, M.A., Alomary, M.N., Alshabrmi, F.M., Palai, S., Deb, P.K. and Devi, R., 2022. Dietary polyphenols and their role in oxidative stress-induced human diseases: Insights into protective effects, antioxidant potentials and mechanism (s) of action. *Frontiers in Pharmacology*, *13*, p.806470. doi: 10.3389/fphar.2022.806470
89. Ruiz-Ojeda, F. J., Rupérez, A. I., Gomez-Llorente, C., Gil, A. and Aguilera, C. M. 2016. Cell models and their application for studying adipogenic differentiation. *International Journal of Molecular Sciences*, 17:1040-1049.
90. Saunders, J., Smith, T. and Stroud, M.2015. Malnutrition and undernutrition. *Medicine*, *43*(2), pp.112-118.
91. Scalbert, A., Johnson, I.T. and Saltmarsh, M. 2005. Polyphenols: antioxidants and beyond. *The American Journal of Clinical Nutrition*; 81: 215S– 217S.
92. Seo, C.R., Yi, B., Oh, S., Kwon, S.M., Kim, S., Song, N.J., Cho, J.Y., Park, K.M., Ahn, J.Y., Hong, J.W. and Kim, M.J.2015. Aqueous extracts of hulled barley containing coumaric acid and ferulic acid inhibit adipogenesis in vitro and obesity in vivo. *Journal of Functional Foods*, *12*, pp.208-218.
93. Shah, R.T., Prasad, K. and Kumar, P. 2016. Maize - A potential source of human nutrition and health: A review. *Cogent Food and Agriculture*, 2 (1): 116995-116704.
94. Shahidi, F. and Ambigaipalan, P. 2015. Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects–A review. *Journal of Functional Foods*, *18*, pp.820-897.
95. Shahidi, F., Danielski, R., Rhein, S.O., Meisel, L.A., Fuentes, J., Speisky, H., Schwember, A.R. and de Camargo, A.C., 2022. Wheat and rice beyond phenolic acids: Genetics, identification database, antioxidant properties, and potential health effects. *Plants*, *11*(23), p.3283.doi: 10.3390/plants11233283
96. Shahidi, F. and Zhong, Y. 2015. Measurement of antioxidant activity. *Journal of Functional Foods*, *18:* 757-781.
97. Sharifi-Rad, M., Anil Kumar, N.V., Zucca, P., Varoni, E.M., Dini, L., Panzarini, E., Rajkovic, J., Tsouh Fokou, P.V., Azzini, E., Peluso, I. and Prakash Mishra, A., 2020. Lifestyle, oxidative stress, and antioxidants: Back and forth in the pathophysiology of chronic diseases. *Frontiers in Physiology*, 11, p.694.doi: 10.3389/fphys.2020.00694
98. Skroza, D., Šimat, V., Vrdoljak, L., Jolić, N., Skelin, A., Čagalj, M., Frleta, R. and Generalić Mekinić, I., 2022. Investigation of antioxidant synergisms and antagonisms among phenolic acids in the model matrices using FRAP and ORAC methods. *Antioxidants*, *11*(9), p.1784.doi: 10.3390/antiox11091784
99. Śliwka, L., Wiktorska, K., Suchocki, P., Milczarek, M., Mielczarek, S., Lubelska, K., Cierpiał, T., Łyżwa, P., Kiełbasiński, P., Jaromin, A. and Flis, A. 2016. The comparison of MTT and CVS assays for the assessment of anticancer agent interactions. *PloS One*, 11(5), p.e0155772.doi: 10.1371/journal.pone.0155772
100. Song, F., Tang, M., Wu, Q., Shen, X., Wang H., Chen H. and Zhao S. 2017. Anti-adipogenic effects of polyphenol extracts of Areca flower tea on 3T3-L1 Preadipocytes. *Food Science and Technology Research*, 23 (5), pp. 705 – 715.
101. Sotler,R, Poljšak, B., Dahmane, R., Jukić, T., Pavan Jukić,D., Rotim, C., Trebše, P., and Starc., A. 2019. Prooxidant activities of antioxidants and their impact on health *Acta Clinica Croatia*, 2019; 58, pp.726-736.
102. Spires, M., Delobelle, P., Sanders, D., Puoane, T., Hoelzel, P. and Swart, R. 2016. Diet-related non-communicable diseases in South Africa: determinants and policy responses. *South African Health Review*, *2016* (1), pp. 35-42.
103. Srinivasan, M., Sudheer, A. R and Menon, V.P. 2007. Ferulic Acid: Therapeutic potential through its antioxidant property. *Journal of Clinical Biochemistry and Nutrition*, 40(2): 92-100.

104. Taofiq, O., González-Paramás, A.M., Barreiro, M.F. and Ferreira, I.C.2017. Hydroxycinnamic acids and their derivatives: Cosmeceutical significance, challenges and future perspectives, a review. Molecules, 22(2), p.281.
105. Taylor,J.R.N., 2003. Fermented foods: Beverages from sorghum and millet. In: Caballero, B., Trugo,L. and Finglas, P.M. (eds). *Encyclopaedia of food sciences and nutrition*. San Diego: Academic Press, pp 2352-2359.
106. Tian, S., Chen, Y.S.Z., Yang, Y. and Wang, Y. 2019. Functional properties of polyphenols in grains and effects of physicochemical processing on polyphenols. *Hindawi Journal of Food Quality,* 2019, pp. 2-7.
107. Tian, L., Zhang, S., Yi, J., Zhu, Z., Cui, L., Decker, E.A. and McClements, D.J. 2022. Factors impacting the antioxidant/prooxidant activity of tea polyphenols on lipids and proteins in oil-in-water emulsions. *Food Science and Technology,156*, p.113024 doi: 10.1016/j.lwt.2021.113024.
108. Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M. and Telser, J. 2007. Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology*, 39(1), pp.44-84.
109. Villaño, D., Fernández-Pachón, M.S., Troncoso, A.M. and García-Parrilla, M.C. 2005. Comparison of antioxidant activity of wine phenolic compounds and metabolites in vitro. *Analytica Chimica Acta*, *538*(1-2), pp.391-398.
110. Wang, S., Moustaid-Moussa, N., Chen, L., Mo, H., Shastri, A., Su, R., Bapat, P., Kwun, I. and Shen, C. 2014. Novel insights of dietary polyphenols and obesity. *The Journal of Nutritional Biochemistry*, 25 (1), pp. 1-18.
111. Wishart, D.S., Feunang, Y.D., Marcu, A., Guo, A.C. and Liang, K. 2018. The Human metabolome database for 2018. *Nucleic Acids Research*, 46 (D1), pp. D608-17.
112. Wolfe, D. 2009. *Superfoods: The food and medicine of the future*. California: North Atlantic Books, p.11-15.
113. World Health Organisation. 2018. *Malnutrition*. [Online] Available at: https://www.who.int/news-room/fact-sheets/detail/malnutrition [Accessed 2 Feb. 2020].
114. World Health Organisation. 2022. *Noncommunicable disease*. [Online] Available at: https://www.who.int/news-room/fact-sheets/detail/noncommunicable-diseases [Accessed 20 September 2022].
115. Xu, H.Q., Liu, J.C., Zhang, Z.Y. and Xu, C.X., 2022. A review on cell damage, viability, and functionality during 3D bioprinting. Military Medical Research, 9(1), p.70 doi: 10.1186/s40779-022-00429-5
116. Yang, J.Y., Della-Fera, M.A., Rayalam, S., Ambati, S., Hartzell, D.L., Park, H.J. and Baile, C.A.2008. Enhanced inhibition of adipogenesis and induction of apoptosis in 3T3-L1 adipocytes with combinations of resveratrol and quercetin. *Life Sciences 82*, pp.1032-1039
117. Yıldız, F., Eciroğlu, H., Övey, İ.S. and Avnioğlu, S., 2023. Effect of combination treatment of protocatechuic acid with 5-fluorouracil and oxaliplatin on colon cancer Caco-2 cell line. *Indian Journal of Experimental Biology*, 61(03) pp, 151-158
118. Young, I.S. and Woodside, J.V. 2001. Antioxidants in health and disease. *Journal of Clinical Pathology*, 54(3), pp.176-186.
119. Zambonin, L., Caliceti, C., Vieceli Dalla Sega, F., Fiorentini, D., Hrelia, S., Landi, L. and Prata, C.2012. Dietary phenolic acids act as effective antioxidants in membrane models and in cultured cells, exhibiting proapoptotic effects in leukaemia cells. *Oxidative Medicine and Cellular Longevity*, *2012*(1), p.839298.doi: 10.1155/2012/839298
120. Zhou, K., Yin, J.J. and Yu, L.L. 2006. ESR determination of the reactions between selected phenolic acids and free radicals or transition metals. *Food Chemistry*, *95*(3), pp.446-457.
121. Zulueta, A., Esteve, M.J. and Frígola, A. 2009. ORAC and TEAC assays comparison to measure the antioxidant capacity of food products. *Food Chemistry*, *114*(1), pp.310-316.

Appendix A: Ethics Clearance



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Faculty of Health Sciences **Research Ethics Committee**

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 18 March 2022 and Expires 18 March 2027.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through June 30, 2025 and Expires 07/28/2026.

18 January 2024

Approval Certificate
Annual Renewal

Dear Miss N Saule,

Ethics Reference No.: 502/2020 – Line 3
Title: □ The antioxidant and anti-adipogenic properties of phenolic acids identified in Mageu.

The **Annual Renewal** as supported by documents received between 2023-12-20 and 2024-01-17 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2024-01-17 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2025-01-18.
- The Research Ethics Committee (REC) must monitor your research continuously. To this end, you must submit as may be applicable for your kind of research:
  - a) annual reports;
  - b) reports requested *ad hoc* by the REC;
  - c) all visitation and audit reports by a regulatory body (e.g. the HPCSA, FDA, SAHPRA) within 10 days of receiving one;
  - d) all routine monitoring reports compiled by the Clinical Research Associate or Site Manager within 10 days of receiving one.
- The REC may select your research study for an audit or a site visitation by the REC.
- The REC may require that you make amendments and take corrective actions.
- The REC may suspend or withdraw approval.
- Please remember to use your protocol number (502/2020) on any documents or correspondence with the Research Ethics Committee regarding your research.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

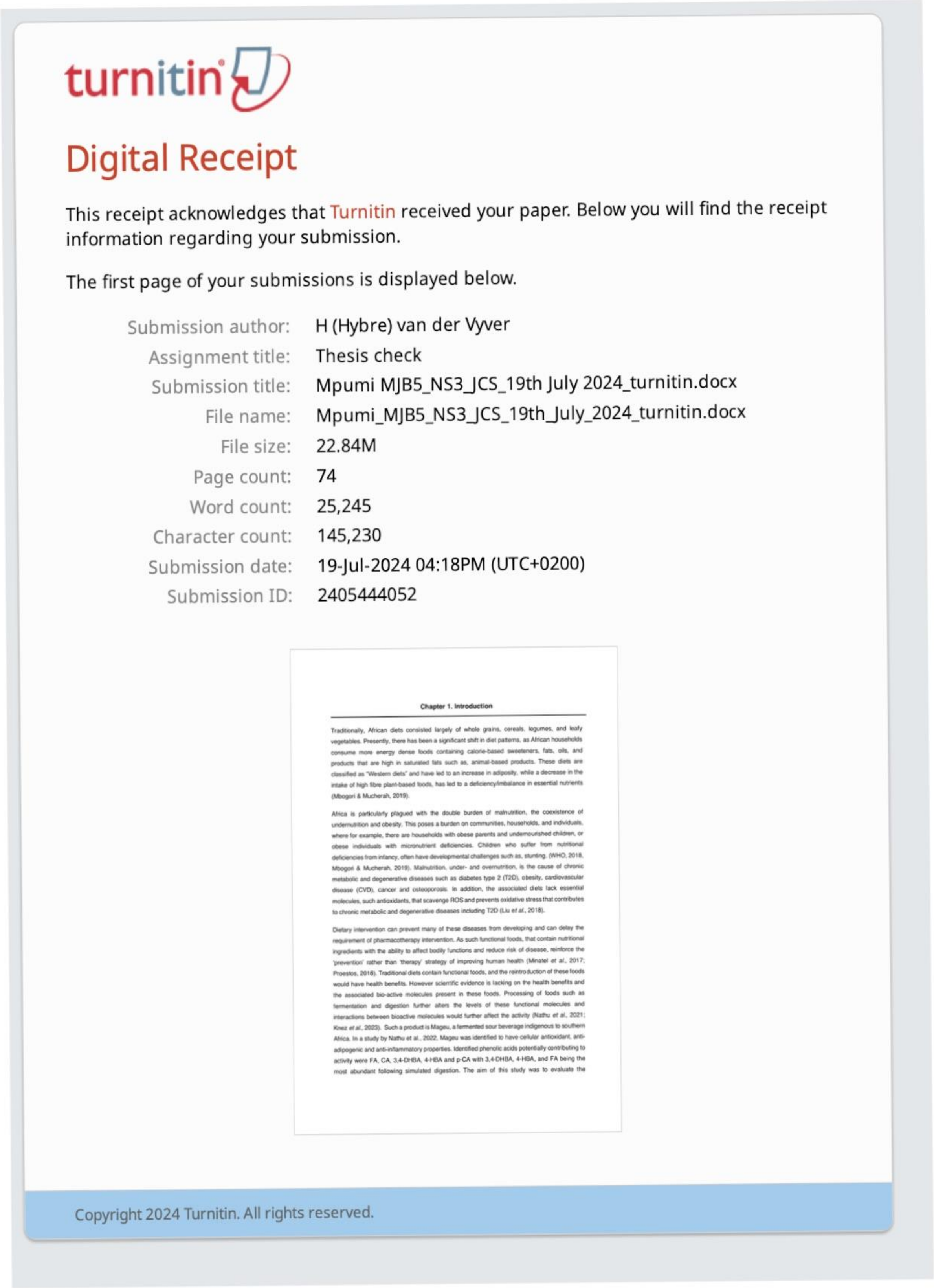
On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
Deputy Chairperson *of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

Research Ethics Committee
Room 4-60, Level 4, Tswelopele Building
University of Pretoria, Private Bag X323
Gezina 0031, South Africa
Tel +27 (0)12 356 3084
Email: deepeka.behari@up.ac.za
www.up.ac.za

Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

Appendix B: Turnitin Digital receipt



turnitin

Digital Receipt

This receipt acknowledges that Turnitin received your paper. Below you will find the receipt information regarding your submission.

The first page of your submissions is displayed below.

Submission author:	H (Hybre) van der Vyver
Assignment title:	Thesis check
Submission title:	Mpumi MJB5_NS3_JCS_19th July 2024_turnitin.docx
File name:	Mpumi_MJB5_NS3_JCS_19th_July_2024_turnitin.docx
File size:	22.84M
Page count:	74
Word count:	25,245
Character count:	145,230
Submission date:	19-Jul-2024 04:18PM (UTC+0200)
Submission ID:	2405444052

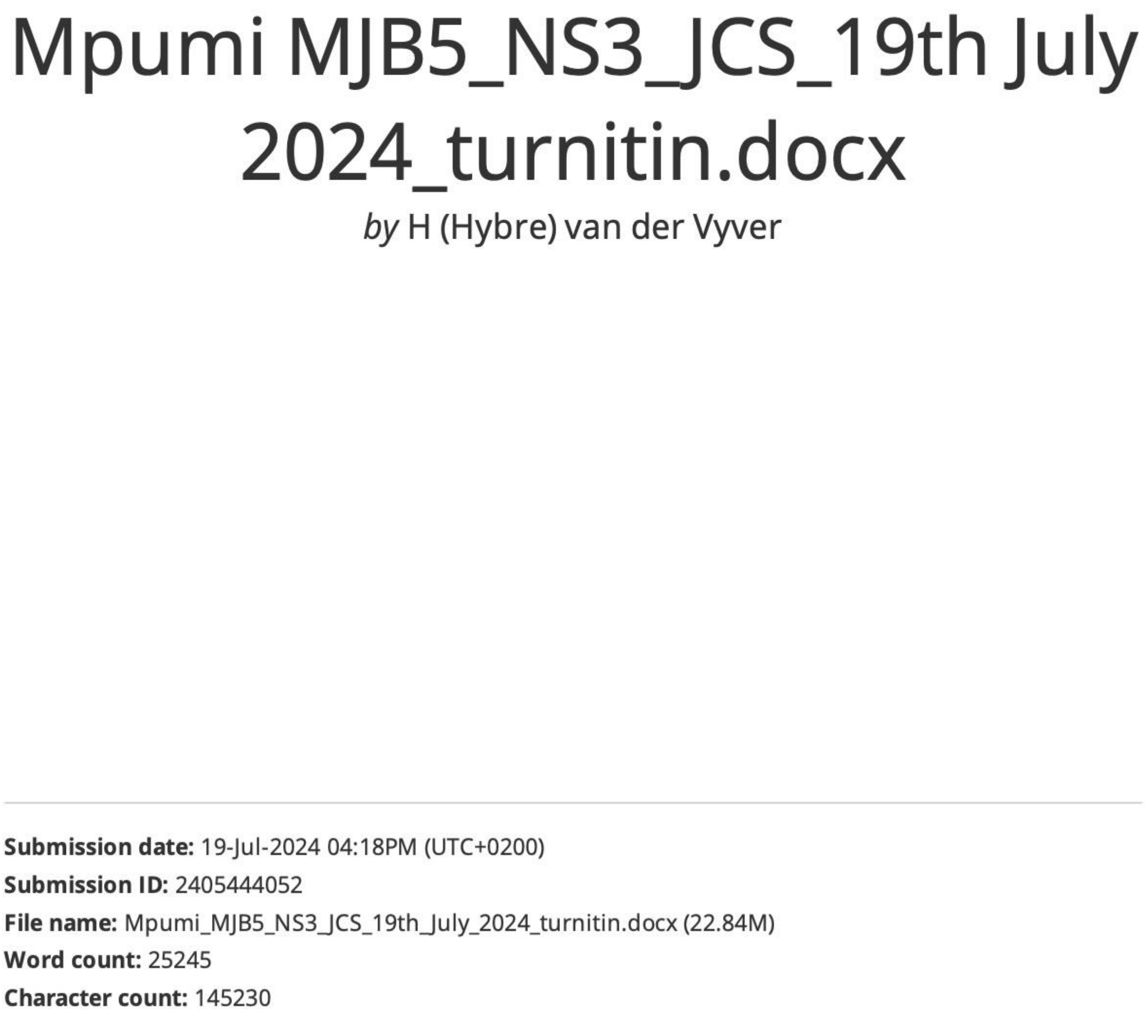
Chapter 1. Introduction

Traditionally, African diets consisted largely of whole grains, cereals, legumes, and leafy vegetables. Presently, there has been a significant shift in diet patterns, as African households consume more energy dense foods containing calorie-based sweeteners, fats, oils, and products that are high in saturated fats such as, animal-based products. These diets are classified as "Western diets" and have led to an increase in adiposity, while a decrease in the intake of high fibre plant-based foods, has led to a deficiency/imbalance in essential nutrients (Mbogori & Mucherah, 2019).

Africa is particularly plagued with the double burden of malnutrition, the coexistence of undernutrition and obesity. This poses a burden on communities, households, and individuals, where for example, there are households with obese parents and undernourished children, or obese individuals with micronutrient deficiencies. Children who suffer from nutritional deficiencies from infancy, often have developmental challenges such as, stunting. (WHO, 2018, Mbogori & Mucherah, 2019). Malnutrition, under- and overnutrition, is the cause of chronic metabolic and degenerative diseases such as diabetes type 2 (T2D), obesity, cardiovascular disease (CVD), cancer and osteoporosis. In addition, the associated diets lack essential molecules, such antioxidants, that scavenge ROS and prevents oxidative stress that contributes to chronic metabolic and degenerative diseases including T2D (Liu *et al.*, 2018).

Dietary intervention can prevent many of these diseases from developing and can delay the requirement of pharmacotherapy intervention. As such functional foods, that contain nutritional ingredients with the ability to affect bodily functions and reduce risk of disease, reinforce the 'prevention' rather than 'therapy' strategy of improving human health (Minatel *et al.*, 2017; Proestos, 2018). Traditional diets contain functional foods, and the reintroduction of these foods would have health benefits. However scientific evidence is lacking on the health benefits and the associated bio-active molecules present in these foods. Processing of foods such as fermentation and digestion further alters the levels of these functional molecules and interactions between bioactive molecules would further affect the activity (Nathu *et al.*, 2021; Knez *et al.*, 2023). Such a product is Mageu, a fermented sour beverage indigenous to southern Africa. In a study by Nathu et al., 2022, Mageu was identified to have cellular antioxidant, anti-adipogenic and anti-inflammatory properties. Identified phenolic acids potentially contributing to activity were FA, CA, 3,4-DHBA, 4-HBA and p-CA with 3,4-DHBA, 4-HBA, and FA being the most abundant following simulated digestion. The aim of this study was to evaluate the

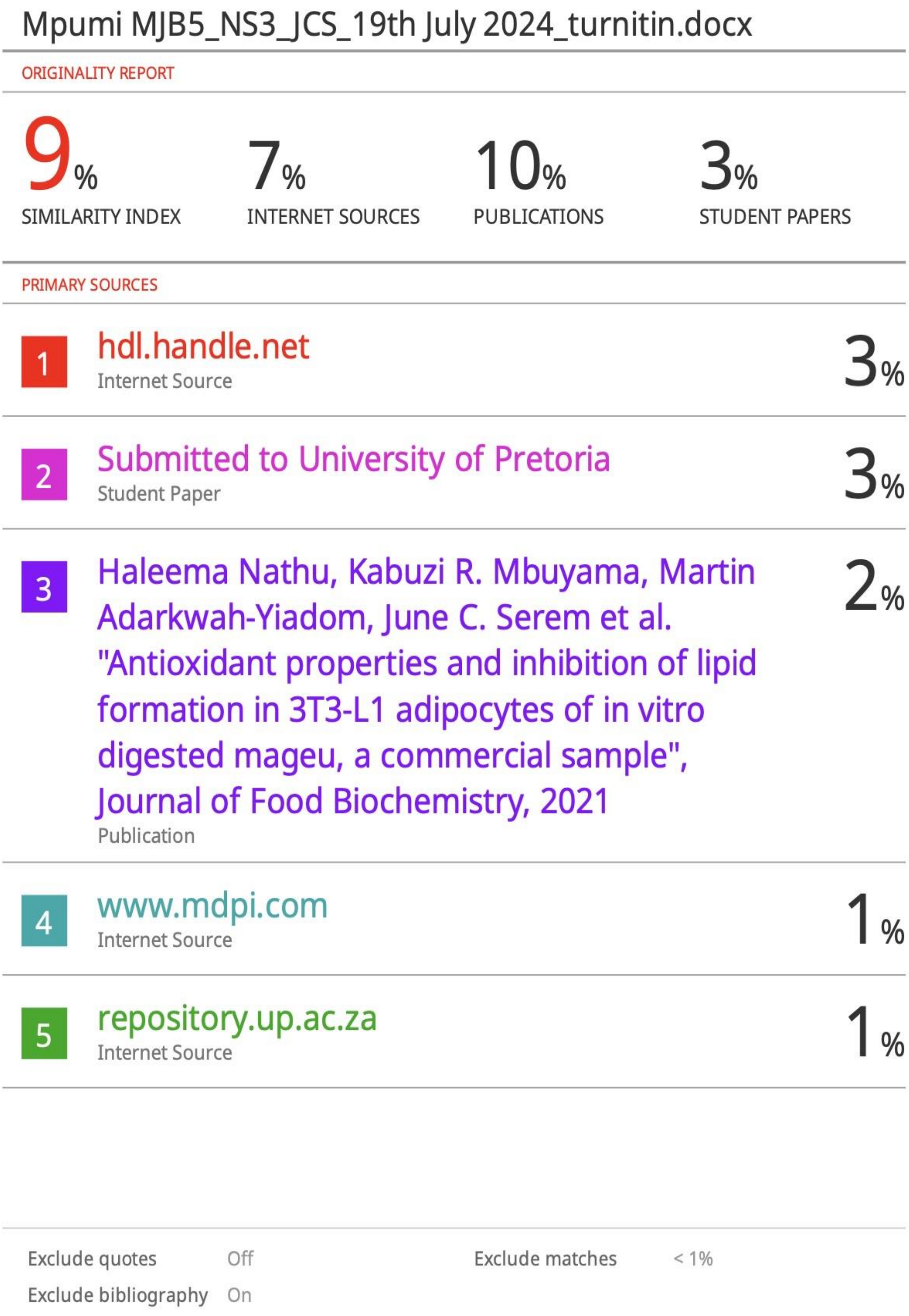
Appendix B: Turnitin Originality report



Mpumi MJB5_NS3_JCS_19th July 2024_turnitin.docx

by H (Hybre) van der Vyver

Submission date: 19-Jul-2024 04:18PM (UTC+0200)
Submission ID: 2405444052
File name: Mpumi_MJB5_NS3_JCS_19th_July_2024_turnitin.docx (22.84M)
Word count: 25245
Character count: 145230



Mpumi MJB5_NS3_JCS_19th July 2024_turnitin.docx

ORIGINALITY REPORT

9%	7%	10%	3%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	hdl.handle.net Internet Source	3%
2	Submitted to University of Pretoria Student Paper	3%
3	Haleema Nathu, Kabuzi R. Mbuyama, Martin Adarkwah-Yiadom, June C. Serem et al. "Antioxidant properties and inhibition of lipid formation in 3T3-L1 adipocytes of in vitro digested mageu, a commercial sample", Journal of Food Biochemistry, 2021 Publication	2%
4	www.mdpi.com Internet Source	1%
5	repository.up.ac.za Internet Source	1%

Exclude quotes Off
Exclude bibliography On
Exclude matches < 1%