EVALUATION OF THE ANTIOXIDANT AND ANTI-DIABESITY POTENTIAL OF *CYCLOPIA MACULATA* USING *IN VITRO* NON-CELL BASED SCREENING MODELS

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A thesis submitted in partial fulfillment of the requirements for the degree of Masters in Herbal Science in the South African Herbal Science and Medical Institute, University of Western Cape, Bellville, South Africa

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September 2014

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KEY WORDS:

Obesity,

Diabetes,

Oxidative stress,

Cyclopia maculata,

Antioxidant capacity,

DPPH, ABTS, ORAC and FRAP

Total polyphenol content,

Lipase inhibition,

α-Glucosidase inhibition,

Effect of fermentation,

HPLC-DAD-BCD.

Declaration

I declare that the thesis *EVALUATION OF THE ANTIOXIDANT AND ANTI-DIABESITY POTENTIAL OF CYCLOPIA MACULATA USING IN VITRO NON-CELL BASED SCREENING MODELS* is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

A.N MATROSE **September 2014**

Signed ……. **UWC Bellville**

Abstract

The simultaneous increase in obesity and type 2 diabetes has resulted in a new term, 'diabesity', to describe individuals suffering from both diseases. Adverse effects associated with the use of the anti-obesity drug, Orlistat™, and the anti-diabetic drug, Acarbose™, both enzyme inhibitors, necessitate the search for alternative anti-diabesity therapeutics with less or no side effects. Furthermore, oxidative stress plays a major role in the pathogenesis of these diseases. The aim of this study was therefore to evaluate the antioxidant and anti-diabesity potential of a hot water extract of *C. maculata* in non-cell based assays and correlate the activities with phenolic composition.

Total antioxidant capacity (TAC) was assessed in terms of free radical scavenging and iron reducing ability. The DPPH, ABTS, ORAC and FRAP assays were employed. Anti-diabesity potential was assessed in terms of the inhibition of the digestive enzymes, α-glucosidase and pancreatic lipase. The effect of fermentation was also determined by analyzing aqueous extracts of unfermented *C. maculata* plant material (n = 9) and their fermented counterparts (n =9). Extraction simulated the industrial scale process used for preparation of food-grade extracts.

Total polyphenol content of *C. maculata* extracts was measured in gallic acid equivalence (GAE) and the unfermented batch of extracts contained an average of 24 g GAE/100 g. This was significantly higher than that of the fermented batch of

extracts (16 g GAE/100 g). HPLC analysis showed highest quantities for mangiferin and isomangiferin. Other compounds in relatively high concentrations were iriflophenone-3-*C*-β-D-glucoside, hesperidin and eriocitrin. The compounds differed in the extent of degradation upon fermentation. Most affected were mangiferin and iriflophenone-3-*C*-β-D-glucoside, i.e. 70 % decrease compared to ≤ 40 % for the other compounds.

Different TAC values were obtained with different assays used, as expected due to different mechanisms and rate of reaction, with the highest TAC value obtained for the ORAC assay in the relative order TAC_{ORAC}>TAC_{ABTS} >TAC_{DPPH}>TAC_{FRAP}, irrespective of state of fermentation. Fermentation decreased TAC values obtained in all assays. The individual polyphenol compound content correlated significantly ($P < 0.0001$) with the TAC of extracts, however, the flavanone glycoside hesperidin showed no detectable antioxidant activity in the on-line HPLC antioxidant assays.

A positive inhibitory activity was obtained against α-glucosidase. The extracts however, were more effective against mammalian α-glucosidase than yeast α-glucosidase. In the respective assays the extracts were, however, significantly weaker α-glucosidase inhibitors than Acarbose™ and (+)-catechin. Fermentation also decreased the activity of α-glucosidase. The lipase inhibitory activity of the extracts was compared with Orlistat™. This drug proved to be highly effective while the *C. maculata* extracts only showed weak inhibition of the enzyme. Once again the extracts of unfermented plant material were more effective than their fermented counterparts.

Based on multiple biological activities of the phenolic compounds in *C. maculata,* this plant might be a useful source of compounds that could provide a lead structure for the development of multiple target-oriented therapeutic modalities for the treatment of diabesity. The major compounds in *C. maculat*a extracts, i.e. mangiferin, isomangiferin and iriflophenone-3-*C*-β-D-glucoside, could be used as chemical markers to obtain a standardized antioxidant nutraceutical. The anti-diabesity activity of these compounds, however, needs further exploration before they can be used as bioactive markers for a nutraceutical product aimed at the anti-diabesity market.

Dedication

I dedicate this master's thesis to my late parents Mr Mona John and Mrs Nomnikelo Victoria Matrose for their love and words of wisdom. I also dedicate this thesis to my siblings Nokwanda, Pamela and Olwethu Matrose for their love, support and encouragement that has got me to where I am today.

Acknowledgement

- I would like to thank God for granting me the opportunity and strength to successfully complete this project;
- I would like to thank Agricultural Research Council (ARC), for allowing me the privilege to further my studies while employed with them;
- I would like to express my sincere gratitude and appreciation to my supervisor **Prof James Syce** for affording me the opportunity to study under his guidance, support and expertise during this study. Thank you for your patience and understanding. I have learnt many valuable lessons from you;
- A special thanks to **Prof Elizabeth Joubert**, my mentor, my boss and co-supervisor for her dedication, commitment, guidance and support. From you I have gained not just research expertise but also wisdom;
- To my second co-supervisor, **Dr Christiaan Malherbe**, thank you for your guidance and support. Without your technical expertise, my practical work would have never reached thesis standards;
- To **Brigette DuPreez** for performing the antioxidant micro-plate analysis;
- **Marieta van der Rijst**, for the statistical analysis of the data and her friendliness and willingness to help with regards to statistical interpretations;
- A word of great appreciation to my ARC supervisor **Dr Dalene de Beer** and ARC management for allowing me some time away from the ARC tasks, so my thesis write up could take priority;
- To all my colleagues at ARC. A special thanks to **George Dico** for his support

and willingness to help;

- Not forgetting **Ilona Kock Steenkamp**, for assisting with the schematic diagrams;
- To **Carin "Pinky" de Wet**, thank you for your cheerfulness and willingness to assist at all time;
- A special thanks to **Dr Aluwaseyi Aboyade**, a post graduate program organizer at South African Herbal Science and Medicine Institute (SAHSMI), for her support and willingness to help;
- **Nokwazi Mbili**, a friend and a sister, your understanding, friendliness, encouragement and support gave me the strength towards completion of this project;
- A word of thanks to all staff of SAHSMI and my fellow MSc students, each one of you played an important role in making me part of the department and keeping me up to date, regardless of my absence from the university. Your willingness to always assist when required was very helpful;
- Last but not least, a special thanks to my pillars of strength, my loving family, without your love and support , I don't know if I would have I made it this far.

List of abbreviations

Abbreviation	Definition
$ABTS^{\bullet+}$	2,2-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid
Abs_{tmax}	Absorbance values at time of maximum
Abs_{t0}	Absorbance values at the start of reaction
A_C	Absorbance of control
A_S	Absorbance of standards or samples
AGCF	α-Glucosidase inhibition with fluorimetric detection
AGLC	α-Glucosidase inhibition with colorimetric detection
ANOVA	Analysis of variance
AUC	Area under the curve
BCD	Bio(chemical) detector
BMI	Body mass index
DAD	Diode-array detector
DMSO	Dimethyl sulphoxide
$DPPH^{\bullet}$	2,2-Diphenyl-1-picrylhydrazyl
FCR	Folin-Ciocalteu Reagent
Fe^{3+}-TPTZ	Ferric-tripyridyl triazine
FFAs	Free fatty acids
FRAP	Ferric reducing/antioxidant power
GAE	Gallic Acid Equivalents
GLM	General Linear Models
HPLC-DAD-BCD	High performance liquid chromatography-diode-array-bio(chemical)-detection
IL-1	Interleukin 1
IL-6	Interleukin 6
LPC	Lipase inhibition with colorimetric detection
LPF	Lipase inhibition with fluorimetric detection
ORAC	Oxygen radical absorbance capacity
MUB	4-Methylumbelliferyl butyrate
4-MUG	4-Methylumbelliferyl α-D-glucopyranoside
4-MUO	4-Methylumbelliferyl oleate

pNPP	*p*-Nitrophenyl palmitate
pNPB	*p*-Nitrophenyl butyrate
pNPA	*p*-Nnitrophenyl acetate
pNPC	*p*-Nitrophenyl caprylate
pNPL	*p*-Nitrophenyl laurate
pNPRP	*p*-Nitrophenyl propionate
PNPG	*p*-Nitrophenyl-α-D-glucopyranoside
PA	Pistagremic acid
PCA	Principal component analysis
PEEK	Poly-ether ketone
PPL	Porcine pancreatic lipase
PTFE	Polytetrafluoroethylene
RATG	Rat α-glucosidase inhibition with fluorimetric detection
ROS	Reactive oxygen species
RTW	Retention time window
SS	Soluble solid
SSC	Soluble solids content
STZ	Streptozotocin
TAC	Total antioxidant capacity
TE/g	Trolox equivalence per gram
TPC	Total polyphenol content
TNF-α	Tumor necrosis factor alpha
T2D	Type 2 diabetes
T2MD	Type 2 diabetes mellitus
VWD	Variable wavelength detector
WHO	World Health Organisation

Table of contents

List of Tables

List of Figures

Chapter 1

Introduction

Obesity is a direct result of an imbalance between energy intake and energy expenditure, which results in excess energy stored in fat cells **(Goedecke *et al*., 2006)**. It is an increasing medical problem globally **(Melnikova and Wages, 2006**; **Birari and Bhutani, 2007)**, with 1.46 billion adults worldwide estimated to have a body mass index (BMI) of greater than25 kg/m^2 in 2008 and of these 205 million men and 297 million women were obese **(Finucane *et al*., 2011)**. South Africa's overweight and obesity prevalence has also increased alarmingly among all its citizens, following the global trend of obesity (**Mollentze, 2006)**. Moreover, insulin resistance, type 2 diabetes, dyslipidaemia, hypertension and coronary heart disease have been reported to be adverse health consequences associated with obesity **(Mollentze, 2006)**.

Obesity elevates the incidence of insulin resistance which leads to development of diabetes **(Hollander, 2007)**. **Shaw *et al*. (2010)** estimated a global prevalence of 285 million diabetic cases in 2010, and this figure is predicted to reach 439 million by 2030. Obesity and diabetes often occur simultaneously and therefore are termed diabesity. Diabesity is a common term that reflects both diseases aetiological and clinical presentation and or also defined as a combination of type 2 diabetes mellitus (T2MD) and obesity **(Astrup and Finer, 2000; Tharakan *et al*., 2011)**.

According to **Fernandez-Sanchez *et al*. (2011)**, obesity, inflammation, increased cellular oxidative stress and diabetes are directly linked. Furthermore, a significant correlation between lipid peroxidation (a marker of oxidative injury) and body mass index (BMI) of obese subjects, suggests a direct link betweenobesity and increased oxidative stress **(Furukawa *et al*., 2004)**. Increasing experimental and clinical evidences suggest that oxidative stress may also play a major role in the pathogenesis of both type 1 and type 2 diabetes **(Maritim *et al*., 2003)**.

The most appropriate approach for prevention of obesity is with lifestyle modifications but therapeutic strategies include anti-obesity agents and surgery **(Sergent *et al*., 2012)**. Various drugs with different mechanism of action for treatment of obesity are on the market or in development, and one of the mechanisms of action is through the inhibition of the nutrient digestive enzyme, pancreatic lipase **(Adan, 2013)**, a dietary enzyme responsible for digestion of approximately 50-70 % of dietary triglycerides into monoacylglycerides and free fatty acids which are absorbed by enterocytes **(Sergent *et al*., 2012)**. The ability of any compound/substance to inhibit pancreatic lipase is a widely employed mechanism to determine its anti-obesity potential (**Birari and Bhutani, 2007)**. For instance Orlistat™, a clinically approved drug for the treatment of obesity, inhibits pancreatic lipase **(Khedidja and Abderrahman, 2011)**. The mechanism by which this drug exerts its effect is by inactivating the hydrolysis of dietary fat and preventing the absorption of dietaryfats by approximately 30 %, thus

reducing the calorie intake of an obese patient **(Li *et al*., 2009)**. However, side effects associated with the use of Orlistat™ have been reported which include amongst others oily stools, faecal incontinence and flatulence, **(Melnikova and Wages, 2006**; **Birari and Bhutani, 2007)**.Therefore pancreatic lipase inhibitors with less or no side effects are needed. As reviewed by **Birari and Bhutani (2007)** phytochemicals of plant origin such as saponins and polyphenolics were found to inhibit pancreatic lipase, rendering them as alternative strategies for the development of anti-obesity therapeutics. However, these strategies may also exhibit adverse effects on cell survival parameters as compounds such as saponins have been reported to induce cell apoptosis **(Li *et al*., 2013)** and would therefore require toxicology studies before further development.

Similarly, treatment of diabetes type 2 can be achieved through inhibition of α-glucosidase (a digestive enzyme responsible for breaking down of complex carbohydrates thus leading to increased blood glucose levels) **(Hollander, 2007)**. Therefore, inhibition of this enzyme prevents postprandial increase in blood glucose levels **(Hollander, 2007)**. Acarbose™ is the clinically approved anti-diabetic drug that inhibits α-glucosidase to exert its therapeutic effects **(Silvio and Inzucchi, 2002**). However, adverse effects such as flatulence and diarrhea (both resulting from increased bacterial fermentation as a result of a delayed carbohydrates uptake in the gut) and abdominal discomfort have been reported with the use of Acarbose™ (**Silvio and Inzucchi, 2002; Hollander, 2007)**. Therefore, new α-glucosidase inhibitors with less/no side effects are needed **(Birari and Bhutani, 2007)**. The potential of natural products is

unexplored and might be an alternative strategy for development of anti-diabetic therapeutics **(Sergent *et al*., 2012)**. Furthermore, **Prabhakar and Doble (2011)** noted that, apart from the currently available anti-diabetic treatments, herbal medicines have also been recommended as therapeutics due to their effectiveness, fewer side effects and low costs. A review by **Pandey and Rizvi (2009)** on plant polyphenols as dietary antioxidants in human health and disease has revealed that these compounds provide a significant protection against diabetes through inhibition of glucose absorption in the gut.

Apart from their potential role as lipase and α-glucosidase inhibitors *in vivo* (**Birari and Bhutani, 2007; Pandey and Rizvi, 2009)**, polyphenols have the ability to modulate oxidative stress, one of the underlying factors contributing to obesity and diabetes **(Fernandez-Sanchez*et al*., 2011)**. These compounds reduce oxidative stress through their free radical scavenging ability and, in so doing also protect the cells from oxidative damage **(Soobratteee*t al*., 2005)**. Moreover, **Bagchi *et al*. (1997)** discovered that flavonoids such as proanthocyanidins provide a significant protection against free radical cellular damage and free radical induced peroxidation.

The current study investigated the anti-diabesity potential of *Cyclopia maculata*, a *Cyclopia s*pecies that grows naturally in the Overberg, and specifically in the Genadendal area, where it is locally consumed as a herbal infusion. Honeybush *(Cyclopia species)* is one of South Africa's commercialized herbal teas, with *C. subternata, C. intermedia* and *C. genistoides* comprising most of the annual

production **(Joubert *et al*., 2011)**.Growing demand for this herbal tea, to some extent as a result of its healthy image, has raised interest for other species such as *C. maculata*. However, the commercial potential of *C. maculata* is still unexploited and as a result investigation of its phenolic composition is limited to date. **Joubert *et al*. (2003)** showed that *C. maculata* contains high levels of the xanthones such as mangiferin and isomangiferin, and the flavanone, hesperidin. Their study reported that the plant material of *C. maculata* (on drymass basis) contained 1.63 %, 0.32 % and 0.72 % of mangiferin, isomangiferin and hesperidin, respectively. However, other compounds such as vicenin-2, scolymoside, iriflophenone-3-C-β-D-glucoside, and phloretin-3',5'-di-C-β-D-glucoside have also recently been identified in this species **(Schulze, 2013)**.

Several studies have demonstrated a number of bioactivities associated with mangiferin and/or hesperidin, amongst which are, antioxidant, immunomodulatory, anti-allergic, anti-inflammatory and anti-diabetic properties **(Garg *et al*., 2001; Wauthoz *et al*., 2007)**. Furthermore, an animal model study conducted by **Mahmoud *et al*. (2012)** has shown hesperidin to attenuate hyperglycemia-mediated oxidative stress and pro-inflammatory cytokine production in type 2 diabetes. This flavanone has been reported to have lipase inhibitory effects **(Kawaguchi *et al*., 1997)**. Mangiferin has shown to attenuate oxidative stress induced cellular damage **(Marquez *et al*., 2012)**. Moreover, as evaluated by **Pardo-Andreu *et al*. (2008)**, in atherosclerosis-prone hypercholesterolemic mouse, it also prevents mitochondrial oxidative stress. A study by **Muruganandan *et al*. (2005)** showed that mangiferin exhibited

anti-diabetic activity by significantly lowering fasting plasma glucose levels at different time intervals in streptozotocin (STZ) diabetic rats. **Phoboo *et al*. (2013)** demonstrated α-glucosidase inhibitory effects of mangiferin, however, it was less effective than the clinical drug Acarbose™, a known α-glucosidase inhibitor (**Phoboo *et al*., 2013)**. Mangiferin also exhibit lipase inhibitory effects as determined by **Yoshikawa *et al*. (2002)**. It is well established that mangiferin, isomangiferin and to a lesser extent hesperidin are also potent free radial scavengers **(Leiro *et al*., 2003; Hubbe, 2000)**.

Commercially, the honeybush herbal tea is available on local and international markets in both fermented (oxidized) and unfermented (green; unoxidised) form. A study by **Joubert*et al*. (2008b)** determined that fermentation (i.e. high temperature oxidation processing mainly employed to develop and improve quality and colour of the tea) caused a decrease in total polyphenol content (particularly xanthones) in *Cyclopia* plant material. A decrease in free radical scavenging ability of honeybush aqueous extracts resulting from the oxidation of phenolic compound during fermentation has also been observed **(Hubbe, 2000; Joubert *et al*., 2008a,b)**.

The present study investigated the antioxidant and anti-diabesity potential of *Cyclopia maculata* using *in vitro* non-cell based screening models. The focus fell

on hot water extracts as this type of extract is produced for the food and nutraceutical industries as food ingredient and supplement, respectively.

The specific objectives of the study were:

- To characterise the phenolic composition of *C. maculata* extracts through total polyphenol and HPLC analysis of individual compounds;
- To determine anti-oxidant capacity of *C. maculata* extracts using different antioxidant assays (DPPH$^{\bullet}$, ABTS$^{\bullet+}$, FRAP and ORAC);
- To determine anti-diabesity activity of *C. maculata* extracts through inhibition of pancreatic lipase and α-glucosidase;
- To determine the effect of fermentation on *C. maculata* extracts on its phenolic composition, antioxidant activity and anti-diabesity activity; and finally;
- To identify antioxidant and anti-diabesity bioactives in *C. maculata* extracts using high performance liquid chromatography diode-array detection coupled with (bio)chemical detection (HPLC-DAD-BCD).

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CHAPTER 2

Literature review

2.1 Introduction

This chapter provides an overview of the prevalence of diabesity, risk factors, and commercially available therapeutic agents (Orlistat™ and Acarbose™) for treatment of obesity and diabetes. The mechanism of action, advantages and disadvantages of these drugs are discussed as background to a general overview of natural products used for treatment of diabesity. The emphasis will be on polyphenols as this diverse group of plant constituents are increasingly investigated for anti-diabetic and anti-obesity effects. Of importance, in the context of the study, is their ability to inhibit intestinal α-glucosidase and pancreatic lipase. Oxidative stress, one of the health conditions associated with diabesity, and the potential of polyphenols through their antioxidant activity to modulate oxidative stress, will also be discussed. The focus of this study will fall on *Cyclopia maculata*. The phenolic composition of *Cyclopia species* investigated to date will be reviewed as species contain similar chemical composition (and since little information on the composition of *C. maculata* is available). This will serve to provide insight into the type of compounds that could potentially contribute to bioactivity of *C. maculata* extracts. The effect of "fermentation" (oxidation) on the phenolic composition of *Cyclopia species* and subsequently their bioactivities such as antioxidant, anti-obesity and anti-diabetic effects, will

also be discussed. Principles of the different methods employed in the study will be discussed and limitations highlighted.

2.2 Obesity

Obesity is a growing epidemic globally (**Melnikova and Wages, 2006**;**Birari and Bhutani, 2007)**, with 1.46 billion adults worldwide estimated to be overweight in 2008, and of these, 205 million men and 297 million women were obese (**Finucane *et al*., 2011)**. According to the World Health Organisation (WHO) standards, a body-mass index (BMI) of 25.0 kg/m^2 or higher is categorized as overweight and a BMI of 30.0 kg/m^2 or more as obese **(WHO, 2013)**. BMI is defined as a person's weight in kilograms divided by the square of the height in meters (kg/m^2) **(Baboota *et al*., 2013)**. Obesity is defined as excessive fat build-up in the body that may impair health and decrease life expectancy **(WHO, 2013; Savini *et a*l., 2013)**. This disease occurs as a result ofan imbalance between energy intake and energy expenditure **(Goedecke *et al*., 2006)**. Consumption of energy dense, nutrient-poor foods that are high in saturated fats and sugars, and lack of exercise are the key risk factors contributing to the obesity epidemic **(Cooke and Bloom, 2006)**.

The amount of fat stored in the liver is determined by the balance in fatty acid uptake, fatty acid synthesis, triglyceride synthesis, fatty acid oxidation and triglyceride export. Changes in these conditions could result in excessive fat accumulation (increased levels of adipose tissue), which therefore increases

morbidity risks, causing metabolic abnormalities such as obesity which is often accompanied by hypertension, impaired glucose tolerance and insulin resistance. Moreover, major complications associated with obesity are insulin resistance and diabetes type 2 **(Keller, 2006)**. Therefore the incidence of obesity is intimately linked to diabetes as well as other side effects, such as oxidative stress and inflammation **(Stienstra *et al*., 2006)**.The presence of excessive adipose tissue has been identified as a source of pro-inflammatory cytokines, tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and interleukin 1 (IL-1). These cytokines are potent stimulators of the production of reactive oxygen species (ROS) so that high levels of cytokines could be the reason for increased oxidative stress in obese individuals **(Fernandez-Sanchez *et al*., 2011)**.The excessive adipose tissue also results in elevated levels of plasma free fatty acids (FFAs), which decrease insulin stimulated glucose uptake, leading to insulin resistance **(Guenther *et al*., 2011)**. The direct link between obesity and diabetes has led to the collective term, “diabesity” (a common term that reflects both their etiological and clinical presentation, also defined as a combination of type 2 diabetes (T2D) mellitus and obesity) **(Astrup and Finer, 2000; Tharakan *et al*., 2011)**.

Even though life style interventions are the recommended approach for treatment of obesity **(Sergent *et al*., 2012; Adan, 2013),** targeting specific enzymes or brain circuits that are implicated in obesity with pharmaceutical drugs has been proven to be efficient treatments **(Adan, 2013)**. High levels of FFAs in the plasma can, amongst others, be regulated by inhibiting fat digestion in the small

intestine, thereby reducing body fat accumulation. This can be achieved by inhibiting pancreatic lipase, which is a key enzyme in lipid metabolism as it is responsible for the absorption of dietary triglycerides and phospholipids **(Slanc *et al.*, 2009).** This enzyme hydrolyses 50-70 % of dietary fats into fatty acids and monoglycerides. Inhibition of pancreatic lipase results in inefficient triglyceride digestion and subsequently their excretion in the faeces **(Birari and Bhutani, 2007)**. Therefore, pancreatic lipase inhibitors are potential anti-obesity agents **(Birari and Bhutani, 2007)**. Orlistat™ (Xenical™), a saturated derivatives of lipstatin, a natural product (Figure 1) isolated from the bacterium *Streptomyces toxytricini*, is a clinically approved pharmaceutical drug for the treatment of obesity.

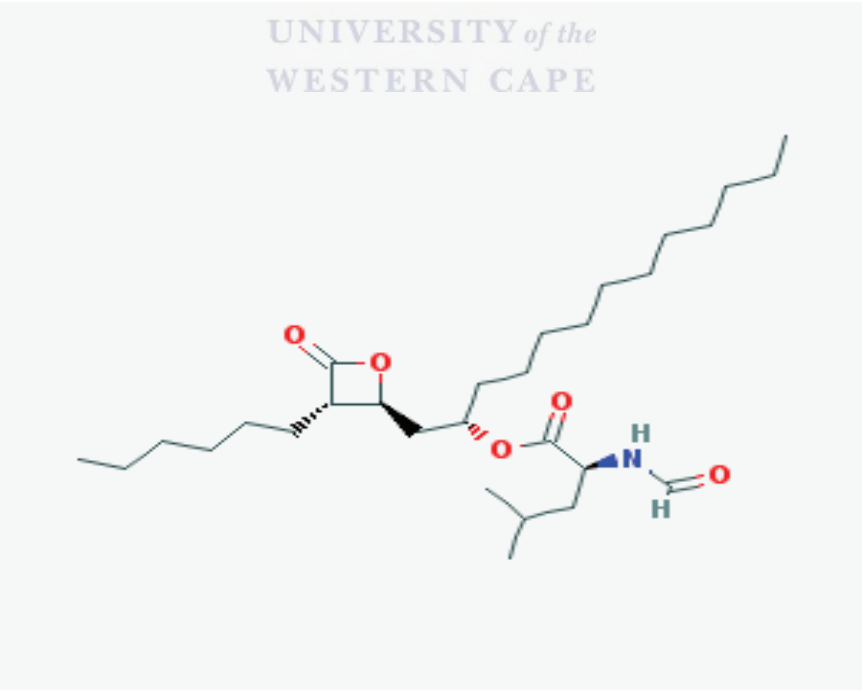


Figure 1. Chemical structure of Orlistat™ [http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=3034010].

The mechanism of action of Orlistat™(Figure 2) is inhibition of pancreatic lipase, which prevents breaking down of ingested fats **(Barbier *et al*., 2004; Patel, 2011)**,thus blocking the absorption of about 25 % of dietary fat **(Hill *et al*., 1999)**. Orlistat™ binds to the active site of pancreatic lipase through its reactive β-lactone ring, leading to an ester bond with the serine hydroxyl group of the catalytic triad of pancreatic lipase. It forms an enzyme inhibitor complex of an acyl-enzyme type resulting in irreversible inhibition of the enzyme **(Tsujita *et al*., 2006)**. As a result, approximately one-quarter of consumed fat passes through the gastrointestinal tract and is eliminated.

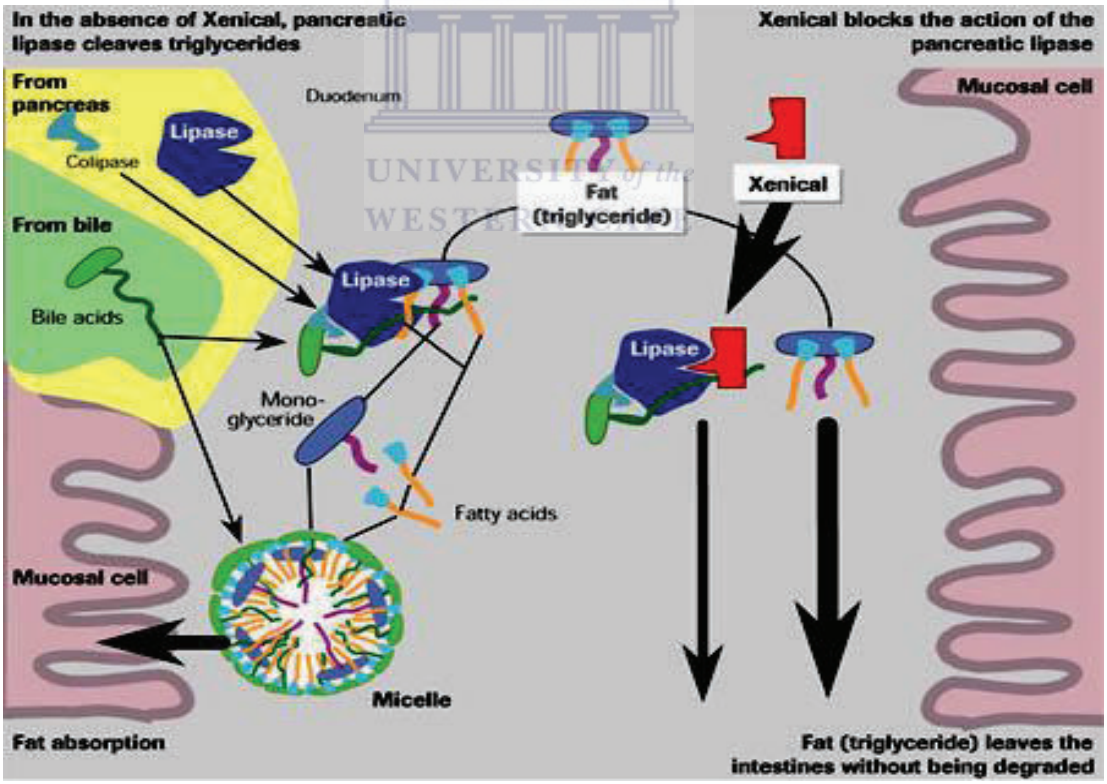


Figure 2. Pancreatic lipase inhibition by Orlistat™ (Xenical™: trade name for Orlistat™ by Roche): A unique mechanism for treatment of obesity **[Patel, 2011]**. Orlistat™ binds to active site of lipase thus blocking the cleavage of triglycerides.

The use of Orlistat™ has been associated with a number of adverse effects, such as oily stool, oily spotting, faecal urgency, increased defecation, flatulence and faecal incontinence (mostly resulting from the unabsorbed fats that are excreted in the faeces) **(Barbier *et al*., 2004; Birari and Bhutani, 2007)**. In addition to that an *in vivo* study by **Ellrichman *et al*. (2008)** in humansdemonstrated changes in gastrointestinal hormone concentrations (such as glucagon-like peptide-1-(7-36)-amide, cholecystokinin and peptide YY) which may raise appetite sensations and increase food consumption. This should therefore also be considered as potential side effects in the use of Orlistat™ for the treatment of obesity. However, its success as a potent pancreatic lipase inhibitor has prompted research for the identification of new pancreatic lipase inhibitors that lack or prevent some of these side effects **(Barbier *et al*., 2004; Birari and Bhutani, 2007; Martins *et al*., 2010**). At present, natural products are being explored for their potential as anti-obesity drugs **(Yun, 2010)**.

2.2.1 Phytochemicals evaluated for anti-obesity potential

Natural products provide a vast pool of pancreatic lipase inhibitors with potential of being developed into anti-obesity agents, as reviewed by **Cavaliere *et al*. (2001)** and **Birari and Bhutani (2007)**. Table 1 summarises information of a selection of plants of which their extracts and secondary metabolites (some of which are polyphenolic compounds) have been shown to have potential as lipase inhibitors. Several of the compounds resemble Orlistat™ in that they irreversibly bind to lipase and inactivate it through formation of a stable covalent intermediate

with serine, in the active site of lipase **(Yun, 2010**), whereas some plant metabolites such as crocin (a glycosylated carotenoid and the major active constituent of *Gardenia jasminoids*) bind covalently, but reversibly modify serine **(Birari and Bhutani, 2007)**.

Table 1 Plants, extracts and constituents with lipase inhibitory activity

Genus/ species	Common name	Type of compounds	Active component	Activity relative to Orlistat™	References
Panax japonicus rhizomes		Saponin	Chikusetsusaponin	<	Han *et al*., 2005
Salix matsudana	Corkscrew willow	Polyphenols	Crude extract	Not compared	Han *et al*., 1999
Rosmarinus officinalis	Rosemary		Methanolic extract (IC_{50} = 13.8 µg/mL)	>	Bustanji *et al*., 2010
Glycyrrhize urolensis	Sweet root	Aromatic ketones (forms flavonoid)	Licocholcone A	<	Won *et al.,* 2007; Zheng *et al*., 2010
Citrus unshiu	Naartjie	Flavonoid	Hesperidin	Not compared	Kawaguchi *et al.,* 1997
Rosmarinus officinalis	Rosemary	Phenolic acids	Chlorogenic acid (IC_{50} = 96.5 µg/mL); caffeic acid (IC_{50} = 32.5 µg/mL)	<	Bustanji *et al*., 2010
Mangifera Indica	Mango	Polyphenols	Crude extract	Not compared	Moreno *et al*., 2006
Filipendula kamtschatica	Giant meadowsweet	Flavonoid (flavanone)	3-*O*-β-Xylopyranosyl-(1-2)-*O*- β-galactopyranoside Quercetin (IC_{50} = 300 µM)	<	Kato *et al.,* 2012
Filipendula kamtschatica	Giant meadowsweet	Chlorogenic acid	2-*O*-Caffeoyl-4-*O*-galloyl-L-threonic acid (IC_{50} = 246 µM)	<	Kato *et al.,* 2012

Table 1 (continued) Plants, extracts and constituents with lipase inhibitory activity

Genus/ species	Common name	Type of compounds	Active component	Activity relative to Orlistat™	References
Filipendula kamtschatica	Giant meadowsweet	Chlorogenic acid	3-*O*-Caffeoyl-4-*O*-galloyl-L-threonic acid (IC_{50} = 26 µM)	<	Kato *et al.,* 2012
Ilex paraguariensis	*Yerba mate*	Polyphenols	Crude extract	Not compared	Martins *et al*., 2010
Camellia sinensis	Green tea	Catechins (flavanol)	EGCG and ECG	Not compared	Koo and Noh, 2007
Platycodon grandiflorum	Hakone white	Saponin	Platycodin D	<	Zheng *et al*., 2010
Prunella vulgaris			Crude extract	<	Zheng *et al*., 2010
Rheum palmatum			Crude extract	<	Zheng *et al*., 2010
Dioscorea nipponica	Chuan long shu yu	Saponin	Dioscin	Not compared	Zheng *et al*., 2010
Nelumbo nucifera	Lotus	Phenolic constituents	Crude extract	Not compared	Zheng *et al*., 2010
Pure compound		Flavonoid	Luteolin	<	Zheng *et al*., 2010
Arachis hypogaea	Peanut shell	Flavonoid	Luteolin	<	Yamamoto *et al*., 2000

> and < indicate more and less effective than Orlistat™

The inhibitory potency of the extracts is generally significantly weaker than that of Orlistat™, since the extracts do not contain only active but also inactive substances **(Yun, 2010)**. An *in vitro* study by **Zheng *et al*. (2010)** showed that crude extracts of *Prunella vulgaris* and *Rheum palmatum* exhibited a significantly low percentage inhibition (<10 %) at a concentration of 10 µg/mL when evaluated for their pancreatic inhibition, whereas Orlistat™ showed approximately 65 % inhibition at the same concentration. Their findings were in agreement with the report by **Yun (2010)** and other authorswith regards to the potency of plant

extracts. However, purified fractions and compounds from crude extracts can also be less potent than Orlistat™. **Zheng *et al*. (2010)** demonstrated that the flavonoid, luteolin (isolated from promising Chinese herbs), exhibited weaker lipase inhibition activity (17 % inhibition) than Orlistat™ (87 % inhibition) at the same concentration (25 µg/mL). On the other hand, some crude plant extracts have been shown to possess higher lipase inhibitory activity than their isolated compounds. For instance, a methanolic extract from *Rosmarinus officinalis* (rosemary) showed higher lipase inhibitory activity than its phenolic acids, chlorogenic acid and caffeic acid (IC_{50} values of 13.8, 96.5 and 32.6 µg/mL, respectively) **(Bustanji *et al*., 2010)**. In the same assay Orlistat™ was shown to have an IC_{50} value of 0.65 µg/mL. Thus both crude extracts and isolated compounds were much less effective than Orlistat™.

In nature, there is a vast of number of phenolic compounds, ranging from relatively simple compounds, such as phenolic acids to complex polymers such as tannins. Flavonoids are the major phenolic compounds of plant extracts and generally the most active constituents as their structures allow binding to enzymes and scavenging of radicals, amongst others **(Fraga *et al*., 2010; Xiao *et al.,* 2013a, b)**. They are represented by a benzene ring (A) in their structure, condensed with a heterocyclic six-membered pyran or pyrone ring (C), which is in 2 or 3 position and carries a phenyl ring (B) as a substituent (Figure 4). Structurally flavonoids can differ greatly or in many cases slightly, however, these differences have a huge impact on the potency of the active compounds.

A study by **You *et al*. (2012)** comparing the lipase inhibitory activity of the flavanol catechin and the flavonol quercetin showed the latter compound to be ca 30 times more effective in inhibiting lipase (IC_{50} = 83.66 µg/mL *vs* 2500.98 µg/mL, respectively). These compounds are structurally similar except for the C_2-C_3 double bond and C_4 keto group present in quercetin. **Nakai *et al*. (2005)** showed that flavanols require galloyl moieties within their chemical structures and/or polymerization for enhanced pancreatic lipase inhibition. A study by **Wu *et al*. (2013)** investigating the binding interactions of the flavanol (-)-epigallocatechin-3-gallate (EGCG) with lipase, revealed non-covalent binding of EGCG to lipase, which alters the molecular conformation of the enzyme, resulting in a decreased enzyme catalytic activity.

Compounds with similar chemical structure, except for the presence and/or positioning of moieties, may have varied inhibitory potencies. Such a case was demonstrated by **Lee *at al*. (2010)** for the aglycone luteolin and its glycosides, orientin and isoorientin. The inhibitory activity of luteolin against pancreatic lipase was much weaker than those of its *C*-glycosylated derivatives, orientin and isoorientin. Furthermore, *C*-glycosylation of luteolin at position C-6 (isoorientin) resulted in a more potent compound than when glycosylated at position C-8 (orientin). The type of glycoside moiety could also play a role. Luteolin 6-*C*-β-D-boivinopyranoside was shown to be less effective than the 6-*C*-β-D-glucopyranoside, isoorientin **(Lee *et al*., 2010)**.

Kato *et al*. (2012) demonstrated that the position of the moiety is also important for other type of phenolic compounds. The IC_{50} values of the threonic acids, 2-*O*-caffeoyl-4-*O*-galloyl-L-threonic acid and its isomer 3-*O*-caffeoyl-4-*O*-galloyl-L-threonic acid were respectively 246 μM and 26 μM (Table 1). These compounds are regio-isomers containing a diacylglycerol-like structure. In addition to this keto structural feature being important for activity, the position of the caffeoyl groups greatly affects the potency of these compounds, with the 3-*O*-caffeoyl isomer having ca 10 fold higher potency than the 2-*O*-caffeoyl isomer. Even though the inhibitory effect of 3-*O*-caffeoyl-4-*O*-galloyl-L-threonic acid was significantly higher than that of its regio-isomer, it showed a significantly weaker lipase activity than that of Orlistat™ (IC_{50} = 0.22 μM).

In conclusion structural features of compounds are important for activity, and even for the same type of compound, minor modifications can have a major effect on their lipase inhibitory activity

2.3 Diabetes

Diabetes, as is the case for obesity, is a seriously increasing medical problem globally **(Larsen *et al*., 2010)**. Obesity and/or lack of physical activity were identified as two of the main determining factors in the development of insulin resistance that precede the diagnosis of type 2 diabetes (T2D) **(Astrup, 2001; Hamilton *et al*., 2007)**. More than 90 % of diabetes cases are T2D **(Larsen *et***

***al*., 2010)**. In 2010, the estimated number of adults affected by T2D worldwide was 285 million. By 2030 it was predicted to affect 439 million people globally **(Shaw *et al*., 2010)**, but in 2006, T2D was already projected to rank as 9th leading cause of death in low-income countries **(Mathers and Loncar, 2006)**. This can be attributed to growth and ageing of the population as well as urbanisation associated with increasing trends towards unhealthy diets, obesity and sedentary lifestyles **(Green *et al*., 2003)**.

Diabetes is defined as a health condition that is generally characterised by metabolic defects in production and utilisation of glucose by the body which result in elevated blood glucose **(Larsen *et al*., 2010)**. According to **Schinner *et al*. (2005)** the major pathophysiological event contributing to the development of T2D, besides β-cell failure, is the resistance of target cells to insulin (a hormone responsible for metabolism of carbohydrates, lipids, and proteins). Insulin resistance is a physiological condition in which cells fail to respond to the normal actions of the hormone, therefore leading to insufficient insulin-stimulated glucose uptake **(Reaven, 1988)**. The inadequate use of insulin by the cells leads to postprandialhyperglycemia **(Larsen *et al*., 2010)**, which is defined as a rapid increase of blood glucose level after food consumption **(Sakulnarmrat and Konczak, 2012)**.

The increase in blood glucose can be reduced through inhibition of enzymes involved in carbohydrate digestion, specifically intestinal α-glucosidase, as this is

the main enzyme responsible for the release of glucose from foods **(Boath *et al*., 2012)**. Inhibition of α-glucosidase is thus considered as one of the main approaches underlying management of T2D **(Van de Laar, 2008)**. Inhibitors of intestinal α-glucosidase may be effective in retarding carbohydrate digestion, thus preventing excess glucose absorption in the small intestine **(Tadera *et al*., 2006)**.

α-Glucosidase is targeted by proprietary drugs such as Acarbose™ (Figure 3), which are prescribed to control blood glucose levels in T2D after ingestion of starch containing meals **(Boath *et al*., 2012)**.

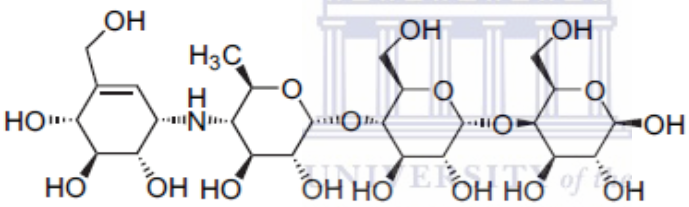


Figure 3. Chemical structure of Acarbose ™ **[De Melo *et al*., 2006]**.

Acarbose™ reduces postprandial hyperglycemia by interfering with the digestion of dietary carbohydrates **(De Melo *et al*., 2006)**. In the small intestine, starch is digested to oligosaccharides by amylase, and further digested to glucose by membrane-bound α-glucosidase. Acarbose™ competitively binds to the oligosaccharide binding site of α-glucosidase, thus preventing binding and enzymatic hydrolysis of the oligosaccharide substrate (Figure 4). α-Glucosidase inhibition therefore represents a pharmacologic approach for modifying the

digestion and absorption of dietary carbohydrates as an adjunct to dietary changes **(Bischoff, 1994)**.

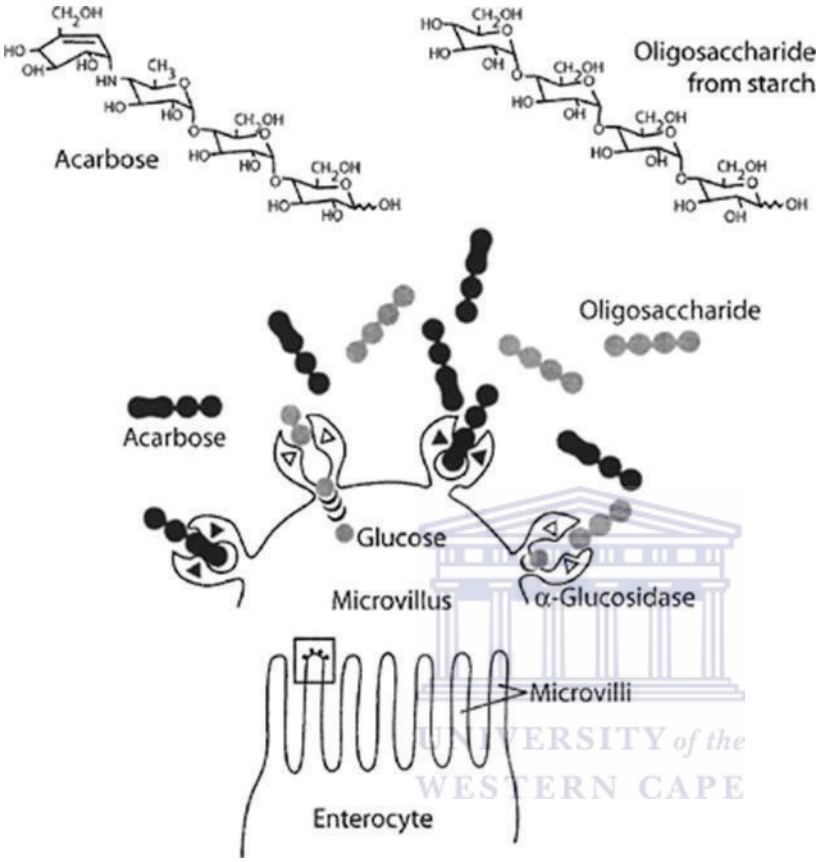


Figure 4. α-Glucosidase inhibition by Acarbose ™ **[Bischoff H, 1994]**.

α-Glucosidase inhibitors, Voglibose™ and Miglitol™, are also clinically used for treatment of diabetes, but Acarbose™,due to its efficacy, is the most widely used anti-diabetes drug of this type **(Van de Laar *et al*., 2005; Ismail and Deshmukh, 2012)**. Since it delays carbohydrate uptake, bacterial fermentation in the gut is increased, resulting in flatulence, diarrhea and abdominal discomfort **(Silvio and Inzucchi, 2002; Hollander, 2007)**. Therefore, new α-glucosidase inhibitors with

less or no side effects are needed **(Birari and Bhutani, 2007)**. Natural and synthesized α-glucosidase inhibitors have become an attractive therapeutic approach for treatment of postprandial hyperglycemia **(Garlapati *et al*., 2013)**.

2.3.1 Phytochemicals evaluated for anti-diabetes potential

A wide array of medicinal plants and their active constituents play a role in the prevention and treatment of diabetes **(Prabhakar and Doble, 2008)**. Many plants extracts and secondary metabolites have been reported as potential α-glucosidase inhibitors. Several examples are presented in Table 2. The source of the enzyme used to evaluate this activity is also indicated in the same table as the activity depends on the source of enzyme used the in vitro assay **(Kim *et al*., 2008a; Hogan *et al*., 2010)**. The mechanism of action through which crude plant extracts or in some cases purified compounds exerts their anti-diabetic effects has been revealed to be same as that of Acarbose™ **(Yoshikawa *et al*., 1997; Chen, 2007)**. For the purpose of this discussion, the activity of a number of plant extracts in comparison to the anti-diabetic drug, Acarbose™ will be highlighted.

Gynura medica, a species belonging to the *Compositae* family, has been found (amongst other medicinal plants) to show good hypoglycemic activity in diabetic animal models. Its ethyl acetate extract inhibits yeast α-glucosidase more effectively than Acarbose™. This drug is not very effective against yeast α-glucosidase, but very effective against mammalian α-glucosidase

(Uddin *et al*., 2012). Many other plants of this genus were also found to inhibit the key enzymes relevant to T2D, including α-glucosidase and α-amylase **(Tan *et al*., 2013)**. The inhibitory activity of a methanol extract of *Cassia auriculata* against α-glucosidase was reported to be relatively comparable to that of Acarbose™, showing ED_{50}values of 4.9 mg/kg and 3.1 mg/kg, respectively **(Abesundara *et al.,* 2004)**. Comparison of different extracts of *Gynura divaricata* (L.) DC, a traditional Chinese herb used in folk medicine for treatment of diabetes, showed them to inhibit yeast α-glucosidase in a dose-dependent manner. All extracts were less effective than Acarbose™. The drug at 0.2 mg/mL inhibited 47 % of the enzyme activity whereas the water extract at 1.25 mg/mL inhibited of 39 % **(Wu *et al*., 2011)**.

Jo *et al*. (2011) evaluated the *in vitro* mammalian α-glucosidase inhibitory activity of skin/pulp and seed extracts of *Schizandra chinensis* fruit. On the basis of the results their postprandial blood glucose lowering effect was subsequently compared to Acarbose™ in vivo, showing significantly reduction in postprandial hyperglycemia caused by sucrose loading for the pulp/skin extract, but to a lesser extent than that observed for the Acarbose™ administered group.

Despite their resemblance to Acarbose™ in their mode of action, purified compounds of plant extracts have a greater potential as anti-diabetes agents than Acarbose™, since many are stronger α-glucosidase inhibitors. **Yoshikawa*et al*. (1997)** demonstrated that salacinol (a compound isolated from

Salacia reticulata) competitively inhibited intestinal α-glucosidase (Table 2). The inhibitory activity of salacinol against maltase (IC_{50} = 3.2 µg/mL) and sucrase (IC_{50} = 0.84 µg/mL) was found to be nearly equal to that of Acarbose™ (IC_{50} value not presented), while its activity was more potent against isomaltase (IC_{50} = 0.59 µg/mL). It was later determined that salacinol showed an even stronger inhibition of the increase in serum glucose levels in rats fed high sucrose diet than the drug **(Yoshikawa *et al*., 1997)**.

Prabhakar and Doble (2008) and other authors noted that active constituents of plant extracts include various types of constituents, including flavonoids, alkaloids, glycosides, polysaccharides, steroids, carbohydrates, glycopeptides, terpenoids, amino acids and inorganic ions. Phenolic acids, flavonoids and polysaccharides were identified as the major hypoglycaemic active components of the *Gynura genus* **(Tan *et al*., 2013)**. **Sakulnarmrat and Konczak *(*2012)** demonstrated that flavonoid-rich fractions of Australian herbs (*Anise myrtle*, *Lemon myrtle* and *Tasmania pepper leaf)* exhibited pronounced yeast α-glucosidase inhibitory activities *in vitro* (Table 2). Luteolin, the flavone aglycone, was reported to be more potent than Acarbose™ **(Kim *et al*., 2000; Nicolle *et al*., 2011)**.

According to **Gunawan-Puteri *et al*. (2011)**, **Wu *et al*. (2011)**, **Tan *et al*. (2013)** and others, flavonoids are the major compounds found in many medicinal plants and are reported as the main compounds responsible for the activities against

the key enzymes relevant to T2D such as α-glucosidase and α-amylase. The following discussion will therefore focus on flavonoids and their structural configuration in relation to their α-glucosidase inhibitory activity. As previously indicated, many different flavonoids are present in plants. Certain structural features are important for α-glucosidase inhibitory activity as reviewed by **Xiao *et al*. (2013a)**. The main structural features and their effect are summarized in Figure 5 and 6. The number of hydrogen bonds and glycosylation, amongst others, play an important role in the binding of flavonoids to the active sites of digestion enzymes such as α- amylase and α- glucosidase **(Xiao *et al*., 2013a, b)**.

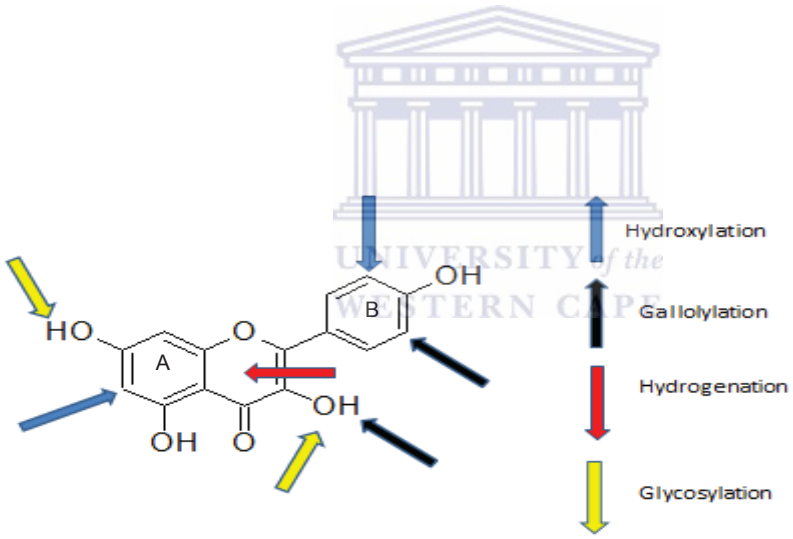


Figure 5. The potential sites of the flavonoids affecting the inhibitory effects against α-glucosidase are schematically illustrated. The up and down arrows represent increasing and decreasing inhibition, respectively **[Xiao *et al*., 2013b]**.

A hydroxyl group on the A ring enhances the α-glucosidase inhibitory effects of flavonoids. **Gao *et al*. (2004)** observed a dramatic loss in activity due to removal of a hydroxyl group at positions of 5, 6, or 7 of baicalein (5, 6, 7-trihydroxyflavone). Hydroxylation of the B-ring is also important with compounds

containing a catechol group being more effective than their 3'-deoxy counterparts. Luteolin was demonstrated to be more effective than apigenin, orientin more effective than vitexin, and isoorientin more effective than isovitexin **(Li *et al*., 2009**). Furthermore, as for lipase inhibition, the presence and position of the sugar moiety affect activity. In this case the aglycone was shown to be more effective than the glycosides, opposite to the effect demonstrated for lipase inhibition (as previously discussed in section 2.2.1). Glycosylation at the C-8 position (orientin and vitexin) also had a greater impact on activity than glycosylation at C-6 (isoorientin and isovitexin) with the C-6 glycosides thus being more effective **(Li *et al*., 2009)**. However, another study, using α-glucosidase from rat intestine instead of yeast α-glucosidase showed vitexin to be more effective than isovitexin **(Shibano *et al*., 2008)**.

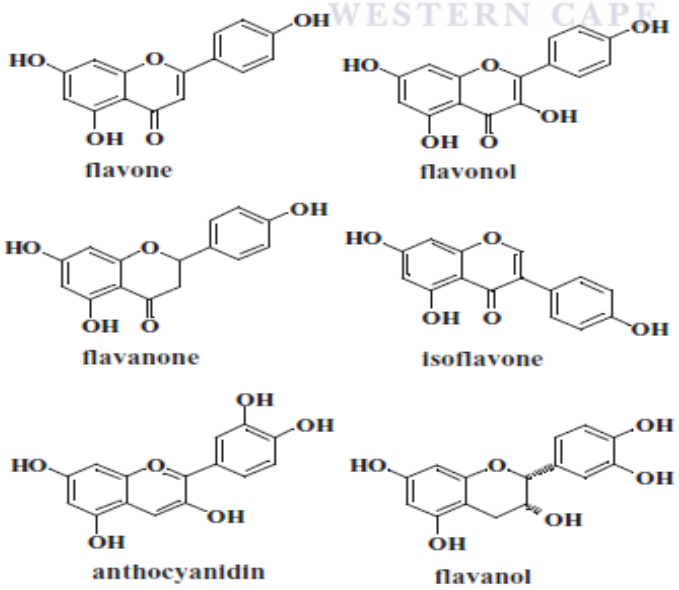


Figure 6. Chemical structures of flavonoids representing structural features enhancing the α- glucosidase inhibitory activity **[Xiao *et al*., 2013a]**.

The presence and number of moieties such as methoxy groups also determine activity. 5, 7, 3', 4'-Tetramethoxyflavone was shown to be less effective than 5, 7, 4'-trimethoxyflavone, due to the presence of the additional methoxy group at the 3-position **(Azuma *et al*., 2011)**. Saturation of the 2, 3-double bond of the C-ring of flavones lowered α-glucosidase inhibition **(Tadera *et al*., 2006)**. It is postulated that loss of planarity plays a role **(Xiao *et al*., 2013b)**. Conflicting results for the effect of hydroxylation of the C-ring have been noted **(Xiao *et al*., 2013b)**.

In conclusion, structural configuration plays a major role in the α-glucosidase inhibitory activity of flavonoids. Despite their specificity in compound-enzyme interaction, polyphenols have high efficacy for α-glucosidase inhibition. It is thus evident that polyphenol-rich plant extracts have potential as α-glucosidase inhibitors. Knowledge of their phenolic composition would aid in identifying potential candidates.

Table 2 Plants, extracts and constituents with α-glucosidase inhibitory activity

Genus/ species	Common name	Phenolic compounds	Active component	Source of enzyme	Activity relative to Acarbose ™	References
Gynura medica		Flavonoids, phenolic acids, cerebrosides, polysaccharides, alkaloids, terpenoids and sterols	3,5-Dicaffeoylquinic acid methyl Ester	Yeast	>	Tan *et al*., 2013
Gynura medica		Flavonols and phenolic acids	Ethyl acetate extract	Yeast	>	Tan *et al*., 2013
Lonicera japonica	Japanese honeysuckle	Flavonoid	Luteolin	Yeast	>	Kim *et al*., 2000
Anemarrhena asphodeloides Bunge		Saponin, xanthone,	Sarsasapogenin, mangiferin, neomangifrin.	Not specified; Rice (Ichiki *et al*., 2007)	Not compared	Ichiki *et al*., 2007

Table 2 (continued) Plants, extracts and constituents with α-glucosidase inhibitory activity

Genus/ species	Common name	Phenolic compounds	Active component	Source of enzyme	Activity relative to Acarbose ™	References
Salacia reticulata Wight	Kotalahimbatu	Xanthone, glycoside with a sulphate group, ester	Mangiferin, salacinol, kotalanol, epigallocatechin	Yeast	>	Yoshikawa *et al.,* 2002
Salacia reticulata	Saptarangi	Xanthone glycoside with a sulphate group, ester	Mangiferin, salacinol, kotalanol, epigallocatechin	Yeast	>	Yoshikawa *et al*., 2002
Salacia reticulata	Kotalahimbutu	Glycoside with a sulphate group	Salacinol	Rat (*In vivo*)	>	Yoshikawa *et al*., 1997
Anetholea anisata	Anise myrtle	Polyphenolic rich fractions	Ellagic acid, ellagic acid derivatives quercetin, hesperetin	Rat intestinal acetone powder	Not compared	Sakulnarmrat *et al*., 2012
Backhousia citriodora	Lemon myrtle	Polyphenolic rich fractions	Ellagic acid, ellagic acid derivatives quercetin, hesperetin	Rat intestinal acetone powder	Not compared	Sakulnarmrat *et al*., 2012
Tasmannia lanceolata Tasmannia pepper leaf	Mountain pepper/ Tasmannia pepper leaf	Polyphenolic rich fractions	Chlorogenic acid, quercetin 3-rutinoside (rutin)	Rat intestinal acetone powder	Not compared	Sakulnarmrat *et al.,* 2012
Kaempferia parviflora	Black galingale	Flavones	5,7,3',4'-Tetramethoxyflavone (IC_{50} = 20.4 μM), 5,7,4'-Trimethoxyflavone (IC_{50} = 54.3 μM), 3,5,7,3',4'-pentamethoxyflavone (IC_{50} = 64.3 μM)	Rat intestinal acetone powder	Not compared	Azuma *et al*., 2011
Gynura divaricata	Vary across regions as well as languages. Ref:*eol.org/pages/ 6242973/*		Water extract and organic solvent fractions	Yeast α-glucosidase	<	Wu *et al*., 2011

> and < indicate more and less effective than Acarbose™

2.4 Oxidative stress – link to diabesity

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and the ability of a biological system to readily detoxify the reactive intermediates or to repair the resulting cellular damage **(Sies, 1997)**. ROS are molecules or atoms possessing an unpaired electron

which is free to react with other molecules thus causing cellular injury or cell death **(Wong *et al*., 2006; Bisbal *et al*., 2010)**. ROS, produced during normal aerobic respiration, glucose and lipid metabolism, cellular stress and immune responses **(Halliwell, 1991; Kim *et al*., 2008b)**, are an important part of the normal physiological functions of the body. Disturbance in the normal redox state of cells can cause toxic effects through the production of high levels of peroxides and free radicals **(Maritim *et al*., 2003; Dudonne *et al*., 2009)**. An imbalance of the antioxidant defence system of the body and free radical formation may lead to the development of chronic diseases such as cancers, cardiovascular diseases, diabetes, neurodegenerative diseases, rheumatoid arthritis and increased oxidative stress **(Wong *et al*., 2006; Kajaria *et al* ., 2012)**. **Maritim *et al*. (2003)** noted that free radicals are formed disproportionately in diabetes by glucose oxidation, non-enzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins. Moreover, it has been recognized that there is direct association between obesity and increased oxidative stress **(Keaney *et al*., 2003; Higdon and Frei, 2003)**. Therefore, antioxidants are also important in treatment of diabesity.

Halliwell and Gutteridge (1989) stated that an antioxidant is any substance that when present at low concentrations, compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate. This definition includes compounds of a non-enzymatic as well as an enzymatic nature **(Sies, 1997)**. Numerous medicinal plants have been exploited for their

antioxidant activity and polyphenols (flavonoids) have been identified as major constituents of plant extracts **(Liu, 2004)**. This class of phytochemicals is well-known for their free radical scavenging properties **(Xiao *et al.,* 2013a, b)**.

2.4.1 Structure-activity relationship of antioxidants

Polyphenols are the most abundant antioxidants in human diet and are the most common and widespread constituents in the plant kingdom **(Xiao *et al*., 2013 a, b)**. Flavonoids are the most potent antioxidants of the polyphenol sub groups **(Nijveldt *et al*., 2001)**.This group of compounds is characterised by aromatic rings in their chemical structure, consisting of one or more hydroxyl groups attached to the aromatic rings **(Liu, 2004)**. The C2=C3 bond on the C-ring, a dihydroxylgroup (catechol-type) or three adjacent hydroxyl group (pyrogallol-type) on the B-ring, and the presence of C-5, and C-7 hydroxyl groups on the A-ring are listed as the main requirements for antioxidant and antiradical activity of flavonoids **(Nijveldt *et al*., 2001; Woodman *et al*., 2005; Xiao *et al*., 2013a, b)**. The presence of a catechol group on the B-ring is reported to be a major determinant for high radical scavenging activity of many compounds and an additional OH at C-5 results in even higher activity **(Cos *et al*., 1998; Woodman *et al*., 2005)**. These structural features (Figure 3) enable easy electron donation, thus breaking the free radical chain reactions **(Fraga *et al*., 2010)**, rendering more stable and less-reactive radicals and therefore prevent cellular injury caused by free radicals (Figure 5) **(Nijveldt *et al*., 2001)**.

The flavonol quercetin, amongst others, is an example of a good antioxidant, owing its potency to fulfilling the general structural requirements for a potential antioxidant (Figure 7) **(Woodman *et al*., 2005)**.

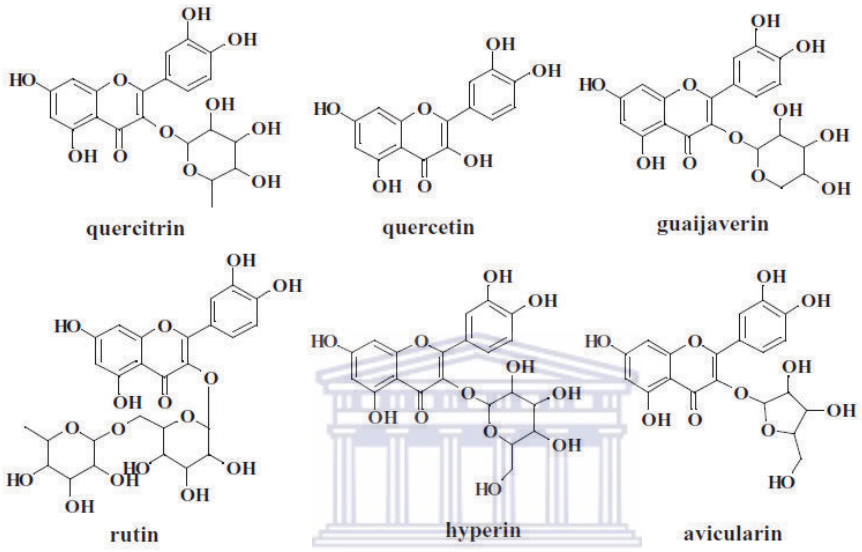


Figure 7. Chemical structures of quercetin and its glycosides **[Xiao *et al*., 2013a]**.

Therefore polyphenol-rich plant extracts or regular consumption of a polyphenol rich diet could contribute a major improvement to human health **(Liu, 2004)**. In addition to their lower cost, fewer side effects and effectiveness, interests in using natural products as starting materials for drug discovery has benefitted many therapeutic areas **(Hashim *et al*., 2012)**. Phytochemicals have been proven to contribute a variety of health promoting effects to human diet, such as antioxidant, anti-inflammatory, anti-cancer, anti-diabetic, anti-viral, anti-bacterial properties **(Liu, 2004; Mckay and Blumberg, 2007)**. In addition to that, **Liu (2004)** further noted that the additive and synergistic effects of phytochemicals in

fruits and vegetables are responsible for their antioxidant potency. Moreover, honeybush extracts and individual compounds that were identified as major constituents in *C. maculata*, have been reported as good antioxidants **(Sato *et al*., 1992; Joubert *et al*., 2008b)**. This aspect will be discussed under section 2.5 as the current study will also focus on the health modulating properties of a honeybush species (*C. maculata*), with regards to its antioxidant activity.

2.5 *Cyclopia species (*honeybush tea)

Cyclopia (*Fabaceae*) is a genus consisting of more than 20 species of woody legumes endemic to the fynbos region of South Africa **(De Nysschen *et al*., 1996)**, growing in the coastal districts of the Western and Eastern Cape provinces **(Du Toit *et al*., 1998; Joubert *et al*., 2011)**. The species are characterised by their woody stems and hard-shelled seeds. The different species have different leaf shapes and sizes (Figure 8), but all have trifoliate leaves and indented calyx single-flowered inflorescences and sweet scented bright yellow flowers **(Bond and Goldblatt, 1984; Schutte *et al*., 1995)**.

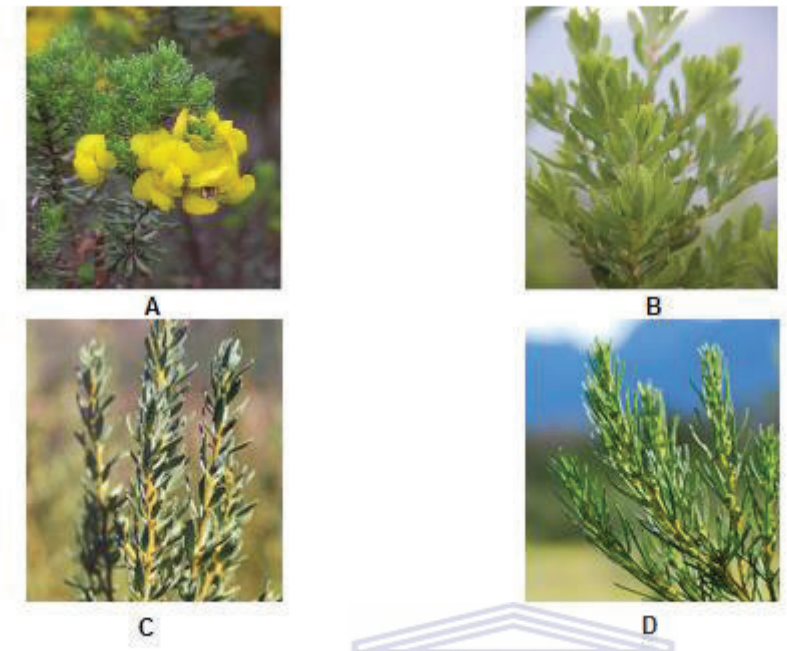


Figure 8. *Cyclopia* species (A) *C. genistoides*, (B) *C. subternata*, (C) *C. intermedia*, (D) *C. maculata*. Species A, B and C are commercially available and species D is still under development (photos supplied by **E. Joubert**, ARC Infruitec-Nietvoorbij, Stellenbosch).

The most commonly used and currently also the major commercialised species are *C. intermedia, C. subternata* and *C. genistoides* **(Joubert *et al*., 2011)**. Concerted efforts to cultivate and establish a honeybush industry started during the mid-1990s so that production is still small. Most of the annual production is exported to countries in Europe. Other countries importing large quantities are United States of America and Japan **(Joubert *et al*., 2011)**. Currently demand exceeds production **(Erasmus, 2012)**.Growing demand for this herbal tea, to some extent as a result of its healthy image, has raised commercial interest for other species **(Joubert *et al*., 2011)**. One of these is *C. maculata*, a lesser known species of which the commercial potential has not yet been developed.

*Cyclopia maculata*grows naturally in the Overberg, amongst others in the Genadendal area, where it is locally consumed as herbal infusion.

Traditionally the leaves of several *Cyclopia* species were used to brew honeybush tea **(De Nysschen *et al*., 1996)**. Anecdotal evidence suggested that regular consumption of the tea could have health benefiting effects **(Du Toit and Joubert, 1998; Joubert *et al*., 2008)**. **Joubert *et al*. (2011)** noted that the first mention of the use of a *Cyclopia* species as a herbal tea was by **Green (1881)**, who referred to "Cape tea". The leafy shoots and flowers of *Cyclopia* species were fermented and dried to prepare a tea that was traditionally used as a restorative and an expectorant in chronic catarrh, pulmonary tuberculosis, as well as a health drink for digestive and stomach ailments **(Joubert *et al*., 2011)**. With a history of medicinal use evident, interest in their phenolic composition led to investigations, starting with fermented *C. intermedia* and unfermented *C. subternata*, due to their commercial prominence at the time **(Ferreira *et al*., 1998; Kamara *et al*., 2003, 2004)**.

2.5.1 Chemical composition of *Cyclopia* species

In-depth studies on the phenolic composition of *Cyclopia* species have been carried out on *C. intermedia*, *C. subternata* and *C. genistoides* (**Ferreira *et al*., 1998; Kamara *et al*., 2003 and 2004; De Beer *et al*., 2012; Kokotkiewicz *et al*., 2012and 2013**), revealing qualitative differences. Several classes of compounds have been identified, i.e. flavanones, flavonols, flavones, isoflavones,

coumestans, benzophenones, dihydrochalcones and xanthones. A number of common compounds are listed in Table 3.

Table 3 Chemical compounds previously detected in *Cyclopia species*

	C. genistoides	*C. subternata*	*C. intermedia*	*C. maculata*	*References*
Mangiferin	√	√	√	√	Ferreira *et al*. (1998), Kamara *et al*. (2004, Joubert *et al.* (2003; 2008), Kokotkiewicz*et al*. (2013)
Isomangiferin	√	√	Nd	√	Ferreira *et al*. (1998), Joubert *et al*. (2008b), De Beer *et al*. (2009), Kokotkiewicz*et al*. (2013)
Eriocitrin	√	√	Nd	√	Joubert. *et al* (2008b)
Luteolin	√	√	Nd	√	Joubert *et al.* (2003; 2008b)
Naringenin	Nd	Nd	√	Nd	Ferreira *et al*. (1998)
Eriodictyol	Nd	Nd	√	√	Ferreira *et al*. (1998)
Narirutin	√	√	√	Nd	Ferreira *et al*. (1998), Joubert *et al.* (2008)
Hesperidin	√	√	√	√	Ferreira *et al*. (1998), Joubert *et al.* (2008), Kokotkiewicz*et al*. (2013)
Hesperetin	√	√	√	Nd	Ferreira *et al*. (1998), Joubert *et al.* (2008)

√: detected, Nd: not detected

Quantitative analysis performed on *Cyclopia* species (*C. intermedia, C. genistoides, C. subternata* and *C. sessiliflora*) identified the xanthones, mangiferin and isomangiferin, and the flavanone, hesperidin as the major polyphenols present in honeybush **(Joubert *et al*., 2003; Joubert *et al*., 2008b; De Beer and Joubert, 2010)**. Even though little information on the composition of *Cyclopia maculata* is available, the plant material (on dry mass basis) has been reported to contain 1.63 %, 0.32 % and 0.72 % of mangiferin, isomangiferin and hesperidin, respectively **(Joubert *et al*., 2003)**.

Quantitative analysis of hot water extracts of several *Cyclopia* species showed large variation in the content of the active compounds between and within species **(Joubert *et al*., 2008b; De Beer and Joubert, 2010)**. Table 4 summarises data of individual constituents and total polyphenol content as determined by **Joubert *et al.* (2008b**). Given the qualitative and quantitative differences in phenolic composition, differences in biological activities between *Cyclopia* species can be expected.

Table 4 Variation in phenolic content of *Cyclopia* species

Compounds	***Cyclopia intermedia***	***Cyclopia subternata***	***Cyclopia genistoides***	***Cyclopia sessiliflora***
Mangiferin	0.23±0.05	0.08±0.02	4.29±1.20	0.19±0.04
Isomangiferin	nd	nd	0.94±0.15	nd
Eriocitrin	nd	0.26±0.07	nd	0.13±0.04
Narirutin	0.02±0.00	0.04±0.01	0.20±0.06	nd
Hesperidin	0.45±0.08	0.27±0.08	0.47±0.01	0.45±0.03
Hesperetin	0.06±0.03	nd	nd	nd
Luteolin	nd	0.01	0.01	nd
Total polyphenols	16.26±1.39	17.49±1.54	22.01±0.92	17.10±1.44

Joubert et al. (2008b); values as percentage of dried extract; nd: not detected. Total polyphenol content expressed as gram gallic acid equivalents/100g dried extract as determined with the Folin-Ciocalteu reagent.

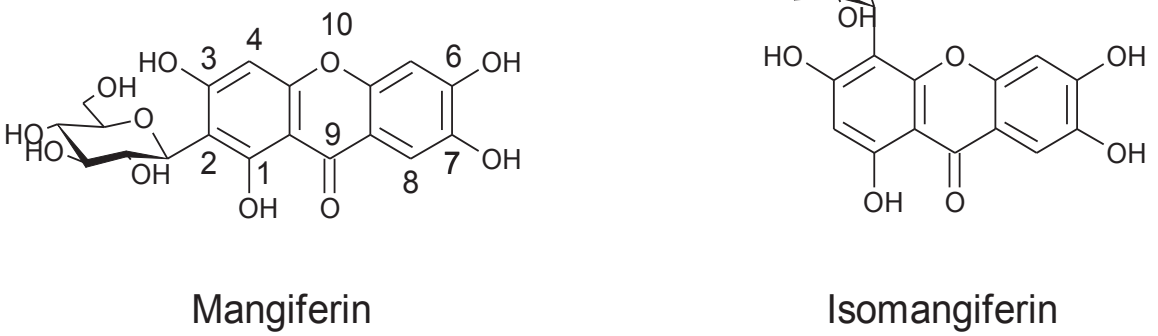
2.5.2 Potential of *Cyclopia species* as anti-diabesity agent

Generally, many of the compounds present in the leaves of *Cyclopia species* are known to have various health promoting properties. Several of the compounds

contain one or more of the structural features (discussed in sections 2.2, 2.3 and 2.4) required for inhibition of the enzymes, pancreatic lipase and α-glucosidase, and antioxidant activity, indicating the potential of honeybush as anti-diabesity nutraceutical.

The xanthones are of interest as they are the major class of compounds detected in *Cyclopia* species (**De Beer *et al.,* 2010)**. Xanthones comprise an important class of oxygenated heterocycles whose role is well-known in medicinal chemistry. The biological activities of this class of compounds are associated with their tricyclic scaffold but vary depending on the nature and/or position of the different substituents **(Pinto *et al*., 2005)**. The chemical structure of xanthones is based on a C6-C3-C6 skeleton, where two phenyl rings are connected via a unit composed of three carbons (Figures 9) and two or more hydroxyl groups attached to each phenyl ring **(Liu *et al*., 2006)**. Even though little is known about the mechanism of action of xanthones for α-glucosidase inhibition, it has been demonstrated that three or more phenolic hydroxyl groups are fundamental for significant inhibitory effects. The position of the hydroxyl groups are also important, with the hydroxyl group at C-7 more active than that at C-6, which was more active than that at C-8 **(Liu *et al*., 2006)**.The inhibitory activity is significantly enhanced by the conjugated π-system **(Liu *et al*., 2007)**. For example, **Liu *et al*. (2007)** showed that a compound having one more conjugated aromatic ring was at least 17-fold more effective than the compound having a smaller d π-system.

Mangiferin is known to be associated with a vast diversity of pharmacological properties, including antioxidant, anti-allergic anti-tumour, immunomodulatory, anti-inflammatory, anti-diabetic, lipolytic, antibone resorption, monoamine oxidase-inhibiting and anti-parasitic properties **(Bhattacharya *et al*., 1997; Liu *et al*., 2007; Wauthoz *et al*., 2007; Vyas *et al*., 2012)**. Moreover, mangiferin is known to be a potent free radical scavenger **(Sato *et al*., 1992; Joubert *et al*., 2008b)**. Inhibitory effects against pancreatic lipase and α-glucosidase have been reported for mangiferin **(Yoshikawa *et al*., 2002; Liu *et al*., 2006)**, supporting its potential as treatment for diabetes mellitus **(Ichiki *et al*., 1998)** and for lowering body weight **(Yoshimi *et al*., 2001)**. Moreover, a decrease in blood glucose and decreased insulin resistance were exhibited after oral administration of mangiferin in an in vivo mouse T2D model, providing further evidence of its anti-diabetic effect **(Muira *et al*., 2001)**.



Mangiferin Isomangiferin

Figure 9. Structures of mangiferin (major honeybush xanthone) and isomangiferin (minor honeybush xanthone)

Hesperidin, another major constituent of honeybush, has been recognised for its antioxidant properties. This flavanone has been found to reduce superoxide in electron transfer plus concerted proton transfer reaction *in vitro* **(Jovanovic *et al.*, 1994)**. **Wilmsen *et al*. (2005)** evaluated this activity in both chemical (using DPPH) and biological (using methylviologen; 1,1'-dimethyl-4-4'-bipyridinium dichloride) systems and the antioxidant activity of hesperidin was shown to be similar to that of Trolox. This activity was also exhibited in an *in vivo* study by **Fraga *et al.* (1987)** in liver homogenates for hydroperoxide-induced chemiluminescence.

Furthermore, porcine pancreatic lipase inhibitory effects (IC_{50}=32 µg/mL) have been reported for hesperidin **(Kawaguchi *et al*., 1997)**, suggesting that the compound possesses anti-obesity properties. Moreover, a study by **Zhang *et al*. (2012)** revealed that a hesperidin derivative exhibited anti-hyperglycemic activity through inhibition of the enzyme α-glucosidase (55.02 % inhibition at 10 mM hesperidin). Hesperidin and naringinin have been reported to attenuate hyperglycemia-mediated oxidative stress and pro-inflammatory cytokine production in high fat fed/streptozotocin-induced type 2 diabetic rats **(Mahmoud *et al*., 2012)**.

Eriocitrin (another flavanone present in substantial amounts in some *Cyclopia* species) has been shown to suppress oxidative stress **(Minato *et al*., 2003)** and compounds such as luteolin, eriodictyol, hesperetin and naringinin have shown

antioxidant and antiradical potential in various *in vitro* and *in vivo* studies **(Sato *et al*., 1992; Joyeux *et al*., 1995; Haraguchi *et al*., 1996)**. These findings suggest that the presence of these flavonoids in *Cyclopia* extracts could contribute therapeutic effects in management of diabesity by this herbal tea as the link between oxidative stress, inflammation and diabesity has been noted **(Furukawa *et al*., 2004; Maritim *et al*., 2003; Fernandez-Sanchez *et al*., 2011)**.

The success of herbal teas such as honeybush relies on the fermented form **(Du Toit *et al*., 1998; Joubert *et al*., 2011)**. The plant material, comprising leaves and stems, and sometimes flowers as well, were traditionally fermented (a key step in the production of tea, whereby the plant material undergoes high temperature oxidation) for colour, flavour and taste development. However, a study conducted by **Joubert *et al*. (2008b)** found that the extracts of fermented *Cyclopia* species contained lower quantities of total polyphenols and had significantly lower anti-oxidant capacities than extracts of unfermented plant material. Their study also showed a significant decrease in the content of the individual phenolic compounds, thus rendering the extracts less potent in the fermented state with regards to antioxidant activity. Like many medicinal plants, the therapeutic effects of *Cyclopia* are attributed to the presence of polyphenols, which are known to be susceptible to oxidation and degradation at high temperatures. Hence the current study will also investigate the effect of fermentation on the anti-diabesity potential of *C. maculata.*

2.6 Principles of laboratory assays employed in investigation of anti-diabesity and antioxidant potential of plant products

2.6.1 Anti-diabesity assays

2.6.1.1 Pancreatic lipase inhibition assay

Lipases, are secreted by the pancreas in the presence of fat, hydrolyses dietary triglycerides into monoglycerides and fatty acids. Lipase inhibitors covalently bind to the active site of lipase (serine), preventing the enzyme from binding to the said substrate (triglycerides) **(Margesin *et al*., 2002)**. Chromogenic substrates of the *p*-nitrophenyl ester type such as *p*-nitrophenyl palmitate (pNPP) **(Kanwar *et al*., 2005)**, *p*-nitrophenyl butyrate (pNPB) **(Kim *et al*., 2012)**, *p*-nitrophenyl acetate (pNPA), *p*-nitrophenyl caprylate (pNPC), *p*-nitrophenyl laurate (pNPL) and *p*-nitrophenyl propionate (pNPRP) are widely used for determination of lipase activity **(Margesin *et al*., 2002)**. Other probes such as the micellar solutions of triolein and 4-methylumbelliferyl oleate (4-MUO), fluorescent probes are also used for determination of lipase activity **(Margesin *et al*., 2002)**. Due to their ease of use, pNPB and 4-MUO are commonly used substrates **(Kato *et al*., 2012)**. Moreover, the assay methods that employ these two substrates (pNPB and 4-MUO) have been reported to show three to four order or even higher activity compared to the assays that use the micellar solution of triolein as a substrate **(Ikeda *et al*., 2005; Kusano *et al*., 2008)**.The principle of the assay is based on the hydrolysis of the substrate such as pNPB to release *p*-nitrophenol as an end product which is detected through

subsequent measurement of absorbance at 405 nm (Figure 10) **(Kanwar *et al*., 2005; Slanc *et al*., 2009; Kim *et al*., 2010a)**. One unit of the enzyme is defined as micromole(s) of *p*-nitrophenol released by hydrolysis of the substrate by one milliliters of enzyme under assay conditions **(Kanwar *et al*., 2005)**. In any assay that involves the use of a chromogenic substrate blocking of residual/unquenched enzymatic activity is essential to avoid the false increase in colour intensity and for maintaining reproducibility of the results. Appropriate methods to block lipase activity are lacking **(Kanwar *et al*., 2005)**. However, a number of studies have reported the use of either, sodium carbonate **(Rapp and Backaus, 1992; Dosanjh and Kaur, 2002)**, acetone, ethanol oracetone: methanol mixture for the above-mentioned purpose **(Kanwar *et al*., 2005)**.

Colorimetric methods are easy and the quickest assays that can be employed to determine lipase inhibition activity of plant extracts **(Margesin *et al*., 2002)**, however, a disadvantage associated with this type of assay is the colour interference by plant extracts. Fluorimetric methods that involve the use of butyrylesters of 7-hydroxy-4-methylcoumarin or 4-methylumbelliferyl butyrate (MUB) as substrates **(Andlauer *et al.,* 2009)** are alternative methods for determination of lipase inhibitory activity of plant extracts to account for any colour interference. The enzyme converts the substrate to a reaction product (Figure 10) that fluoresces when excited by light of a particular wavelength (excitation 365 nm and emission 460 nm). However, as the same as the colorimetric substrates, these types of substrates are also insoluble in water, thus

requiring the use of a minimum portion of organic solvent such as dimethyl sulphoxide (DMSO) in water to dissolve, which in turn could affect the activity of the enzyme. Despite the above mentioned limitation, the fluorescent method is reported to be more sensitive and specific than the colorimetric methods **(Cooper and Morgan, 1981; Margesin *et al*., 2002)**.

2.6.1.2 α-Glucosidase inhibition assay

The α-glucosidase inhibition assay is carried out using enzymes from different sources such as yeast α-glucosidase **(Liu *et al*., 2007)** or rat intestinal acetone powder **(Boath *et al*., 2012)**, and in some cases microbial α-glucosidases from other sources than yeast are used **(Kim *et al*., 2008a)**. Due to its accessibility and low cost, yeast α-glucosidase is the most widely used enzyme **(Liu *et al*., 2007)**. However, it has been reported that most yeast α-glucosidase inhibitors did not show inhibitory activity against mammalian α-glucosidase due to the difference in molecular recognition of the binding site of the enzymes **(Gao and Kawabata, 2004; Jo *et al*., 2011)**.*p*-Nitrophenyl α-D-glucopyranoside (pNPG) is the most widely used substrate for colorimetric detection (Figure 10) at 405 nm **(Liu *et al*., 2007; Boath *et al*., 2012)**. To block the residual/unquenched enzymatic activity sodium carbonate can be used **(Kim *et al*., 2010b)**. For the same reason(s) stated in 2.6.1.1, fluorimetric methods are preferred for determination of α-glucosidase inhibitory effects of plant extracts. In this case (Figure 10), 4-methylumbelliferyl α-D-glucopyranoside (4-MUG) is a suitable

substrate for this type of assay **(Azuma *et al*., 2011)** and the principle of the method is similar to the one discussed in 2.6.1.1.

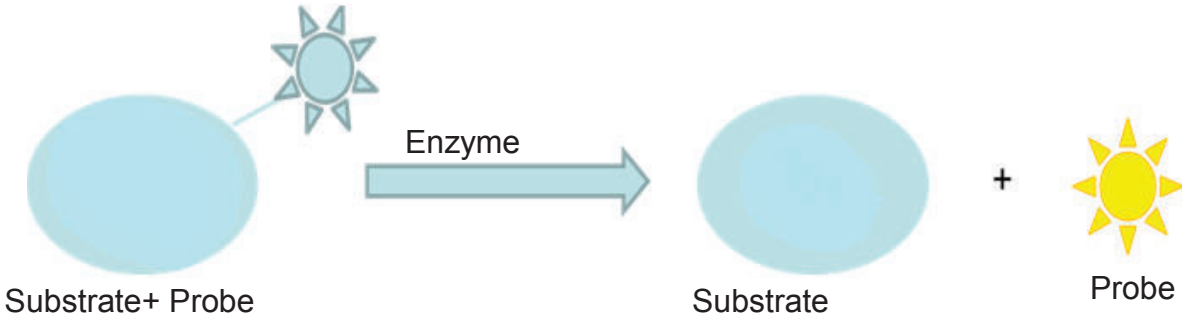


Figure 10. Enzyme reacts with substrate to release probe. The probe could be chromogenic or fluorescent, depending on the assay (supplied by **CJ Malherbe**, ARC Infruitec-Nietvoorbij, Stellenbosch).

2.6.2 Folin–Ciocalteau Reagent (FCR) method for total polyphenol determination

The literature describes a number of methods for determination of total phenolics, including the Prussian Blue assay by **Price and Butler (1977)** and the Folin-Denis method by **Folin and Denis (1912)**, which was later modified in 1927 by **Folin and Ciocalteu (Schofield *et al*., 2001)**. Subsequently, these methods have been modified numerous times and the version of the Folin-Ciocalteu method adapted by **Singleton and Rossi (1965)** is the most commonly used method for determination of the total polyphenol content of plant extracts. The Folin-Ciocalteu assay is based on the oxidation of phenolic compounds in

alkaline medium by molybdenum and tungsten to form a blue-coloured complex. The intensity of the complex is measured spectrophotometrically at 765 nm **(Arthur *et al*., 2011; Bajcan *et al*., 2013)**, and the total polyphenol content of analysed samples is calculated as the amount of gallic acid equivalents **(Bajcan *et al*., 2013)**.

This method offers several advantages. Firstly, the FCR can be purchased readily mixed. Secondly, the assay is quick and easy as the visual colour changes from intense yellow to blue in the presence of phenols in the reaction mixture. Thirdly, even though the initial methods were described using test tubes or cuvettes **(Singleton and Rossi, 1965)**, the method has been adapted for use in 96-well micro-plates **(Zhang *et al*., 2006)**. Therefore, many samples could be tested simultaneously. Lastly, the total polyphenol content of the tested plant extracts could be expressed in terms of gallic acid equivalence. The historical use of gallic acid is owed to its affordability, water solubility, easy crystallization from water and stability in dry from **(Singleton *et al*., 1999)**.

2.6.3 Antioxidant assays

Antioxidants act by several mechanisms and no one assay can capture the different modes of action of an antioxidant **(Niki and Noguchi, 2000; Badarinath *et al*., 2010)**.The use of multiple assays could help compare antioxidants from different food products since different assays act on different principles **(Prior *et al*., 2005)**. Moreover, various antioxidants with different reactivity and selectivity

towards substrates are involved in oxidative stress *in vivo* **(Niki and Noguchi, 2000)**. The current study will employ four of the antioxidant methods (ferric reducing/antioxidant power (FRAP), 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and the oxygen radical absorbance capacity (ORAC) assay) to determine the antioxidant potential of *C. maculata*. The following section describes the principles, strengths and limitations of the individual methods that are commonly used to determine the antioxidant activity of plants extracts and constituents.

2.6.3.1 DPPH radical scavenging method

The $DPPH^{\bullet}$ scavenging assay was first described by **Blois in 1958** and was later modified slightly by numerous researchers **(Brand-Williams *et al*., 1995; Li *et al*., 2007; Chan *et al*., 2007; Tirzitis and Bartosz, 2010; Krishnaiah *et al*., 2010)**. It is one of the most extensively used antioxidant assays for plant samples. $DPPH^{\bullet}$ is a stable free radical that reacts with compounds that can donate a hydrogen atom **(Tirzitis and Bartosz, 2010)**. This method is based on the measurement of the reducing ability of antioxidants towards $DPPH^{\bullet}$ **(Prior *et al*., 2005)**. This ability can be evaluated by electron spin resonance or by colour change from purple to yellow if/when an extract/antioxidant is able to reduce $DPPH^{\bullet}$ **(Brand-Williams *et al*., 1995; Li *et al*., 2007; Tirzitis and Bartosz, 2010)** leading to a decrease in absorbance which is measured spectrophotometrically at 515 nm **(Bondet *et al*., 1997; Krishnaiah *et al*., 2010)**. The assay is known to be sensitive enough to detect active ingredients at low

concentration **(Hsu *et al*., 2005)**. The visual colour change also serves as an advantage in that one can easily see when the reaction has occurred. This stable free radical is commercially available and therefore it does not have to be generated before the assay like other radicals such as $ABTS^{\bullet+}$ **(Prior *et al*., 2005; Tirzitis and Bartosz, 2010)**. In spite of the wide use of this method, complications, which may cause incorrect results, have been reported. The $DPPH^{\bullet}$ scavenging assay is pH-dependant. Therefore, partial ionization of the tested compounds can affect the rate of their reaction with the free radical **(Musialik and Litwinienko, 2005)**. In addition to that this method requires the use of organic solvents as $DPPH^{\bullet}$ is insoluble in water. Moreover, $DPPH^{\bullet}$ is an organic nitrogenous radical, which bears no similarity to the highly reactive and transient peroxyl radicals involved in lipid peroxidation **(Huang *et al*., 2005)**.

2.6.3.2 ABTS radical cation scavenging method

This method was first developed by **Rice-Evans and Miller (1994)** and was then modified by **Re *et al*. (1999)**. Originally, $ABTS^{\bullet+}$ was generated by the activation of metmyoglobin with hydrogen peroxide. The method was improved by reacting ABTS with potassium persulfate to generate $ABTS^{\bullet+}$ **(Re *et al*., 1999)**. The blue/green pre-formed $ABTS^{\bullet+}$ is reduced in the presence of hydrogen donating antioxidants. $ABTS^{\bullet+}$ is reported to have absorption maxima at wavelengths 645 nm, 734 nm and 918 nm, however, the decrease in this radical absorbance is commonly measured spectrophotometrically at 415 nm **(Re *et al*., 1999; Krishnaiah *et al*., 2010)**. At this wavelength coloured plant extracts can interfere

with measurement. Thermodynamically a compound requires a redox potential lower than that of $ABTS^{\bullet+}$ in order to reduce it, and many phenolic compounds meet this requirement and can thus react with $ABTS^{\bullet+}$ **(Van den Berg *et al*., 1999)**. The effect of antioxidant concentration and duration of the inhibition of the radical absorption of the cation are taken into account when the antioxidant activity is determined. $ABTS^{\bullet+}$ is soluble in both aqueous and organic solvents, and it is not affected by ionic strength, therefore, this decolourisation assay can be used to measures total antioxidant capacity of both lipophilic and hydrophilic substances **(Re *et al*., 1999; Pellegrini *et al*., 1999)**. Moreover, $ABTS^{\bullet+}$ can also be used to determine the effects of pH on antioxidant mechanisms, as it is can be used over a wide pH range. **Cano *et al*. (1998)** demonstrated that $ABTS^{\bullet+}$ was stable from pH 3.0 to pH 6.5 but optimal at pH 4.5. However, even though frequently used, the radical is noted to be unstable at pH values of above 7.5 **(Cano *et al*., 1998)**. In addition to that, like $DPPH^{\bullet}$, $ABTS^{\bullet+}$ is not found in mammalian biology and thus represents a non-physiological radical source **(Prior *et al*., 2005; Tirzitis and Bartosz, 2010)**.

2.6.3.3 Ferric Reducing Antioxidant Power (FRAP)

The original FRAP method was developed to measure reducing power in plasma **(Benzi and Strain, 1996)**, but the assay has been adapted to determine antioxidants in plant extracts **(Benzi and Szeto, 1999)**. The principle of this assay is based on the reduction of a ferric-tripyridyl triazine (Fe^{3+}-TPTZ) complex to its ferrous coloured form in the presence of antioxidants **(Benzie and Strain,**

1996). The Fe^{3+}-TPTZ complex is reduced to an intense blue ferrous under acidic conditions. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentrations. The activity can also be expressed in either ascorbic acid equivalence **(Aparadh *et al*., 2012)** or Trolox equivalence **(Benzie and Strain, 1996; Arthur *et al*. 2011)**. Moreover, the reaction is reported to be non-specific, therefore, any half-reaction which has a less-positive redox potential under the same reaction conditions than Fe^{3+} /Fe^{2+}-TPTZ will influence the reduction of the complex **(Benzie and Strain, 1996)**. The FRAP assay is simple, speedy, inexpensive and reagents are simple to prepare, giving highly reproducible results with no specialized equipment required. Moreover, in contrast to other tests for antioxidant power, the FRAP assay is reported to be robust and does not require specialised equipment, thus can be performed using automated, semi-automatic or manual methods **(Benzie and Strain, 1996; Prior *et al*., 2005)**. **Pulido *et al*. (2000)** showed that reaction time is important and the use of 4 and 30 min reaction times could be applied to differentiate between active and less active antioxidants. As for other antioxidant methods, the antioxidant efficiency of polyphenols seems to depend on the extent of hydroxylation and conjugation **(Pulido *et al*., 2000)**.

2.6.3.4 Oxygen Reduced Absorbance Capacity (ORAC)

The original work on the development of the ORAC assay was by **Glazer (1990)**, and the method was further developed by numerous researchers **(Cao *et al*.,**

1993; Ghiselli *et al*., 1995; Huang *et al*., 2002) and others. ORAC is a "biological" assay based on the inhibition of peroxyl-radical-induced oxidation that is initiated by decomposition of azo-compounds at high temperatures **(Ou *et al*., 2001; Ganske and Dell, 2006)**. It presents a hydrogen atom transfer reaction mechanism, which is most relevant to human biology **(Prior *et al*., 2005)**. The antioxidant scavenges the peroxy radical thus delaying decay in fluorescence (Figure 11) **(Prior *et al*., 2005)**. ORAC values are usually reported as Trolox equivalents **(Prior *et al*., 2005)**. The earlier version(s) of the method which involve the use of B-phycoerythrin (a protein isolated from *Porphyridium cruentum*) **(Prior *et al*., 2005)** as a probe are reported to be associated with a number of drawbacks. They are noted to be time-consuming and labour-intensive **(Cao *et al*., 1993)**. The improved assay that employs fluorescein as a probe was demonstrated to be robust and compatible with lipid soluble antioxidants **(Huang *et al*., 2002)**.

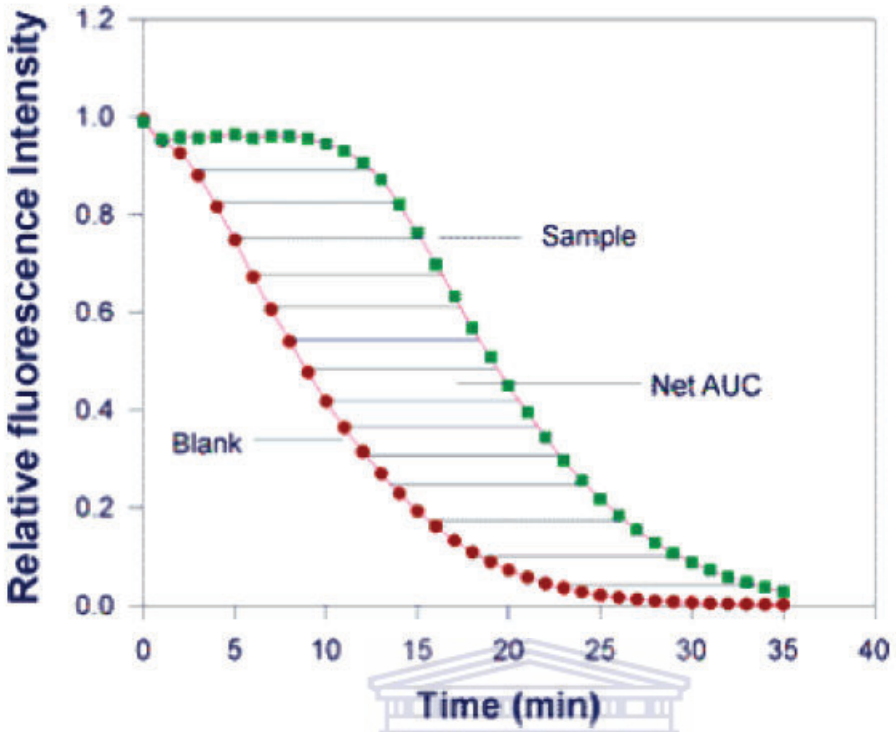


Figure 11. Diagram illustrating the delay in fluorescence decay over time due to antioxidant activity tested by the ORAC method **[Prior*et al*., 2005]**.

2.6.4 On-line HPLC (bio)chemical detection assays

In the search for bioactive compounds from plant products, several attempts have been made to accelerate the isolation, identification and bioactivity evaluation process. Coupling of an HPLC system with on-line post-column detection (Figure 12) of radical scavenging compounds or other bioactive compounds such as enzyme inhibitors (Figure 13) has emerged as an appropriate approach to achieve this objective **(Malherbe *et al*., 2012)**. These (bio)chemical detection (BCD) assays can be defined as the detection of

bioactives based on (bio)chemical reactions or simulated (bio)chemical reactions **(Malherbe *et al.*, 2012)**. Moreover, the system provides both the chromatographic profile and corresponding fingerprint of bioactive compound(s) that may not have been previously identified (unknown compounds) in a particular extract **(Kusznierewicz *et al.*, 2011)**.

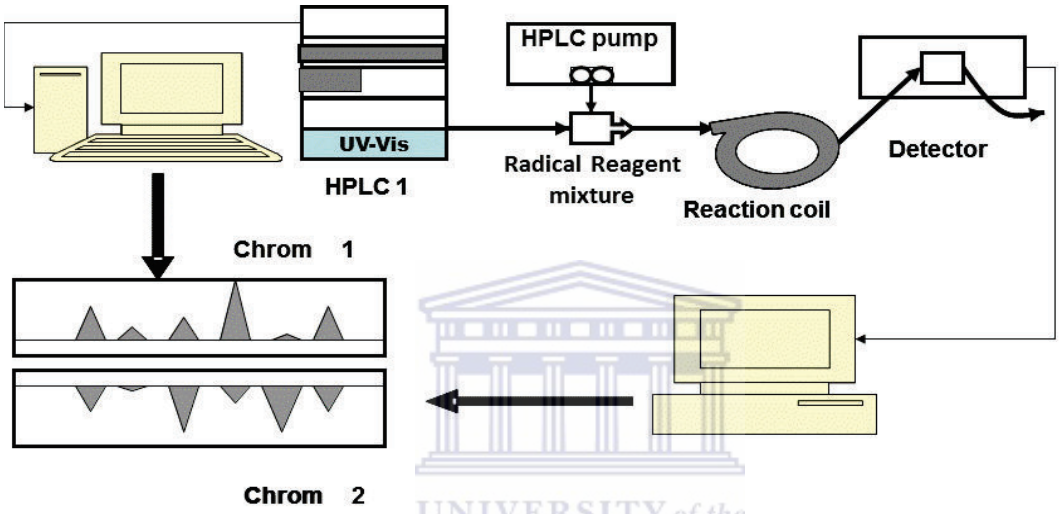


Figure 12. HPLC system coupled with chemical detection (free radical scavenging) (supplied by **CJ Malherbe**, ARC Infruitec-Nietvoorbij, Stellenbosch).

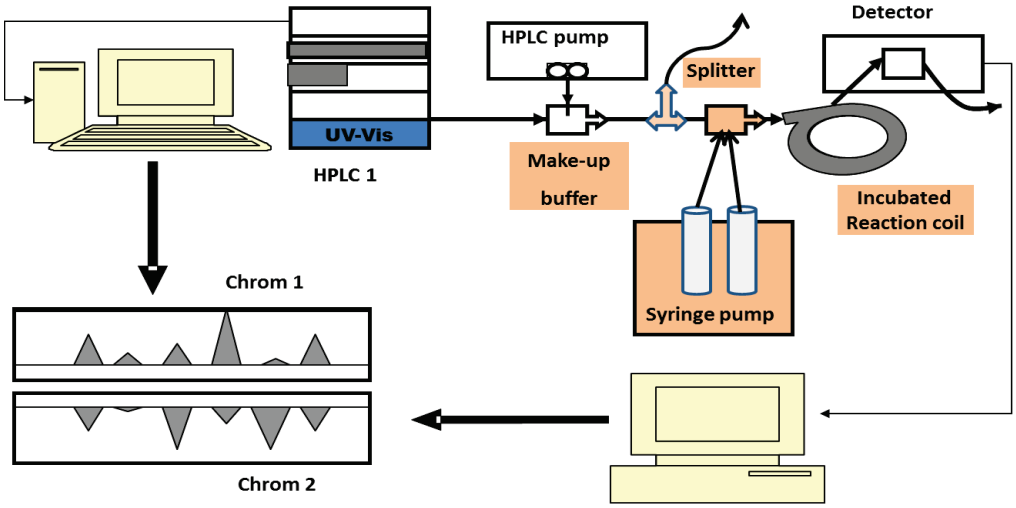


Figure 13. HPLC system coupled with (bio)chemical detection (enzyme inhibition). HPLC effluant combined with make-up buffer, usimg dual syringe pump for enzyme and substrate addition (supplied by **CJ Malherbe**, ARC Infruitec-Nietvoorbij, Stellenbosch).

Several HPLC-on-line BCD methods (based on various assay models) have been studied and developed **(Malherbe *et al*., 2012; Glod *et al*., 2014)**. Amongst these are the antioxidant assays, i.e. luminol-chemiluminescence inhibition, $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ reduction **(Dapkevicius *et al*., 1999; Koleva *et al*., 2001; Nuengchamnon g*et al*., 2005)**. HPLC on-line $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays have been selected for the current study due to their high popularity and other advantages, such as relatively simple set-up and ease of control **(Koleva *et al*., 2001; Niederlander *et al*., 2008)**, and therefore, will be discussed in section 2.6.4.1.

HPLC on-line post-column detection is not only applicable to radical scavenging, but also to enzyme inhibition **(Malherbe *et al*., 2012)**. An on-line method for α-glucosidase inhibition has been developed by adapting the microplate colorimetric assay to on-line format **(Li *et al*., 2010)**. In many cases, the bioactives can be identified by comparison with standard compounds. By combining the HLPC on-line system with mass spectrometry (MS), the molecular weight of the separated compound(s) can be obtained to aid identification **(Nuengchamnong and Ingkaninan, 2009)**. These methods aim at rapid pinpointing of bioactives in complex mixtures without the need for lengthy isolation procedures. It is therefore a useful de-replication tool when screening for new bioactive compounds in plant extracts.

2.6.4.1 On-line HPLC radical scavenging assays

A basic instrumental configuration is required for all on-line HPLC-BCD methods. However, it can be optimised to suit a particular assay type **(Malherbe *et al*., 2012)**. Reduction of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ by the antioxidant, either through hydrogen transfer or electron transfer, leads to a significant change in the UV-Vis absorption of the compound **(Niederlander *et al*., 2008)**. These methods are usually used in a qualitative approach, thus they are suitable for screening of antioxidants **(Arthur *et al*., 2011)**.

For the HPLC on-line $DPPH^{\bullet}$ method, the HPLC separated analytes react with $DPPH^{\bullet}$ post-column and the reduction is detected as a negative peak by a UV-Visdetector at 515 nm (Figure 14). The method is suitable for both isocratic and gradient HPLC analyses with mobile phase compositions ranging from 10 to 90 % organic solvent in water or buffer (pH 3 - 6). The method is simple, has a broad applicability, and uses common but optimized instruments, inexpensive and stable reagents, and a time-saving and non-laborious experimental protocol. It can also be used for quantitative analysis to identify compounds with highest or low antioxidant activity **(Koleva *et al*., 2000; Niederlander *et al*., 2008; Arthur *et al*., 2011; De Beer *et al*., 2011)**. **Arthur *et al*. (2011)** used a quantitative approach to determine the antioxidant activity of four phenylethanoid glycosides of similar structure, differing in the position of the caffeoyl group and type of sugar.

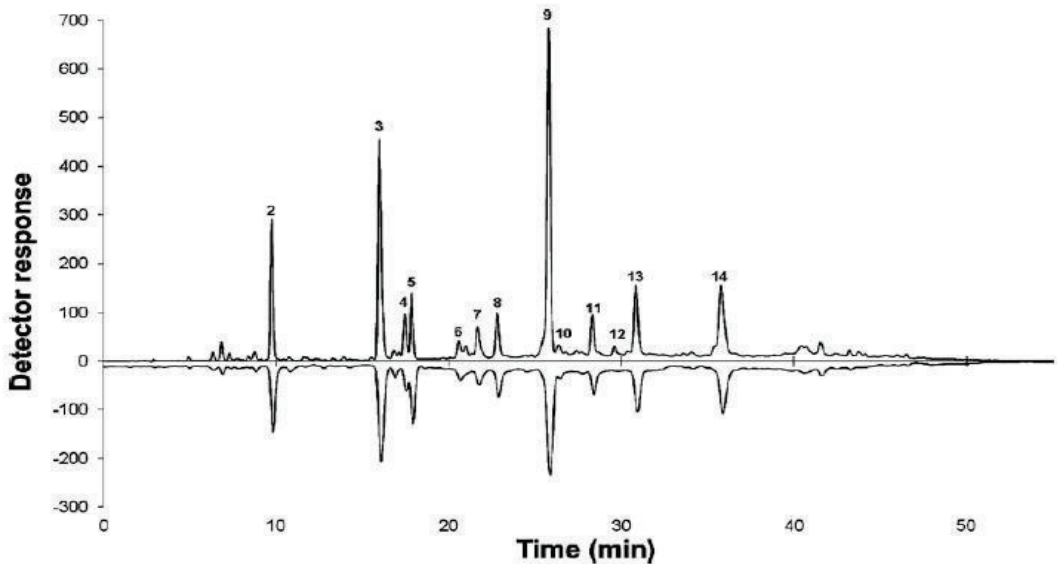


Figure 14. HPLC-DAD-BCD chromatogram of an *Athrixia phylicoides* extract showing the UV-vis chromatogram at the top and the radical scavenging chromatogram as negative peaks at the bottom **[De Beer *et al*., 2011]**.

For the HPLC on-line ABTS$^{\bullet+}$ method the HPLC-separated analytes react post-column with the preformed ABTS$^{\bullet+}$, and the induced bleaching is also detected as a negative peak, either at 414, 600 or 734 nm**(Koleva *et al*., 2001; Niederlander *et al*., 2008; Lee *et al*., 2012)**. The HPLC-ABTS$^{\bullet+}$method is also reported to be suitable for both isocratic and gradient HPLC runs using mobile phases containing 100 % organic solvent or its solution in water, weak acids, or buffers (pH 3-7.4) **(Koleva *et al*., 2001)**. Moreover, the poor stability of the radical at higher pH can be avoided due to the short reaction time of the assay. The method can be used to identify compounds with the highest antioxidant potency in a complex mixture **(De Beer *et al*., 2011)**.

The HPLC-ABTS$^{\bullet+}$ assay is more sensitive than the HPLC-DPPH$^{\bullet}$ assay as the ABTS$^{\bullet+}$ is more water-soluble than DPPH$^{\bullet}$. As a result it is the most widely used for the evaluation of water-soluble antioxidants **(Niederlander *et al*., 2008)**. Moreover, this method is described as a complimentary extension of the HPLC-DPPH$^{\bullet}$ on-line assay **(Koleva *et al*., 2001)**. **De Beer *et al*. (2011)** demonstrated that the negative peak areas obtained with the two methods are not the same, indicating difference in reactivity of the compounds towards the two radicals.

Like any other scientific method, HPLC-ABTS$^{\bullet+}$ assay has its strengths and limitations. Even though it provides a fast screening tool, it provides only time-dependent stoichiometry of reaction (moles ABTS$^{\bullet+}$quenched per OH group over a given time), and therefore it does not measure reaction rate, as it misses the critical initial fast reaction and eliminates distinction between fast and slower reacting antioxidants (**Tian *et al*., 2013)**. In addition to that, for both HPLC on-line assays, the reaction temperature and mobile phase pH caused dramatic changes to the activity of antioxidants. **Koleva *et al*. (2000)** demonstrated that a highly acidic system (pH 2.2) caused a drastic reduction in DPPH$^{\bullet}$ absorbance. Different peak areas were observed by **Kusznierewicz *et al*. (2011)** when investigating phenolic compounds, which were analysed at different reaction temperatures 30 °C-130 °C (post-column derivatisation carried out with ABTS$^{\bullet+}$ and DPPH$^{\bullet}$).

2.6.4.2 Enzyme inhibition HPLC on-line assays

There are no on-line methods that have been published to date for lipase inhibition. However, a HPLC-BCD method, adapted from the micro-plate assay, is available for α-glucosidase inhibition, using of yeast α-glucosidase and the probe, *p*-nitrophenyl-α-D-glucopyranoside (PNPG) **(Li *et al*., 2010)**. The assay was developed for screening of α-glucosidase inhibitors in plant extracts. HPLC separation of complex mixtures usually requires a gradient of organic solvents such as methanol or acetonitrile, but high concentrations of organic solvents could lower enzyme activity or could cause a complete inactivation of the enzyme. Therefore, 30 % methanol is reported to be the optimal concentration for the assay conditions. Moreover, volatile acid can be added to the mobile phase to improve separation **(Li *et al*., 2010)**. Acarbose™ has been reported to show a definite negative peak with (bio)chemical detection (at 1 mg/mL). It is however not visible on the UV-vis chromatogram. Plant extracts that were evaluated for α-glucosidase inhibitory effects using the method of **Li *et al*. (2010)** showed negative peaks for some compounds. Recently **Li et al. (2014)** demonstrated inhibitory activity for tannins such as ellagic acid. Furthermore, the same method mentioned was adapted by **Muller *et al*. (2012)**. Their modification of the assay included replacing 2 % acetic acid with 1 % formic acid to account for enzyme sensitivity to high acids. Since Acarbose™ was shown not to be detectable by HPLC-DAD (not UV-visible), catechin, a flavanol, was used as a positive control and to align chromatograms taking into account the delay in post-column reaction **(Muller *et al*., 2012)**. The method was applied to a rooibos

extract and negative peaks were obtained (Figure 15), indicating α-glucosidase inhibitory effects for some compounds. The optimal pH for this assay is reported as pH 6.8 **(Li *et al*., 2010b; Muller *et al*., 2012)**. Peaks observed for enzyme inhibition are not sharp as for free radical scavenging due to the longer reaction times causing peak broadening (Figures 14 and 15). Poor separation of compounds could therefore lead to incorrect identification of an active compound.

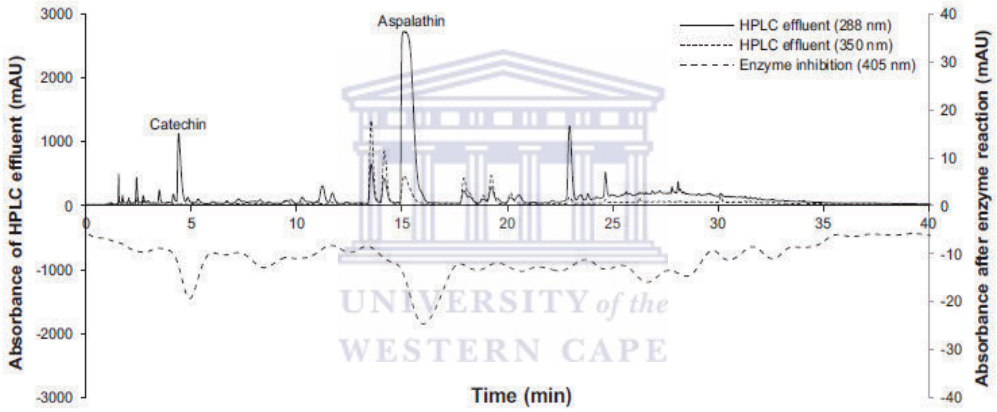


Figure 15. HPLC-DAD-BCD chromatogram of α-glucosidase inhibition by rooibos extract showing the UV-Vis chromatogram at the top and chromatogram depicting enzyme inhibition as negative peaks at the bottom **[Muller *et al*., 2011]**.

In conclusion, due to multiple reaction characteristic and mechanisms, as well as different phase localizations that are involved in an oxidant-antioxidant reaction, no single assay can accurately reflect all radical sources or all antioxidants in a mixed or complex system **(Prior *et al*., 2005)**. More than one assay should thus be used. HPLC-BCD on-line assays could provide additional information as

individual compounds, contributing to the activity of an extract, could be identified. Due to fast reaction times not all active compounds will be identified.

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Chapter 3

Research outlay

3.1 Introduction

This chapter provides the objectives and approach used in this study to evaluate the antioxidant and anti-diabesity potential of *Cyclopia maculata,* using *in vitro* non-cell (chemical) based screening assays. The principles of the individual assays employed are reviewed in Chapter 2.

3.2 Study objectives

The overall aims of this study were to determine the potential of *Cyclopia maculata* aqueous extracts *as* antioxidant, anti-diabetic and anti-obesity agents and to determine the effect of fermentation of the plant material, prior to extraction, on these potential health-promoting properties.

The specific objectives were:

- To characterise the phenolic composition of *C.maculata*extractsthrough total polyphenol and HPLC analysis of individual compounds;
- To determine anti-oxidant capacity of *C.maculata*extracts using different antioxidant assays (DPPH$^{\bullet}$, ABTS$^{\bullet+}$, FRAP and ORAC);
- To determine anti-diabesity activity of *C.maculata*extracts through inhibition of pancreatic lipase and α-glucosidase;

- To determine the effect of fermentation on *C.maculata* extracts on its phenolic composition, antioxidant activity and anti-diabesity activity; and finally;
- To identify antioxidant and anti-diabesity bioactives in *C. maculata* extracts using high performance liquid chromatography diode-array detection coupled with (bio)chemical detection (HPLC-DAD-BCD).

3.3 Study approach

In order to reach the set objectives, procedures as described in the subsequent sections were followed.

3.3.1 Sample preparation

Preparation of plant extracts (from harvest to freeze-dried aqueous extracts) is illustrated in Figure 16. Briefly, shoots from plant seedlings, selected from a natural population in the wild, were harvested to allow for natural variation (n = 9). After cutting into small pieces each batch was divided into two sub-batches (n = 9 per treatment), with one sub-batch subjected to high temperature oxidation ("fermentation") before drying (n = 9) and the other dried without fermentation (n = 9). This allowed for direct comparison of the effect of fermentation on each batch.

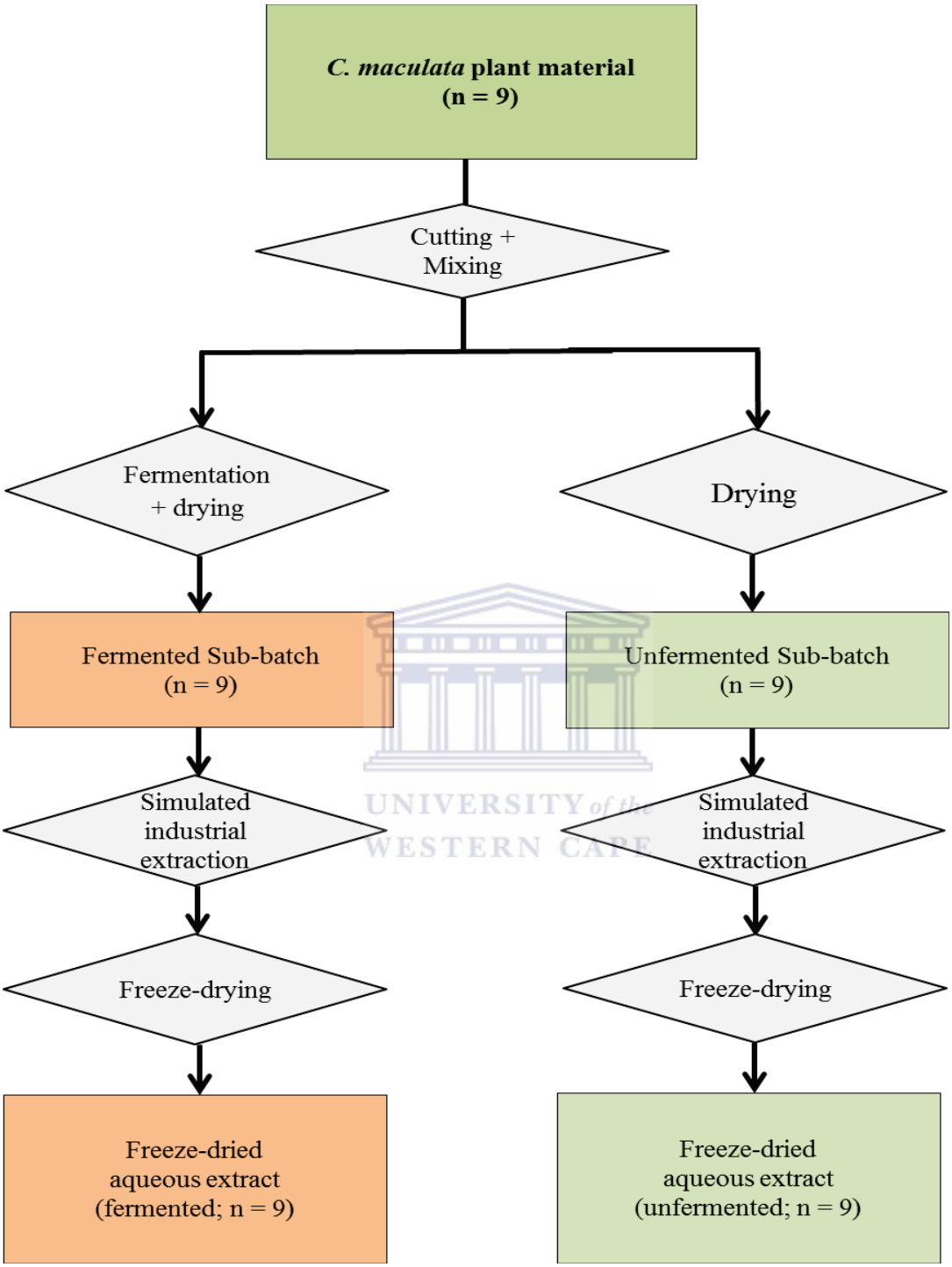

Figure 16. Preparation of extracts (from harvest to freeze-drying). Drying: The cut plant material dried on a forced circulation dring tunnel before divided to sub-batches.

Aqueous extraction of the leaves was performed simulating the industrial scale process used when preparing food-grade extracts. The extracts were then

subjected to freeze-drying and stored at 4 ºC in the dark under desiccation to prevent chemical changes. All analyses were performed on the reconstituted freeze-dried aqueous extracts.

3.3.2 Characterisation of *C. maculata* in terms of composition and activity

For bioactivity characterisation of *C. maculata* extracts, different micro-plate assays (unless otherwise specified) were used, thus enabling end-point or kinetic measurements of multiple samples per experiment. A schematic diagram of different assays employed in this regard is illustrated in Figure 17. Absorbance or fluorescence intensity were used as detection modes depending on which mode was more sensitive for each assay.

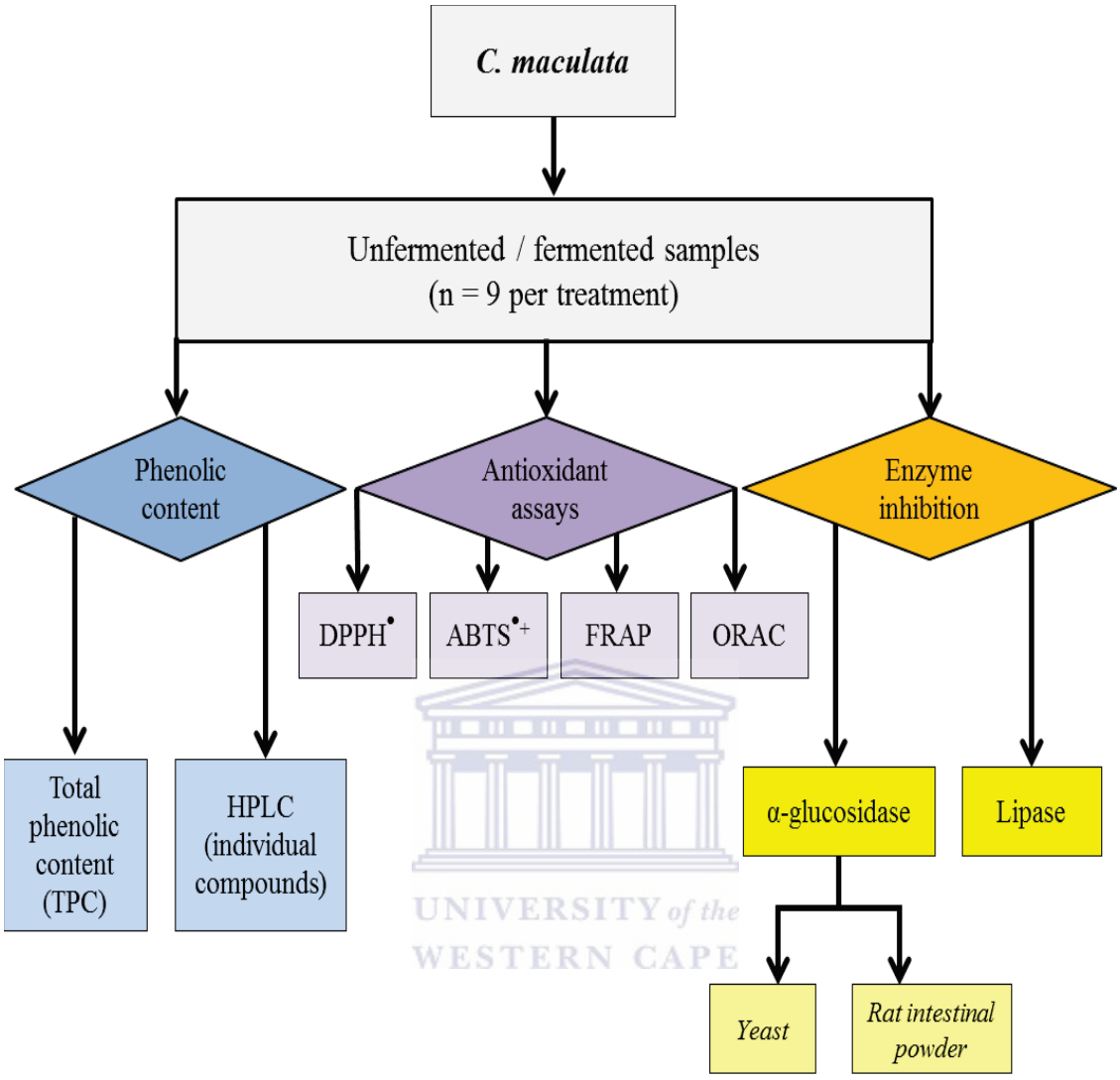

Figure 17. Characterisation of *C. maculata* samples using a battery of assays

3.3.2.1 Phenolic composition

Cyclopia, like most plant species, are rich in polyphenols, which are known to be associated with a number of biological effects. It is thus important to characterise the phenolic composition of the extracts under investigation in this study. The total polyphenol content (TPC) was determined to provide an indication of

differences between samples. For this reason the most commonly used method, incorporating the Folin-Ciocalteu reagent **(Singleton *et al*., 1999)**, was used for determination of TP of *C. maculata* extracts. The TPC was quantified in gallic acid equivalence (GAE). However, TP does not provide information on individual phenolic compounds, and non-phenolic compounds can also react with the Folin-Ciocalteu reagent **(Sanchez-Rangel *et al*., 2013)**. Therefore, the plant extracts were also subjected to HPLC analysis for separation and quantification of individual compounds. The method used was developed for *C. subternata* **(De Beer *et al*., 2009)** so that the quantification focused only on the major compounds, which were adequately separated.

3.3.2.2 Antioxidant activity

Different chemical antioxidant assays are normally used to characterise the antioxidant activity of plant extracts to allow for different mechanisms of action **(Prior *et al*., 2005)**. A number of antioxidant assays (DPPH$^{\bullet}$, ABTS$^{\bullet+}$, FRAP and ORAC) were performed for a meaningful comparison of the antioxidant capacity of *C. maculata* extracts. The principles of these methods and their relative merits were discussed in section 2.6.3.

3.3.2.3 Determination of anti-diabetic potential

One of the therapeutic approaches to treatment of type 2 diabetes (T2D) is to decrease the postprandial hyperglycemia by retarding absorption of glucose. This can be achieved through inhibition of carbohydrate-hydrolyzing enzymes, such as α-glucosidase, as this enzyme plays a key role in digestion of carbohydrates (disaccharides and oligosaccharides), into monosaccharides **(Krentz and Bailey 2005)**. Therefore, the anti-diabetic potential of *C. maculata* extracts was assessed in terms of their ability to inhibit the activity of this enzyme. Two enzyme sources were used for the anti-diabetic assay. Due to its popularity and accessibility **(Hu *et al*., 2013)**, α-glucosidase from *Saccharomyces cerevisiae* was used to evaluate this bioactivity. Thereafter, rat intestinal acetone powder as source of mammalian α-glucosidase was used to determine the physiological relevance of the findings, relative to anti-diabetic effect of the extracts in humans **(Hu *et al*., 2013; Misbah *et al*., 2013)**.

Acarbose™ is the most widely used positive control in α-glucosidase inhibition assays **(Van de Laar *et al*., 2005)**, however, many studies have shown the drug to be a weak microbial α-glucosidase inhibitor (Table 2 : chapter 2), but strong activity against mammalian α-glucosidase has been reported **(Uddin *et al*., 2012)**. Thus (+)-catechin, a known microbial α-glucosidase inhibitor, **(Muller *et al*., 2012)**, or Acarbose™ (for intestinal α-glucosidase) **(Kim *et al*., 2010)** were used as positive controls for the respective assays. These inhibitors were also used to normalize activity between different plates and experimental days.

3.3.2.4 Determination of anti-obesity potential

The anti-obesity potential of the extracts was assessed in terms of their ability to inhibit porcine pancreatic lipase (PPL), as it is the key enzyme in absorption of dietary triacylglycerol through lipid metabolism **(Slanc *et al*., 2009)**. Amongst the different sources of lipases (such as animal, plant or microbial cells), PPL was selected for the current study as this mammalian enzyme has relevance to human digestive system **(Luthi-Peng *et al*., 1992; Tokdar *et al*., 2011)**. Orlistat™ a known potent lipase inhibitor **(Barbier *et al*., 2004)** was used as a positive control and to normalise activity between different plates and experimental days.

3.3.2.5 Substrates used for fluorescence intensity of anti-diabesity methods

The background absorbance of the plant extracts affected the sensitivity of the colorimetric methods that were initially used to evaluate the above mentioned bioactivities, thus necessitating the use of fluorescent probes. To obtain sensitive and specific fluorescent methods **(Cooper and Morgan, 1981; Margesin *et al*., 2002)**, 4-Methylumbelliferyl butyrate (MUB) and 4-methylumbelliferyl α-D-glucopyranoside (MUG) **(Andlauer *et al.,* 2009; Azuma *et al*., 2011)** were chosen as substrates for PPL and α-glucosidase, respectively.

3.3.3 Identification of bioactives of *C. maculata* extracts

From the data obtained from the respective micro-plate assays, samples that showed the highest activity in each assay (from both fermented and unfermented sub-batches) were selected for on-line HPLC-DAD-BCD analysis (Figure 14: chapter 2). Counter partners (lowest activity) of each sample were also subjected to on-line HPLC-DAD-BCD analysis, thus giving obtaining a sample size n = 8 (Figure 18). These methods indicate which peaks on chromatographic fingerprints contribute to the activity of the extracts as observed in the micro-plate assays.

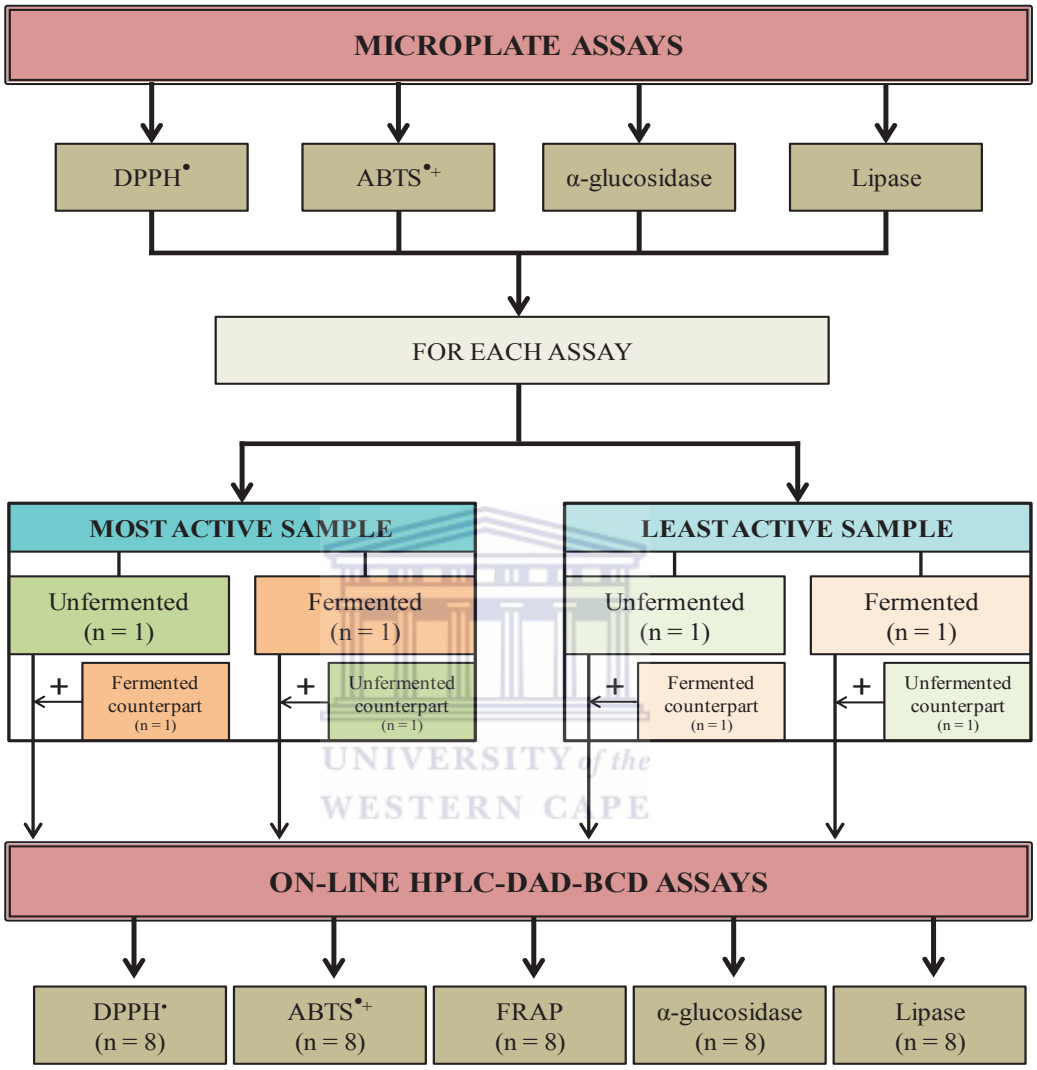

Figure 18. Identification of active constituents for antioxidant activity, lipase and α-glucosidase inhibition from *C. maculata* extracts using the on-line HPLC-DAD-BCD assays.

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Chapter 4

Methods and Materials

4.1 Introduction

This chapter describes the equipment, materials and methods used to evaluate the antioxidant, anti-diabetes and anti-obesity potential of *Cyclopia maculata*. The information is arranged under the following sub-headings:

i) Equipment and material, ii) Reagents and chemicals, and iii) Methods; harvesting and preparation of plant material and preparation of plant extracts, phenolic analysis (total polyphenol and HPL-DAD analysis), determination of antioxidant activity (different micro-plate assays and on-line HPLC-BCD), enzyme assays for determination of anti-diabesity activity of *C. maculata* (micro-plate and on-line HPLC-BCD assays) and iv) data analysis.

4.2 Equipment and materials

4.2.1 Equipment - including consumables

The following equipment and chemicals were used in this study:

- **BioTek Synergy HT multiplate reader** (BioTek Instruments, Winooski, USA),
- **Blue, clearand yellow pipette tips (1000, (300 and 10) and 200 µL)** (Greiner bio-one, Germany),

- **Eppendorf MixMate** (Merck Millipore, Merck KGaA, Darmstadt, Germany),
- **Gilson single channel pipettes** (Gilson, France),
- **Gilson 20-300 µL multi-channel pipette** (Gilson, France),
- **Glassware** (Various suppliers),
- **Hettich Universal centrifuge** (Andreas Hettich GmbH and Co.KG, Germany),
- **High performance liquid chromatograph (HPLC-DAD) with diode-array detector** (Agilent 1200 series HPLC consisting of quaternary pump (G1311A), degassing system (G1379B), diode-array detector (G1315A), auto-sampler (G1329A), controlled by (chemstation software (Rev. B.02.01) (Agilent Technologies, Waldbronn, Germany). A Zorbax Eclipse XDB C18 column (150 x 4.6 mm; 5 µm particle size) (Agilent Technologies, Waldbronn, Germany) was used in the analysis,
- **High performance liquid chromatograph (HPLC-DAD-BCD) with diode-array detector and on-line (bio)chemical detection system** (Agilent 1200 series HPLC consisting of quaternary pump (G1311A), degassing system (G1379B), diode-array detector (G1315A), autosampler (G1329A), controlled by (chemstation software (Rev. B.02.01) (Agilent Technologies, Waldbronn, Germany). A Zorbax Eclipse XDB C18 column (150 x 4.6 mm; 5 µm particle size) (Agilent Technologies, Waldbronn, Germany) was used. HPLC effluent and post-detector reagents were combined through a high pressure mixing tee (U-466) and for enzyme based assays a further micro-splitter (P-470) was used before enzyme and

substrate were added (Idex Health and science, LLC, Oakharbor, Washington, USA). For the antioxidant assays, a 15 m poly-ether ketone (PEEK) reaction coil (0.25 mm i.d) was used, while enzyme assays used a 2 m pre-coil followed by 4 m reaction coil of polytetrafluoroethylene (PTFE) (0.3 mm i.d). Absorbance based assays were monitored using a variable wavelength detector (VWD) (1314B) while a fluorescence detector (FLD) (G1321A) was used for fluorimetric assays. The enzyme and substrate were added with a Harvard PHD 200 Programmable syringe pump (Harvard Apparatus, Holliston, Massachusetts, USA) and reaction coils for enzyme based assays were incubated in a column heater,

- **HR73 Halogen moisture analyzer** (Mettler Toledo, Greifensee, Switzerland),
- **4 mm Millex-HV 0.45 µm PVDF syringe filters** (Merck Millipore, Merck KGaA, Darmstadt, Germany),
- **33 mm Millex-HV 0.45 µm PVDF syringe filters** (Merck Millipore, Merck KGaA, Darmstadt, Germany),
- **Mini-sifter** (Scientific Manufacturing Company, JM Quality Services, Cape Town, South Africa),
- **Parafilm: DEMIS flexible packaging** (Demis, USA),
- **Polypropylene 96-Deep well plate** (Axygen Scientific Inc, USA),
- **Polypropylene 2 mL reaction tubes** (Greiner bio-one, Germany),
- **Reagent reservoir for different reagents** (Axygen Scientific Inc, USA),

- **Ultrasonic processor: 400/600-watt** (Sonics and Materials, Newtown, Connecticut, USA),
- **Virtis Genesis 35ES Freeze-dryer** (Virtis-SP, SP Industries, Pennsylvania, USA),
- **Polystyrene flat bottom 96-well plate** (Greiner bio-one, Germany),
- **Whatman filter paper** (Whatman International, Ltd. Maidstone, U K).

4.2.2 Reagents and chemicals

Analytical grade reagents and chemicals were used, except when noted otherwise. HPLC-grade phenolic standards and solvents, as well as enzymes, substrates and standards (positive controls), are listed separately.

General reagents and chemicals

- **Ascorbic Acid** (Sigma-Aldrich, USA),
- **2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) di-ammonium(ABTS)** (Roche, Switzerland),
- **2,2' -Azobis(2-methylpropanimidamide) dihydrochloride (AAPH)** (Sigma-Aldrich, USA),
- **Dimethyl sulphoxide** (Fluka, Sigma-Aldrich, USA),
- **2,2–Diphenyl-2-picrylhydrazyl radical (DPPH•)** (Sigma-Aldrich, USA),
- **Di-Sodium hydrogen phosphate** (May & Baker, England),
- **(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox)** (Sigma-Aldrich, USA),

- **Ethanol (99 %)** (Servochem, South Africa),
- **Fluorescein disodium** (Fluka, Sigma-Aldrich, USA),
- **Folin-Ciocalteu's phenol reagent** (Merck Millipore, Merck KGaA, Darmstadt, Germany),
- **Gallic acid** (Sigma-Aldrich, USA),
- **Hydrochloric Acid (37 %)** (Merck Millipore, Merck KGaA, Darmstadt, Germany),
- **Iron(III) chloride anhydrous** (Riedel-de Haen, Sigma-Aldrich, USA),
- **Methanol** (Merck Millipore, Merck KGaA, Darmstadt, Germany),
- **Phosphate Buffered saline tablets** (Sigma-Aldrich, USA),
- **Potassium dihydrogen phosphate** (Merck Millipore, Merck KGaA, Darmstadt, Germany),
- **Potassium persulphate** (Sigma-Aldrich, USA),
- **Sodium carbonate** (Merck Millipore, Merck KGaA, Darmstadt, Germany),
- **Tris(hydroxymethyl)aminomethane hydrochloride** (Sigma-Aldrich, USA),
- **2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ)** (Merck Millipore, Merck KGaA, Darmstadt, Germany).

HPLC-gradient grade solvents and calibration standards

- **Acetic acid glacial (>99.85 %)** (Fluka, Sigma-Aldrich, South Africa, Cat. Nr: 33209-2.5L),

- **Acetonitrile-CHROMASOLV™ gradient grade for HPLC (>99.9 %)** (Sigma-Aldrich, USA, Cat. Nr: 34851-2.5L),
- **Citric acid (>99.5 %)** (Sigma-Aldrich, USA, Cat. Nr: CO7598009),
- **Eriocitrin (>98 %)** (Extrasynthese, Genay, France, Cat. Nr: 1110 S),
- **Eriodictyol (>99 %)** (Extrasynthese, Genay, France, Cat. Nr: 1111 S),
- **Formic acid (>98-100 %)** (Merck Millipore, Merck KGaA, Darmstadt, Germany, Cat. Nr: 1.00263.2500),
- **Hesperetin (>99 %)** (Extrasynthese, Genay, France, Cat. Nr: 1115 S),
- **Hesperidin (>98.5 %)** (Sigma-Aldrich, USA, Cat. Nr: 1116 S),
- **Luteolin (>99 %)** (Extrasynthese, Genay, France, Cat. Nr: 1125 S),
- **Mangiferin (100 %)** (Sigma-Aldrich, USA, Cat. Nr: M3547),
- **Methanol–Lichro Solv, Gradient grade for liquid chromatography** (Merck Millipore, Merck KGaA, Darmstadt, Germany, Cat. Nr: 106007),
- **Narirutin (>99 %)** (Extrasynthese, Genay, France, Cat. Nr: 1130 S),
- **Naringenin (>99 %)** (Extrasynthese, Genay, France, Cat. Nr: 1128 S).

Enzymes, substrates and inhibitors

- **Acarbose (>95 %)** (Sigma-Aldrich, USA, Cat. Nr: A8980),
- **(+)-Catechin (>98 %)** (Sigma-Aldrich, USA, Cat. Nr: C-1251),
- **α–Glucosidase from Saccharomyces cerevisiae (215 or 120 units/mg protein tested with p-NPG or maltose, respectively, 23 % protein)** (Sigma-Aldrich, USA, Cat. Nr: G0660-750UN),

- **4-Methylumbelliferyl-α-D-glucopyranoside (4MUG) (>99 %)** (Sigma-Aldrich, USA, Cat. Nr: M9766),
- **4-Methylumbelliferyl butyrate (MUB) (>95 %)** (Sigma-Aldrich, USA, Cat. Nr: 19362),
- **para-Nitrophenyl butyrate (pNPB) (>98 %)** (Sigma-Aldrich, USA, Cat. Nr: N9876),
- **4-Nitrophenyl α-D-glucopyranoside (pNPG) (>99 %)** (Sigma-Aldrich, USA, Cat. Nr: N1377),
- **Orlistat (>98 %)** (Sigma-Aldrich, USA, Cat. Nr: O4139),
- **Rat intestinal acetone powder** (Sigma-Aldrich, USA, Cat. Nr: I1630),
- **Type II crude porcine pancreatic lipase: (42 or 133 units/mg protein tested with triacetin or olive oil, respectively, 56 % protein)** (Sigma-Aldrich, USA, Cat. Nr: L3126).

- ***Abbreviations of enzyme assays, controls and concentrations used:***

➢ **AGLC** = α-Glucosidase with colorimetric probe; (+)-Catechin (control: 17.2 μg/mL).

➢ **AGCF** = α-Glucosidase with fluorimetric probe; (+)-Catechin (control: 200 μg/mL).

➢ **RATG** = Rat intestinal acetone powder; Acarbose™ (control: 202 μg/mL).

➢ **LPC** = Lipase with colorimetric probe; Orlistat™ (control: 25 μg/mL).

➢ **LPF** = Lipase with fluorimetric probe; Orlistat™ (control: 25 μg/mL).

➢ **pNPB** = para-Nitrophenyl butyrate.

- **MUB** = 4-Methylumbelliferyl butyrate.
- **pNPG** = 4-Nitrophenyl α-D-glucopyranoside.
- **4-MUG** = 4-Methylumbelliferyl-α-D-glucopyranoside.

4.3 Methods

4.3.1 Harvesting and preparation of plant material

Nine batches of *C. maculata* shoots (needlelike leaves and stems) (approximately 5 - 8 kg/batch) were harvested from a wild population on the farm Welgedacht, near Riversdale in the Southern Cape region of South Africa. Shoots from more than one bush comprised a batch. The thick stems were removed and the shoots from each batch were cut into small pieces (< 3 mm) with a mechanised fodder cutter, mixed thoroughly and split into two sub batches. These were respectively subjected to controlled drying in a forced circulation drying tunnel at 40 °C for 6 hours to a moisture content less than 10 % directly after cutting to obtain unfermented plant material and to high temperature fermentation (oxidation). For fermentation, the cut plant material (1 kg; 53.9 - 56.4 % moisture content) was moistened with water (1 part water to 4 parts plant material), mixed, placed in a stainless steel container, that was then covered with aluminium foil and placed in a laboratory oven at 90 °C for 16 hours. Following fermentation, the plant material was spread out on a drying rack and dried as described for the unfermented plant material. The moisture content of the plant material was determined, using a

Mettler Toledo HR73 Halogen moisture analyzer. The dried plant material (fermented and unfermented) was passed through a 1.4 mm sieve for 1.5 minutes at 190 rpm using a SMC-Mini-sifter to remove coarse pieces of stem.

4.3.2 Preparation of plant extracts

Hot water extracts of the unfermented and fermented sieved plant material were prepared by extracting 40 g of plant material with 400 mL (1:10 m/v) of freshly boiled deionised water. The mixture was placed in the water bath at 93 °C for 30 minutes with mixing every 5 minutes. The extracts were decanted through a tea strainer into a clean Schott bottle and filtered through Whatman #4 filter paper thereafter. The filtrate was to cool down to room temperature in a water bath and the volume was measured. Thereafter, soluble solids content of the infusions was gravimetrically determined by evaporating duplicate aliquots (5 mL) of each filtrate to dryness in the pre-weighed nickel moisture dishes on a steam bath. The moisture dishes were thereafter dried using a laboratory convection oven at 100 °C for 60 minutes. The moisture dishes were cooled to room temperature in a desiccator, weighed and the soluble solid (SS) content calculated thereafter. The remaining filtrate of each sample was freeze-dried in Virtis Genesis 35ES, with condenser set at-45 °C and a vacuum of 100 mTorr. The freeze-dried filtrates were transferred into labelled 24 mL screw cap amber vials, sealed with parafilm and stored in5 litter jars with silica gel added. The freeze-dried powder samples were stored at 4 °C until analysis.

4.3.3 Preparation of stock solutions

For HPLC analysis (DAD and BCD) and all enzyme assays, stock solutions of the samples at 6 mg/mL concentration were used. These were prepared by dissolving 120 mg of the freeze-dried filtrate with 20 mL of deionised water. For total polyphenol and all antioxidant micro-plate assays, (1 mg/mL) stock solutions were used. These were prepared by dissolving 20 mg of the freeze-dried filtrate with 20 mL of deionised water. All aliquots of stock solutions of *C. maculata* samples were stored at -20 °C in 2 mL reaction tubes and defrosted at room temperature on the day of analysis. All assays were performed in triplicate and results were presented as an average with a percentage error less than 5 %. A Biotek Synergy HT micro-plate reader, equipped with Gen5 Secure software, was used for all micro-plate assays. In the following sections the term 'extracts' will be used although it will refer to filtered extracts. Taking the volume of filtrate recovered into account, the extract yield was calculated (g SS/100g leaves).

4.3.4 Phenolic analysis

4.3.4.1 Total polyphenol analysis

The Folin-Ciocalteu method of **Singleton and Rossi (1965)** adapted for micro-palate reader format by **Arthur *et al*. (2011)** was used for determination of total polyphenol content (TPC) of *C. maculata* extracts. Gallic acid was used to prepare a calibration curve ranging in concentration from 1 mg/L to 10 mg/L. The samples stock solutions (1 mg/mL) were diluted (300 µL sample diluted to a final volume of 1000 µL deionised water solution) to obtain absorbance values within

the range of the calibration curve. Twenty microliters each of the gallic acid standards, samples and the assay control (deionised water) were transferred in triplicate into a 96-well polystyrene flat bottomed micro-plate. Folin-Ciocalteu's reagent (10 x diluted; 100 µL) and sodium carbonate solution (7.5 % w/v; 80 µL) were added into the reaction mixture, followed by mixing of the well contents using an Eppendorf MixMate micro-plate shaker. The micro-plates were incubated at 30 °C for 2 hours to allow for development of a blue-color complex resulting from oxidation of the phenolic compounds. The absorbance was measured at 765 nm and the TPC expressed as mg Gallic Acid Equivalents (GAE) per 100 mL extracts.

4.3.4.2 High performance liquid chromatography-diode-array (HPLC-DAD) analysis

To characterise the phenolic composition of the extracts the HPLC-DAD method of **De Beer and Joubert (2010)** was used to quantify major phenolic compounds in the extracts. An aliquot of each sample was defrosted on the day of analysis. One milliliter of each stock solution (6 mg/mL) was mixed with 100 µL of 10 % (w/v) ascorbic acid. Ascorbic acid was added to the calibration standards and defrosted reconstituted extracts to prevent oxidative degradation of the phenolic compounds. Millipore Millex-HV syringe filters (0.45 µm pore-size) with 4 mm and 33 mm diameter were used to filter calibration standards and plant extracts, respectively, before HPLC-DAD analysis. Gradient elution with acetonitrile (solvent A) and 2 % acetic acid (solvent B) at a flow rate of 0.8 ml/min was as

follows: 0-5 min, 20 % A isocratic;5 - 9 min, 20 – 22 % A; 9 - 10 min, 22 % A isocratic; 10 - 15 min, 22 - 23 A; 15 - 18 min, 23 – 60 % A isocratic; 18 - 20 min, 60 – 20 % A; and 20 - 30 min, 20 % A isocratic. The injection volume for extracts was 10 µL and for standards 10 - 20 µL. The concentration range for calibration standards are presented in Table 5. UV-Vis spectra were recorded for all samples from 200 nm to 550 nm.

Table 5 Concentration range of calibration standards injected

Compounds	**Concentration range (µg/mL)**
Mangiferin	0.036 to 9.093
Luteolin	0.001 to 0.350
Eriocitrin	0.007 to 1.719
Narirutin	0.003 to 0.6400
Hesperidin	0.012 to 3.045
Eriodictyol	0.003 to 0.713
Narigenin	0.003 to 0.672
Hesperetin	0.003 to 0.707

Peaks were tentatively identified by comparing retention times and UV-Vis spectra from HPLC-DAD analysis with those of authentic standards. Mangiferin, isomangiferin and luteolin were quantified at 320 nm, and eriocitrin, eriodictyol, naringinin, narirutin, hesperidin and hesperetin were quantified at 288 nm.

A calibration curve (seven dilution series) was set up for all the available authentic standards. Isomangiferin and iriflophenone-3-*C*-β-D-glucoside were quantified in mangiferin and hesperidin equivalence, respectively. Content was expressed as a 100 mg/ 100 g extract.

4.3.5 Determination of antioxidant activity

4.3.5.1 DPPH• scavenging assay

The method of **Rangkadilok *et al*. (2007)** slightly modified by **Arthur *et al*. (2011)** was used for the free radical scavenging activity determination of *C. maculata* samples. DPPH• was prepared by dissolving 5 mg DPPH• in 100 mL methanol. The solution was covered with foil and sonicated for 5 minutes. The DPPH•-methanol solution was measured at 515 nm and then diluted to obtain an absorbance value between 0.68 - 0.71 for assay standardization purposes. Trolox was used to prepare a calibration curve ranging from 1 µg/mL to 10 µg/mL in the reaction volume. Stock solutions (1 mg/mL) of the samples were further diluted with deionized water (250 µL sample diluted to 1000 µL) to obtain absorbance values within the range of the calibration curve (between 40 and 60 % radical scavenging). Thirty microliters each of the Trolox standards, samples and assay control (deionised water) were transferred in triplicate into a 96 deep-well plate. The DPPH• solution diluted to ca 0.7 absorbance value was added (270 µL), followed by sealing of the deep-well plate with a silicone sealing mat to prevent evaporation of methanol. The contents in deep-well plate were then mixed using an Eppendorf MixMate micro-plate shaker for 30 seconds at

1650 rpm. The micro-plates were incubated in a dark cupboard at room temperature for two hours to allow scavenging of $DPPH^{\bullet}$ by the antioxidants, leading to a decrease in absorbance of the free radical solution. Two hundred microliters of the reaction mixture was then transferred into corresponding wells of a 96-well polystyrene flat bottom micro-plate and the absorbance measured at 515 nm. The radical scavenging ability was expressed as µmole Trolox/g extract, obtained from the calibration curve of the standard. The % inhibition caused by the Trolox standards and the sample extracts was calculated as follows:

% inhibition = $(A_C-A_S)/ A_C *100$, where

A_C = absorbance of control

A_S = absorbance of standards or samples.

The % inhibition of the Trolox standard solutions was plotted against their concentration in the reaction mixture, giving a linear regression curve. The calibration curve was then used to calculate the total antioxidant capacity (TAC_{DDPH}) of a sample in terms of Trolox equivalents (µmoles Trolox/g extract).

4.3.5.2 $ABTS^{\bullet+}$ decolourisation assay

Radical scavenging capacity of *C. maculata* extracts was also determined using the $ABTS^{\bullet+}$ decolourisation assay as described by **Re *et al*. (1999)** and modified for micro-plate use by **Venter (2013)**. An $ABTS^{\bullet+}$ stock solution (7 mM) was

prepared in deionized water (75 mg in 25 mL) and further diluted with 440 µL of 140 mM potassium persulfate to generate the radical. The mixture was stored in a dark cupboard at room temperature for 16 hours to allow development of the radical **(Re *et al*., 1999)** prior to the reaction commencement. Trolox (dissolved in methanol) was used to prepare a standard curve, ranging from 50 µM to 300 µM in the final reaction mixture. Stock solutions of the samples (1 mg/mL) were further diluted to give between 40 % and 60 % bleaching of $ABTS^{\bullet+}$. Twenty microliters of each of the Trolox standards, samples and assay control (deionised water) were transferred in triplicate into a 96-well polystyrene micro-plate and 180 µL of $ABTS^{\bullet+}$ reagent added. The contents in the 96-well polystyrene flat bottom micro-plate were mixed in the Biotek Synergy HT micro-plate reader. Following mixing, the reaction mixture was incubated at 30 °C and absorbance measured after 4 minutes at 734 nm. The percentage inhibition was calculated as described for the $DPPH^{\bullet}$ assay. TAC_{ABTS} was expressed as µmole Trolox equivalents/g extract.

4.3.5.3 FRAP assay

The assay by **Benzie and Strain (1996)** was used as adapted by **Venter (2013)** for use in the micro-plate format to evaluate the ferric reducing antioxidant power (FRAP) of C. *maculata* extracts. The FRAP reagent was freshly prepared using 100 mL of 300 mM acetate buffer (pH 3.6), 10 mL of mM TPTZ in 40 mM HCl, and 10 mL of 20 mM iron (III) chloride ($FeCl_3$). A Trolox stock solution of

1.25 mg/mL was prepared in ethanol and further diluted with deionised water to obtain a calibration curve between 50 µM - 500 µM. *S*ample stock solutions (1 mg/mL) were further diluted as required to obtain absorbance values within the range of the calibration curve. Twenty microliters of the blank (deionised water), standards and extract samples were added in triplicate in their allocated wells in a 96-well micro-plate, followed by the addition of the FRAP reagent. The increase in absorbance due to formation of a coloured TPTZ-Fe^{2+} complex was monitored spectrophotometrically at 593 nm, after a reaction time of 4 minutes at 37 °C. A linear regression plot, prepared for the calibration standard solutions was used to calculate the TAC_{FRAP} value of each sample, expressed as µmole Trolox equivalents/g extract.

4.3.5.4 ORAC assay

To determine oxygen radical absorbance capacity of *C. maculata* aqueous extracts, a method by **Huang *et al*. (2002)** was adapted as modified for use in micro-plate by **Joubert and De Beer (2012)** where 8 x 10^{-2} mM fluorescein disodium solution in 75 mM potassium-phosphate buffer (pH 7.4) was used. AAPH (153 mM) in potassium-phosphate buffer was used as a source of peroxyl radical **(Werber *et al*., 2011)**. Trolox (0.125 mg/mL in methanol) was used to prepare a calibration curve ranging from 5 µM to 30 µM. The stock solutions (1 mg/mL) of the samples were further diluted with deionised water to a final soluble solid concentration of 0.6 - 0.8 µg/mL for optimum inhibition capacity of peroxyl-radical-induced oxidation within the range of the calibration curve. The

Trolox standards, samples and the assay control (deionised water) (25 µL) were each transferred in triplicate into a 96-well of a clear-bottom black micro-plate, and 150 µL of the fluorescein working solution was added to the reaction. The 96-well micro-plate was then incubated in the Biotek Synergy HT micro-plate reader at 37 °C for 10 minutes, and 153 mM AAPH (25 µL) was added to the reaction mixture prior to measurement of fluorescence. The loss of fluorescent intensity was measured in the micro-plate reader over a period of 35 minutes. Excitation was performed at 485 nm with 20 nm band pass and emission was measured at 530 nm with 25 nm band pass. The fluorescence intensity was determined using Gen5™ Data Analysis Software to obtain area under the curve (AUC) and Net AUC (AUC samples – average AUC assay control). A calibration curve, where the Net AUC was plotted against the µmoles Trolox in the reaction volume, was used to quantify the activity of each extract. The activity was thus expressed as µmoles Trolox equivalents/100g soluble solids.

4.3.6 Enzyme inhibition assays

4.3.6.1 Lipase inhibition assay for anti-obesity activity

4.3.6.1.1 Micro-plate colorimetric assay

The method by **Slanc *et al*. (2009)**, was adapted to evaluate pancreatic lipase inhibitory activity of *C. maculata* extracts in the micro-plate. A solution of 3 mg/mL type II crude porcine pancreatic lipase was prepared by dissolving 75 mg of the enzyme in 25 mL deionised water at 37 °C. The crude enzyme solution

was incubated at 37 °C for 30 minutes to extract the enzyme. Millipore Millex-HV syringe filters (0.45 µm pore size) with 4 mm diameter were used to filter the enzyme solution. The appropriate concentration for optimal activity was determined as an absorbance of 0.9 at 405 nm from a range of lipase concentrations (0.1 – 3 mg/mL) after incubation for 20 minutes. Thereafter, the lipase solution was diluted accordingly for the determination of inhibitory activity of the extracts. *para*-Nitrophenyl butyrate (pNPB) (3.33 mM) **(Kim *et al*., 2012)** was used as a substrate for this assay. A stock solution of 2 mg/mL Orlistat™ was prepared and used as positive control at a fixed concentration (25 µg/mL) per well. The composition of the reaction mixture per well (without the substrate) was: 20 µL of plant extract (200 µg/mL), 150 µL of 75 mM Tris-buffer (pH 8.5) and 65 µL of enzyme solution. The reaction mixture was incubated at 37 °C for 15 minutes after which 15 µL of 3.33 mM pNPB was added by the dispenser at the Biotek Synergy HT micro-plate reader and the absorbance was read over 30 minutes at 405 nm. The difference in absorbance between start-point and end-point (t_0 and t_{max}, respectively) of the reaction was used as the measure of the reaction that has taken place. Values for sample blanks, substituting water for the substrate, were subtracted to take absorbance of the sample into account. The Net absorbance and the % inhibition caused by Orlistat™ and the sample extracts were calculated as follows:

Net Abs = $Abs_{tmax} – Abs_{t0}$

% inhibition = $(A_C-A_S)/ A_C *100$, where

A_C = absorbance of control

A_S = absorbance of standards or samples

Abs_{tmax} = Absorbance values at time of maximum

Abs_{t0} = Absorbance values at the start of reaction

4.3.6.1.2 Micro-plate fluorimetric assay

The methods by **Andlauer *et al.* (2009)** and **Slanc *et al*. (2009)** were adapted to micro-plate format. A substrate solution was prepared by dissolving 15 mg of 4-methylumbelliferyl butyrate (MUB) in 10.125 mL DMSO. HPLC grade deionised water was added to the substrate solution to obtain a total volume of 15 mL. A solution of 3 mg/mL type II crude porcine pancreatic lipase, prepared as described in 4.3.6.1.1 was used. The appropriate concentration for optimal activity was determined from a dilution range of lipase concentrations (0.1 - 3 mg/mL). The excitation wavelength was 360 nm with 40 nm band pass while emission was measured at 460 nm with 40 nm band pass. A fluorescence reading between 70000 to 80000 after 30 minutes was selected as optimal and the lipase solution was diluted accordingly for the determination of inhibitory activity of the extracts. The samples were analysed at a final concentration of 480 µg/mL. The composition of the reaction mixture in a micro-well was: 20 µL of control, sample or standard, 125 µL potassium phosphate buffer (pH 7.4), 65 µL lipase solution, (replacing lipase with deionised water for blanks) and 40 µL MUB. Orlistat™ (25 µg/mL) was used as a positive control and to standardise activity between different plates and experimental days. The fluorescence emitted at

460 nm after excitation at 365 nm was recorded over 60 minutes. The values of initial fluorescence (t0) were subtracted from the values at the time of maximum fluorescence (tmax; 4min) to obtain the Net fluorescence and the % inhibition of each sample and control was calculated bellow:

Net Fluorescence (Net FL) = $Fluorescence_{tmax}$– $Fluorescence_{t0}$

% inhibition = 100 x {1 – (Net FL_{sample} – Net FL_{blank})/Net $FL_{control}$}

% activity = 100 - % inhibition.

4.3.6.2 Alpha-glucosidase inhibition for anti-diabetes

4.3.6.2.1 Micro-plate colorimetric assay

For the anti-diabetic potential of *C. maculata,* yeast α-glucosidase inhibitory activity of the extracts was investigated using a method adapted from **Kim *et al*. (2010)**, as described by **Muller *et al*. (2012)**, for micro-plate format. A stock solution of, approximately 2 U/mL α-glucosidase was prepared by using cold HPLC grade deionized water (ca. 4.4 mg in 100 mL). The enzyme solution was stored at 20 °C and defrosted in the refrigerator overnight prior to the experimental day. The substrate, 3 mM *para*-nitrophenyl α-D-glucopyranoside (pNPG), was prepared on the experimental day (18 mg substrate in 20 mL deionised water). +(-)Catechin (17 µg/mL per well) was used as a positive control (a stock solution prepared by dissolving 2.18 mg in 25 mL deionised water). The substrate was replaced with dH_2O for the sample blanks and the sample was

replaced with deionised water for the assay control and reaction mixture. The activity of the enzyme was determined by preparing different dilutions of the stock solution with cold HPLC grade deionised water within activity range from 0.25 to 2 U/mL. The absorbance at 405 nm was monitored during incubation at 37 °C. The dilution that resulted in absorbance between 0.7 to 0.8 after 10 minutes of incubation was selected for sample analysis. A mixture of 200 µg/mL sample, 200 mM potassium phosphate buffer and selected concentration of α-glucosidase, at 20 µL each, was incubated at 37 °C in the Biotek Synergy HT micro-plate reader. After 15 minutes incubation, 40 µL pNPG was added to the reaction mixture. For the assays, absorbance at 405 nm was monitored over 30 minutes. To obtain the net absorbance values, the values of initial absorbance were subtracted from the values of absorbance at the end of the reaction (t0 and t10, respectively). The net absorbance values were used as measure of the reactions that took place during the time over which the assay was monitored. Values for sample blanks, substituting water for the substrate, were subtracted to take absorbance of the sample into account. The net absorbance and the percentage inhibition was calculated as follows:

Net Abs = Abs_{t10} - Abs_{t0} and

% inhibition = $(A_C-A_S)/ A_C *100$, where

A_C = Net absorbance of control

A_S = Net absorbance of standards or samples.

4.3.6.2.2 Micro-plate fluorimetric assay with yeast α-glucosidase

The method by **Azuma *et al.* (2011)** was adapted with minor modifications to comply with micro-plates suitable for enzyme inhibition activity assays. A stock solution of 2 U/mL yeast α-glucosidase was prepared as described in section 4.3.6.2.1. A substrate solution was prepared by dissolving 10.65 mg of 4-methylumbelliferyl-α-D-glucopyranoside (4-MUG) in 2 mL dimethyl sulphoxide (DMSO). Potassium phosphate (200 mM; pH 6.8) was later added to the substrate solution adding up to a total volume of 25 mL. The activity of the enzyme was determined as described for the colorimetric assay. The appropriate concentration for optimal activity was determined based on the fluorescence emitted at 460 nm with 40 nm band pass after excitation at 360 nm with 40 nm band pass. A fluorescence reading between 70000 to 80000 after 60 minutes was selected as optimal activity and α-glucosidase solution was diluted accordingly for the determination of inhibitory activity of the extracts. The extracts were analysed at a final concentration of 500 µg/mL. Catechin (200 µg/mL) was used as a positive control and to standardise activity between different plates and experimental days. The composition of the reaction mixture in a micro-well was similar to that used for the colorimetric assay: 20 µL of 500 µg/mL sample/control/standard, 20 µL potassium phosphate buffer (pH 6.8), 20 µL α-glucosidase solution, (replaced with potassium phosphate for blanks). The micro-plate was incubated at 37 °C in the Biotek Synergy HT micro-plate reader. After 15 minutes incubation, 40 µL of the substrate (4-MUG) was added to each well. The fluorescence emitted was measured at 460 nm after excitation at

360 nm was recorded over 60 minutes. Percentage inhibition of each sample and control was calculated in the following way:

Net Fluorescence (Net FL) = $Fluorescence_{tmax}$– $Fluorescence_{t0}$

Where tmax = 50 min

% inhibition = 100 x {1 – (Net FL_{sample} – Net FL_{blank})/Net $FL_{control}$}

% activity = 100 - % inhibition.

4.3.6.2.3 Micro-plate fluorimetric assay with rat intestinal acetone powder

The method by **Sancheti *et al.* (2011)** was adapted with minor modifications. The same experimental procedure, conditions and sample concentration as described in section 4.3.6.2.2 were used. However, a stock solution of enzyme was prepared by extracting 350 mg of rat intestinal acetone powder with 10 mL 200 mM potassium phosphate (pH 6.8). The extraction process included a sonication sequence of 12 x 30 seconds with 1 minute resting intervals using the Ultrasonic processor. The solution was centrifuged at 10 000 x g for 30 minutes in a Hettich Universal 320R centrifuge (rotor 1615) at 4 °C. The supernatant was filtered with Durapore™ 0.45 µm pore size PVDF syringe filters and kept on ice until analysis. Acarbose™ (200 µg/mL in a well) was used as a positive control and to standardise activity between different plates and experimental days. The reaction mixture per well consisted of 40 µL sample (500 µg/mL)/control/ standard, 20 µL potassium-phosphate buffer (pH 6.8) and 60 µL α-glucosidase solution, (replaced with potassium phosphate for blanks)

was incubated at 37 °C in the Biotek Synergy HT micro-plate reader before 80 µL of the substrate (3 mM 4-MUG) was added to the wells. The net fluorescence and percentage inhibition of extracts and standards were calculated in the following way:

Net Fluorescence (Net FL) = $Fluorescence_{tmax}$– $Fluorescence_{t0}$

Where tmax = 20 min

% inhibition = 100 x {1 – (Net FL_{sample} – Net FL_{blank})/Net $FL_{control}$}

% activity = 100 - % inhibition.

4.3.7 On-line BCD assays

4.3.7.1 Antioxidant assays

4.3.7.1.1 On-line HPLC (DPPH•, $ABTS^{•+}$ and FRAP).

The HPLC system used to identify active compounds responsible for antioxidant activity of *C. maculata* was described in section 4.2.1. Post-column mixing of the HPLC effluent and the radical solution (DPPH•, $ABTS^{•+}$ or FRAP), at an additional flow rate of 0.5 mL/min, was achieved with a high pressure static mixing tee. The reaction coil was made of 15.24 m PEEK tubing (0.25 mm i.d.) resulting in a reaction time of ca. 0.6 minutes.

The DPPH• stock solution (58 mg/L) was prepared in acetonitrile on the day of analysis and kept in a flask protected from light. The working solution, containing

250 mL of a 0.068 mM citric acid phosphate buffer (6.8 mL of 0.01 M citric acid added to 93.2 mL of 0.02 M Na_2HPO_4 and made up to 1000 mL with deionized water) and 750 mL of DPPH in acetonitrile was filtered with Milipore 0.45 µm PVDF-filter by vacuum before use.

The $ABTS^{\bullet+}$ stock solution (7 mM) was prepared as described by **Pelligrini *et al*. (2003)**. A working solution was obtained by mixing 25 mL 7 mM $ABTS^{\bullet+}$ stock solution with 1000 mL of 75 mM potassium phosphate (pH 7.4). The 1 L solution was filtered with Milipore 0.45 µm PVDF-filter by vacuum before use.

The FRAP reagent was prepared as described for the micro-plate FRAP assay (section 4.3.5.3), the mixture contained 100 mL of 300 mM acetate buffer (pH 3.6), 10 mL of mM TPTZ in 40 mM HCl, and 10 mL of 20 mM iron (III) chloride ($FeCl_3$). However, the volumes were adjusted as required for a total volume of 1 L and filtered with Milipore 0.45 µm PVDF-filter by vacuum before use.

The reagents ($DPPH^{\bullet}$, $ABTS^{\bullet+}$ and FRAP) working solutions were kept in the thermostatically controlled cooling bath at 4 °C during analysis. The samples were injected in duplicate at 10 µL and a standard mix of mangiferin and isomangiferin was injected in triplicate at 10 µL. Trolox was used as an internal standard (0.25 mg/mL for $DPPH^{\bullet}$, 1.25 mg/mL for $ABTS^{\bullet+}$ and FRAP) to align the chromatograms and the biograms. Scavenging of $DPPH^{\bullet}$, $ABTS^{\bullet+}$ was detected

as negative peaks at 515 nm, 600 nm, respectively, whereas ferric reduction (FRAP) was detected as positive peaks at 593 nm.

Quantitative on-line ($ABTS^{\bullet+}$ and $DPPH^{\bullet}$) analysis of selected samples was done by injecting 10 µL of the extract of each sample in triplicate. The response of mangiferin and isomangiferin over the expected concentration range was determined in triplicate. For $ABTS^{\bullet+}$ assay, the compounds were injected at a concentration range between 0.114 to 1.364 µg/µL mangiferin and 0.046 to 0.554 µg/µL isomangiferin. In the $DPPH^{\bullet}$ assay, the concentrations between 0.23 to 4.09 µg/µL mangiferin and 0.09 to 1.66 µg/µL isomangiferin were injected. Due to equipment downtime the quantitative analysis of the later compounds could not be performed with the FRAP assay. The response of Trolox was also determined over a concentration range of 0.08 to 1.25 µg/µL for the $ABTS^{\bullet+}$ assay and 0.17 to 3.3 µg/µL for the $DPPH^{\bullet}$. This was not only used to align chromatograms and biograms but also to calculate TEAC values.

4.3.7.2 Enzyme inhibition assays

4.3.7.2.1 Lipase inhibition

In the search of compounds with lipase inhibitory activity from *C. maculata extracts*, a method by **C. J Malherbe, ARC Infruitec-Nietvoorbij, Stellenbosch** (Figure 13; chapter 2) was employed. For the lipase inhibition assay, the HPLC system was coupled with on-line post-column detection of enzyme inhibitors

(Malherbe *et al*., 2012). A stock solution of 3 mg/mL type II crude porcine pancreatic lipase was prepared in 37 °C deionised water on the experimental day and 0.42 mg/mL MUB (in DMSO-water) as prepared in section 4.3.6.1.2 was used as substrate. The enzyme solution was kept at 4 °C during the experiment to prevent degradation **(Granon and Semeriva, 1980)**. Fifty microliters of samples (6 mg/mL) was injected to the HPLC-BCD system. Orlistat™ was used as a positive control and to align chromatograms taking into account the delay in post-column reaction. HPLC effluant was combined with make-up buffer (potassium phosphate: 75 mM (pH 7.4) and 1.32 g/L Tween 20) in a 1:1 ratio using a high pressure mixing tee, Tween 20 was added to prevent secondary binding of enzyme to the reaction coil. The HPLC effluent-makeup buffer mixture was then split using a micro-splitter where 100 µL/minutes was diverted towards the (bio)chemical detection system. To this diverted flow, the enzyme solution was added using a mixing tee, by dual syringe pump at 50 µL/minute and the combination was pre-incubated through a 2 m pre-coil at 37 °C. Immediately after pre-incubation, the substrate solution was added using a mixing tee, by dual syringe pump at 50 µL/minute and the combined mixture passed through 4 m reaction coil at 37 °C. The fluorimetric reaction was recorded at 445 nm emission after excitation at 365 nm. Enzyme inhibition would be indicated as negative peaks.

4.3.7.2.2 α-Glucosidase inhibition

To identify α-glucosidase inhibitors from *C. maculata extracts*, the same equipment and procedure as described in 4.3.7.2.1 was followed. However, 2 U/mL yeast α-glucosidase prepared in 37 °C deionised water was used as enzyme and 0.9 mg/mL pNPG was used as a substrate. The enzyme and substrate solutions were kept at 4 °C during the experiment to prevent degradation **(Unsworth *et al*., 2007)**. Extracts (6 mg/mL) were analyzed using 50 µL injection volume. (+)-Catechin was used as a positive control and to align chromatograms taking into account the delay in post-column reaction. For the same reason as for lipase inhibition assay, the HPLC effluant was combined with make-up buffer (potassium phosphate: 200 mM (pH 6.8) and 1.32 g/L Tween 20) in a 1:1 ratio using a high pressure mixing tee. The colorimetric reaction was recorded by VWD at 405 nm and enzyme inhibitors would have been observed as negative peaks.

4.4 Data and statistical analysis

Data generated by Gen5 Secure software for micro-plates and Chemstation software for HPLC (DAD and BCD) was imported into Microsoft Excel (version 2011) for analysis. Data are expressed as mean of triplicate experiments ± standard deviation. Statistical significant differences between treatments (fermented and unfermented) and the correlation of HPLC with bioactivity data (antioxidant and anti-diabesity) were determined by subjecting the data to *t*-Test analysis of variance (ANOVA) using General Linear Models (GLM) of SAS®

software (Version 9.2, SAS institute Inc, Carry, USA). The correlations were conducted using Pearson methods. A p-value of less than 0.05 ($P < 0.05$) was considered to be statistically significant. A qualitative and quantitative assessment of active peaks on HPLC- DAD-BCD chromatograms were also performed. Principal component analysis (PCA), based on the correlation matrix, was conducted using XLStat (Version 7.5.2, Addinsoft, New York, USA) to determine correlations between attributes and to visualize and elucidate the relationships between the samples and their attributes.

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Chapter 5
Results

5.1 Introduction

Various micro-plate assays were employed to evaluate antioxidant and anti-diabesity potential of aqueous extracts of unfermented and fermented *C. maculata*. Several antioxidant assays were employed to quantify the total antioxidant capacity of the extracts, while anti-diabesity potential was assessed in terms of the inhibition of enzymes relevant to digestion. Characterisation of the phenolic composition of extract entailed quantification of total polyphenol content and the major individual phenolic constituents. On-line HPLC-(bio)chemical methods were employed to gain further insight into the activity of the extracts.

5.2 Characterisation of the phenolic composition of *C. maculata*

The hot water soluble solids content of unfermented and fermented shoots and leaves of *C. maculata* were 19 and 16 %, respectively. The soluble solids content of the plant material decreased on average by 16 %, but large variation, was observed for individual batches, ranging from 11 to 27 % (Table 6).

Table 6. Hot water soluble solids content (SSC) of unfermented and fermented *C. maculata* plant material.

Sample coding	Unfermented	Fermented	% decrease
1	20.70	15.22	27
2	18.40	16.38	11
3	17.57	15.68	11
4	19.62	16.72	15
5	16.99	15.02	12
6	19.16	16.23	15
7	18.61	16.03	14
8	19.51	14.27	27
9	17.57	15.70	11
Average	**19**	**16**	**16**

SSC is expressed as g/100 g plant material. % Decrease: Reduction in soluble solid content of *C. maculata* extracts due to fermentation.

The freeze-dried, hot water extracts were then analysed for their total phenolic content (TPC) at a concentration of 0.3 mg/mL. Gallic acid, most commonly used as standard **(Prior *et al*., 2005)**, was also used as standard in this case to quantify the TPC of *C. maculata* extracts. A very good correlation between the gallic acid concentration and absorbance (R^2 = 0.9997), measured at 765 nm, was observed when using the method of **Singleton and Rossi (1965)**, adapted for use in micro-plate assay format (Addendum; Figure A1). The method was therefore deemed suitable to quantify the TPC of *C. maculata* extracts.

The total polyphenol content values of unfermented extracts varied between 20.7 and 26.0 g GAE/100 g extract, observed for sample 4 and sample 1, respectively. The TPC values of the fermented sampled ranged between 13.9 and 16.7 g GAE/100 g extract (Figure 19), observed for sample 6 and sample 7, respectively. The average total polyphenol content was significantly lower after fermentation ($P < 0.0001$), decreasing from 23.5 to 15.3 g GAE/100 g extract (Table 7). The percentage decrease in TPC of all individual batches exceeded

30 % (Table 8) due to fermentation, accompanied by a decrease in activity of the extracts observed in all measured parameters.

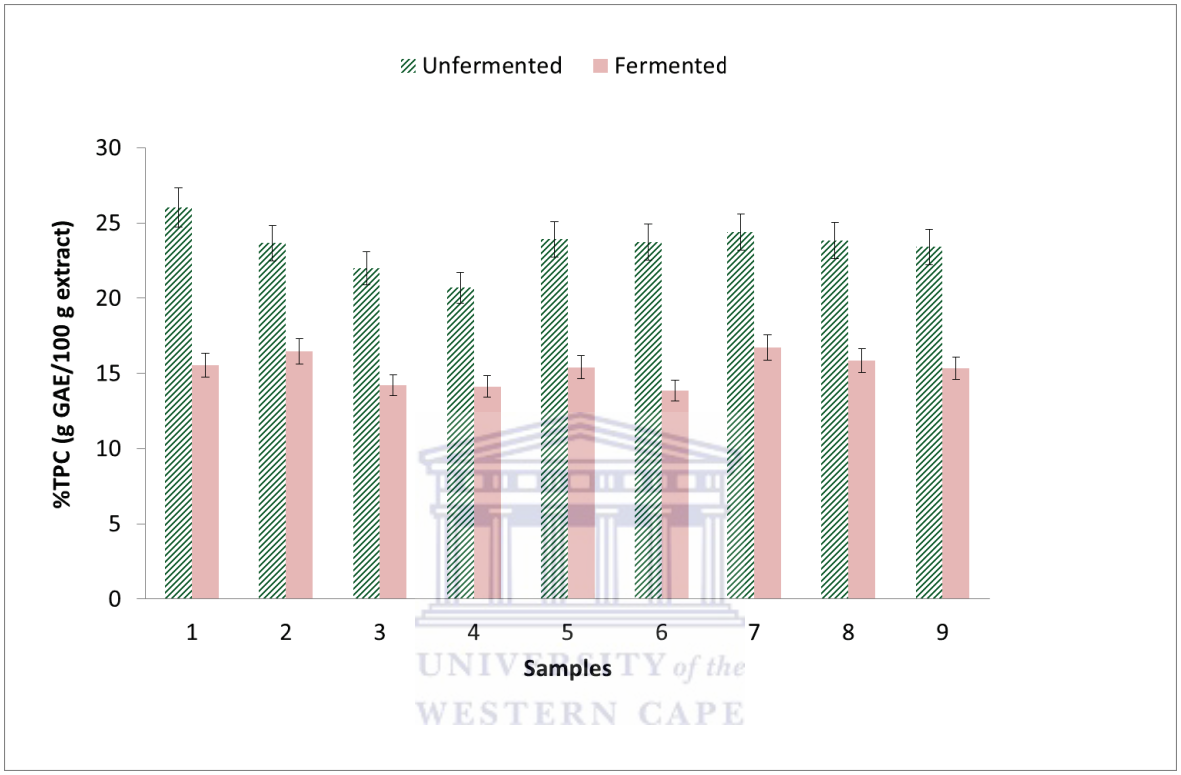

Figure 19. Total polyphenol content (TPC) of aqueous extracts unfermented and fermented *C. maculata* (n = 9) determined by the Folin–Ciocalteau Reagent (FCR) method. TPC is expressed in terms of gallic acid equivalents (GAE).

Table 7. ANOVA of total polyphenol content (TPC) and antioxidant activities (DPPH, FRAP, ORAC and ABTS) of extracts of unfermented and fermented *C. maculata*.

Assay	Units	Unfermented[a,b]	Fermented[a,b]	P value
TPC	g GAE/100 g	23.50 ± 1.49	15.28 ± 1.02	p < 0.0001
DPPH	TAC (µmoles TE/g extract)	1632 ± 213	927 ± 142	p < 0.0001
ABTS	TAC (µmoles TE/g extract)	2301 ± 120	1517 ± 126	p < 0.0001
ORAC	TAC (µmoles TE/g extract)	6808 ± 597	4393 ± 215	p < 0.0001
FRAP	TAC (µmoles TE/g extract)	1078 ± 61	612 ± 48	p < 0.0001

[a]Total antioxidant capacity expressed as µmoles Trolox equivalence/g extract and TPC as gallic acid equivalence/100 g extract; [b] Data are mean ± SD where n = 9.

Table 8. Percentage change of parameters as measured for individual samples of *C. maculata* extracts as affected by fermentation.

Batch	TPC	DPPH	ABTS	ORAC	FRAP	LPC	LPF	AGLC	AGCF	RATGL
1	40	49	39	40	45	41	9	28	16	13
2	30	28	41	37	36	-15	4	50	8	9
3	35	60	37	38	49	5	19	14	18	16
4	32	42	32	40	42	31	-9	-14	17	14
5	36	42	26	41	45	14	5	58	7	11
6	42	47	25	20	52	8	9	106	-6	14
7	32	43	39	32	39	-3	14	83	34	9
8	33	35	38	35	38	25	10	33	4	9
9	34	35	30	32	42	0	24	74	30	12
Average	**35**	**43**	**34**	**35**	**43**	**12**	**9**	**48**	**14**	**12**

Abbreviations: TPC (total polyphenol content), DPPH, ABTS, ORAC, FRAP (antioxidant micro-plate assays), LPC (lipase inhibition with colorimetric probe), LPF (lipase inhibition with fluorimetric probe), AGLC (yeast α-glucosidase inhibition with colorimetric probe), AGCF (yeast α-glucosidase inhibition with fluorimetric probe), RATGL (rat α-glucosidase inhibition with fluorimetric probe).

HPLC analysis was also conducted to quantify individual phenolic compounds present in the extracts of unfermented and fermented *C. maculata*. The individual compounds were identified according to retention time and UV-Vis spectra, compared to those of authentic standards (Figure 20). Typical HPLC chromatograms of unfermented and fermented *C. maculata* extracts at 288 and 320 nm are depicted in Figure 21a,b. Absorbance was measured at two wavelengths to take into account the different type of phenolic compounds, with flavanones and benzophenones giving higher absorbance at 288 nm, while xanthones (mangiferin and isomangiferin) have higher absorbance at 320 nm **(De Beer and Joubert, 2010; Malherbe *et al.*, 2014)**. The HPLC chromatographic

fingerprints of unfermented and fermented *C. maculata* were similar, except that peak areas were smaller for fermented *C. maculata*.

Major phenolic compounds identified were iriflophenone-3-*C*-β-D-glucoside, mangiferin, isomangiferin, hesperidin and eriocitrin. The contents of these compounds in the respective samples are depicted in Figure 20. The xanthones, mangiferin and its regio-isomer, isomangiferin, were shown to be present at the highest quantities in both the aqueous extracts of unfermented and fermented *C. maculata*. The mangiferin levels varied from 4421 to 6357 mg/100 g extract (samples 4 and 3, respectively) in the unfermented batch of extracts and from 740 to 1560 mg/100 g extract (samples 4 and 7) in the fermented batch of extracts. Isomangiferin levels were lower, i.e. in the unfermented extracts varying from 1363 to 1879 mg/100 g extract and 650 to 984 mg/100 g extract of the fermented extracts.

Much lower quantities of iriflophenone-3-*C*-β-D-glucoside, eriocitrin and hesperidin (< 900 mg/100 g extract) were present in all extracts. Of these compounds, the eriocitrin and hesperidin contents were the lowest and highest, respectively. The unfermented extracts contained significantly more of the xanthones, benzophenone and flavonoids than the fermented extract ($P < 0.0001$; Table 9). Mangiferin and iriflophenone-3-*C*-β-D-glucoside contents decreased by more than 70 % due fermentation, while a decrease of ≥ 40 % was observed for isomangiferin, eriocitrin and hesperidin (Table 9).

For the wavelengths selected to quantify the major peaks, the HPLC chromatograms do not show the presence of new compounds such as polymers that could have formed during fermentation. Chromatograms at 420 and 450 nm were also used (Figure 21b) to detect brown polymers **(Cilliers *et al*., 1990; Theron, 2012)**. The HPLC chromatogram at 450 nm showed no defined peaks, while the xanthones, benzophenone and flavanones, absorbing at 288 and 320 nm, still gave detectable peaks at 420 nm, even at very low absorbance units. Interesting to note was that the "hump" starting to elute at 10 minutes also gave very low absorbance at 420 and 450 nm. The "hump" considered to be brown polymeric compounds was estimated in both type of extracts on the basis of peak area at 450 nm for retention time window (RTW) of 10 to 20 minutes. Absorbance at 450 nm was chosen as no peaks of the individual compounds (eriocitrin and hesperidin) that was visible at 420 nm, were observed (Figure 21b).

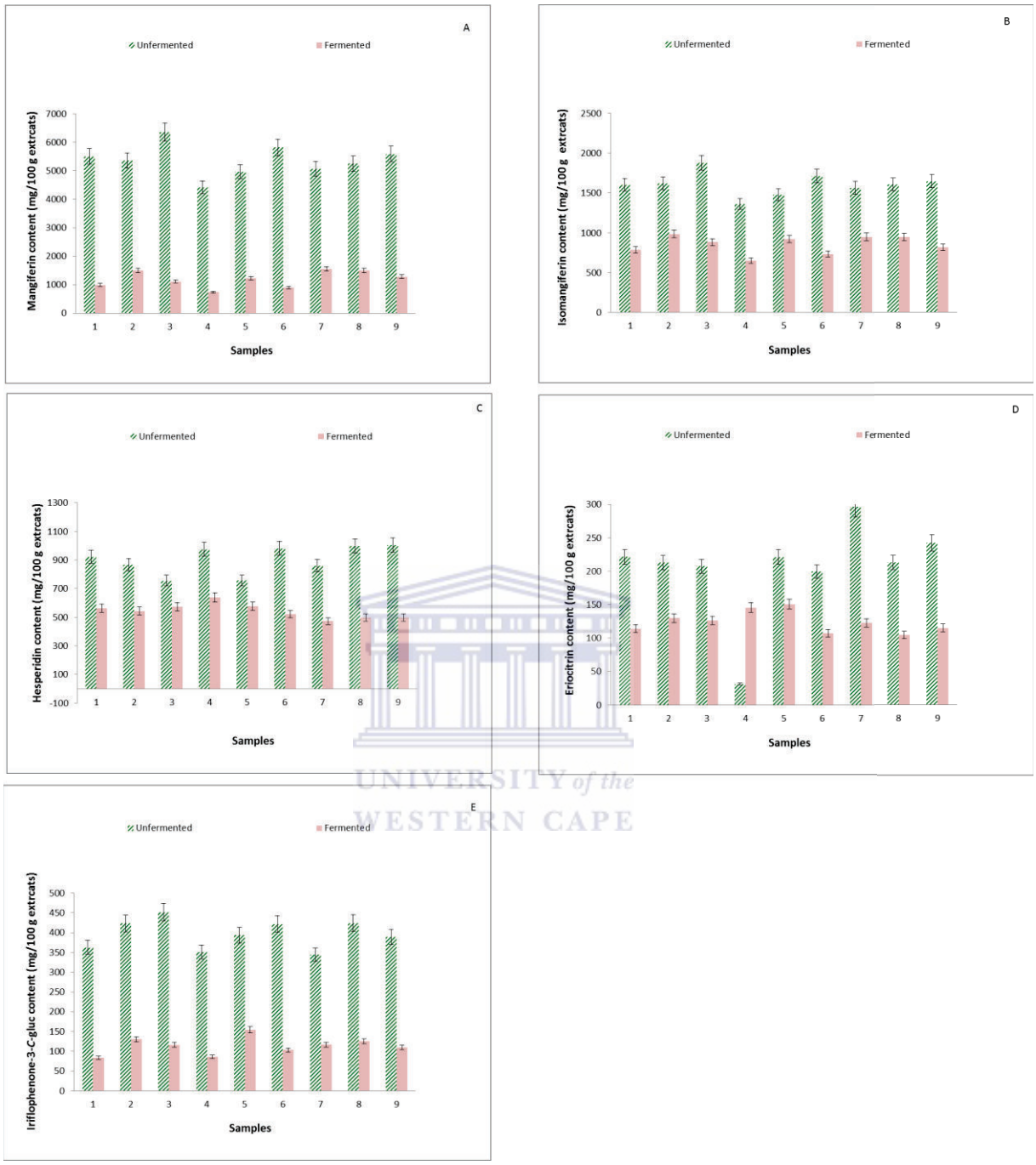

Figure 20. Levels of phenolic compounds present in aqueous extracts of unfermented and fermented *C. maculata* (n = 9) as quantified by HPLC at 288 and 320 nm. Data expressed in mg/100 g extract.

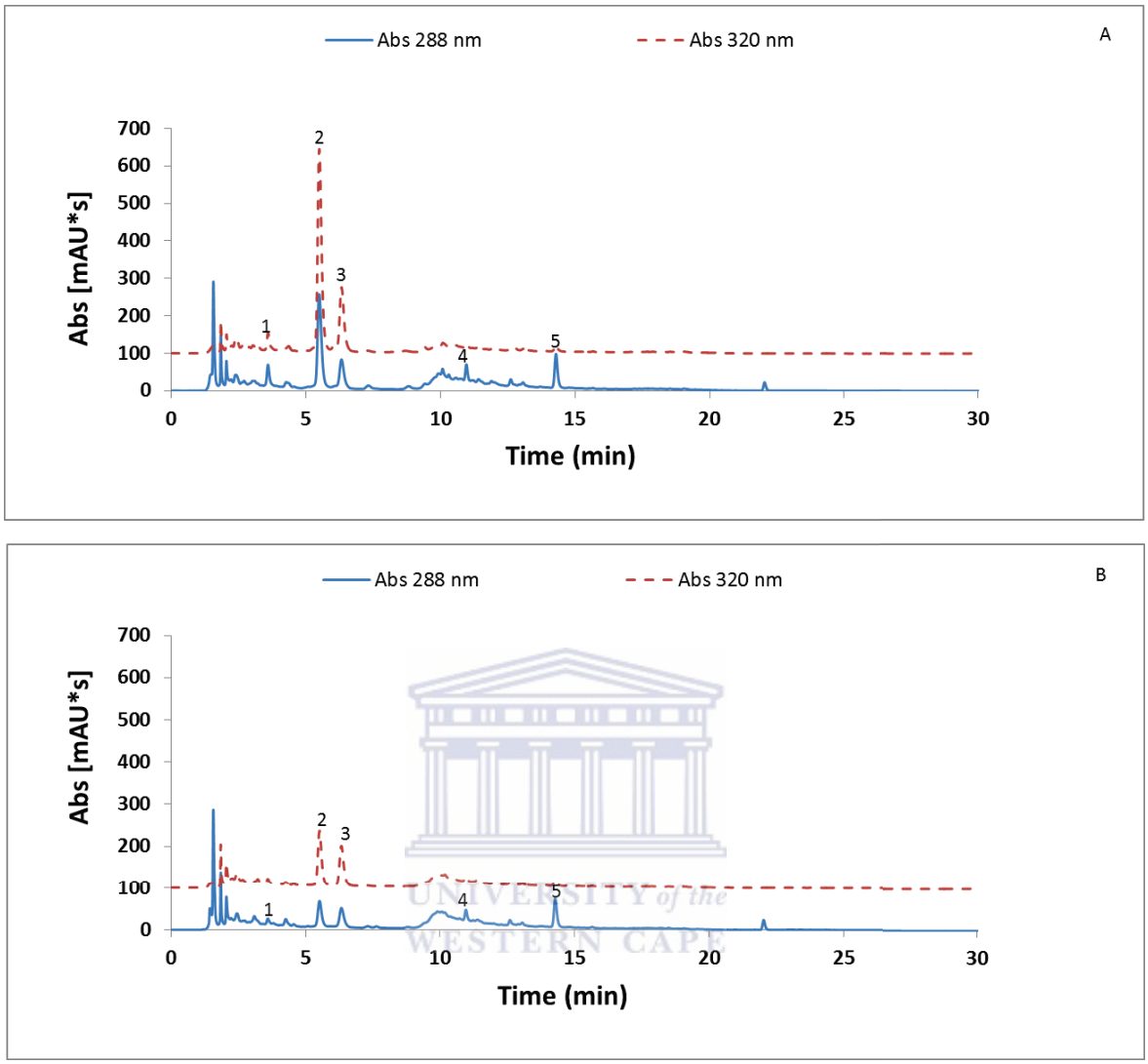

Figure 21a. HPLC diode-array chromatogram of an unfermented (A) and fermented (B) *C. maculata* extract (sample 5) at 288 and 320 nm (**1**: iriflophenone-3-*C*-β-D-glucoside, **2**: mangiferin, **3**: isomangiferin, **4**: eriocitrin, **5**: hesperidin). The chromatograms at 320 nm are slight off-set for illustrative purposes. For both 10 µL of a 5.5 mg/mL stock solution of the extract was injected.

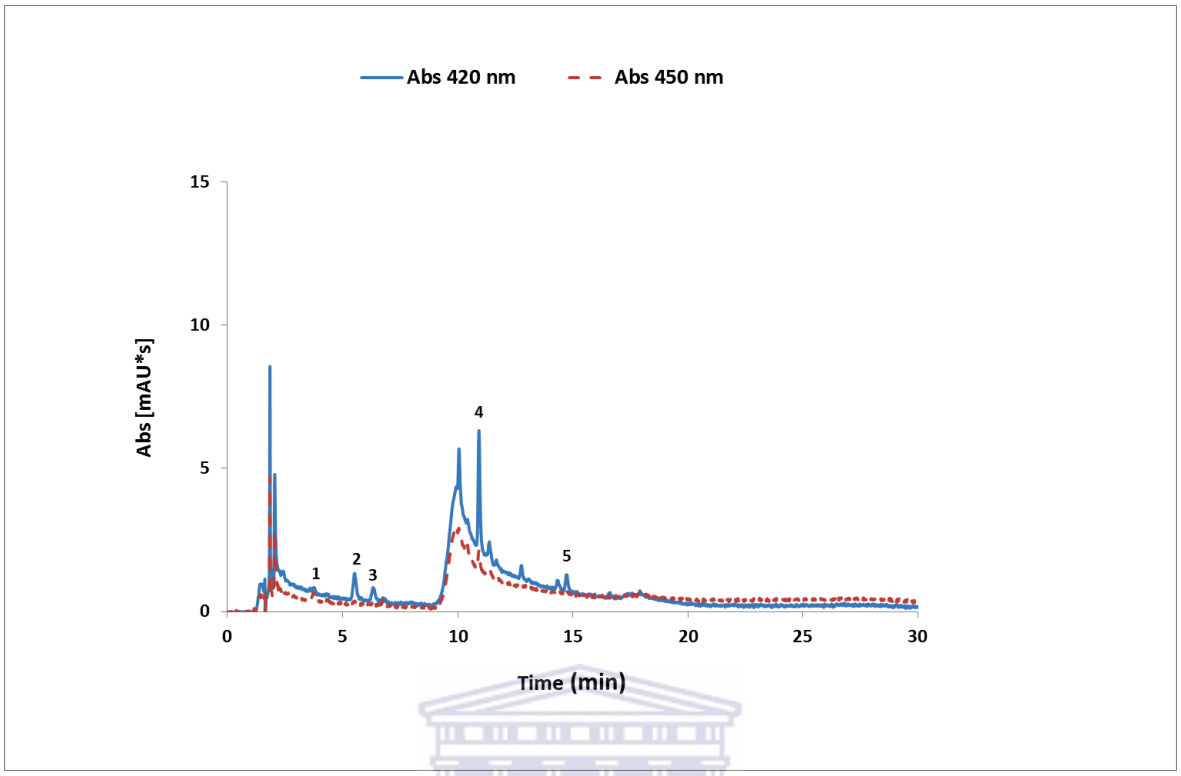

Figure 21b. HPLC diode-array chromatogram of an extract of fermented *C. maculata* (sample 5) at 420 and 450 nm (**1**: iriflophenone-3-*C*-β-D-glucoside, **2**: mangiferin, **3**: isomangiferin, **4**: eriocitrin, **5**: hesperidin). For both 10 µL of a 5.5 mg/mL stock solution of the extract was injected.

Table 9. ANOVA of individual phenolic compounds and polymers as quantified by HPLC-DAD, and changes in contents as affected by fermentation.

Compound	Unfermented[a,b]	Fermented[a,b]	% Change [C]	P value
Mangiferin	5374 ± 548	1202 ± 292	-77	p < 0.0001
Isomangiferin	1608 ± 144	852 ± 113	-47	p < 0.0001
Iriflophenone-3-*C*-β-D-glucoside	396 ± 37	114 ± 22	-71	p < 0.0001
Eriocitrin	230 ± 30	124 ± 16	-46	p < 0.0156
Hesperidin	903 ± 98	543 ± 51	-40	p < 0.0001
Polymers[d]	284 ± 30	504 ± 36	78[c]	p < 0.0001

[a] Content expressed as mg/100 g extract, except for the polymers ; [b] Data are mean ± SD where n = 9. [c] Increase only observed for polymers. [d] Polymers indicate the fraction absorbing at 450 nm and eluting between retention times 10 and 20 minutes. Content expressed as the peak area in mAU*s. The superscript +/- indicate the increase or decrease in levels of compounds.

5.3 Total antioxidant capacity of fermented and unfermented *C. maculata*

The total antioxidant capacity (TAC) of *C. maculata* extracts was determined using multiple micro-plate methods to allow a more comprehensive evaluation as a single method cannot accurately reflect all the antioxidants in a complex mixture **(Prior *et al*., 2005; Apak *et al*., 2007)**. For this reason methods measuring the scavenging of radicals ($DPPH^{\bullet}$, $ABTS^{\bullet+}$ and peroxyl) were employed. The FRAP assay was employed to measure ferric-to-ferrous reduction. All methods used Trolox as standard so that results could be directly compared **(Zucca *et al*., 2013)**.

5.3.1 $DPPH^{\bullet}$ Assay

Scavenging of $DPPH^{\bullet}$ by Trolox as measured by a decrease in absorbance at 515 nm, showed a good linear relationship (R^2 = 0.9998; Addendum; Figure A2) over a concentration range of 5 to 40 µM in the reaction mixture. The samples were analyzed at final concentrations of 15 and 25 µg/mL for extracts of unfermented and fermented *C. maculata*, respectively. The $DPPH^{\bullet}$ scavenging activity of individual *C. maculata* extracts is shown in Figure 22. Sample 5 showed the highest TAC_{DPPH} values (1786 µmoles TE/g extract) from the unfermented group of extracts and sample 8 was the most active sample after fermentation (1117 µmoles TE/g extract). The lowest TAC_{DPPH} values were observed for sample 2 and 3 for unfermented and fermented extracts, respectively. The unfermented and fermented extracts showed average TAC_{DPPH}

values of 1632 and 927 µmoles TE/g extract, respectively (Table7), with an intra-batch variation (%RSD) of 12 and 14 %, respectively.

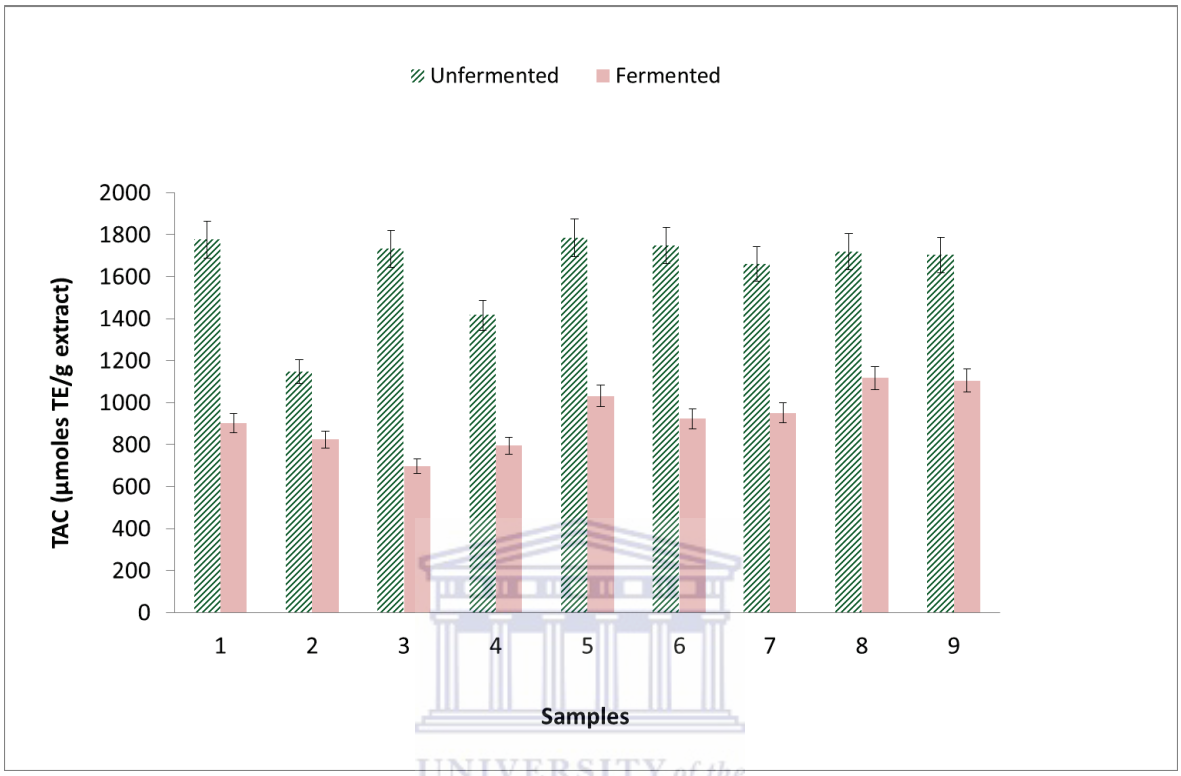

Figure 22. DPPH• scavenging activity of aqueous extracts of unfermented and fermented *C. maculata* (n = 9). Total antioxidant capacity (TAC) is expressed as µmoles Trolox equivalents (TE)/g extract.

The decrease in DPPH• scavenging with fermentation was highly significant ($P < 0.0001$; Table 7) with the percentage decrease observed for individual samples varying between 28 for sample 2 and 60 % for sample 3 (Table 8).

5.3.2 ABTS•+ Assay

Reaction of ABTS•+ with Trolox as measured by a decrease in absorbance at 734 nm showed a good linear relationship ($R^2 = 0.9991$; Addendum; Figure A3) over a concentration range of 50 to 300 µM in the reaction mixture. The samples

were analysed at final concentrations of 80 and 100 µg/mL for extracts of unfermented and fermented *C. maculata*, respectively. As shown in Figure 23, significant $ABTS^{\bullet+}$ quenching activity was found for all samples, ranging from 2488 to 2094 µmoles TE/g extract for unfermented samples and 1758 and 1360 µmoles TE/g extract for fermented samples. Samples with the highest TAC_{ABTS} values in the two respective groups were samples 2 and 5, while the samples with the lowest TAC_{ABTS} values were 6 and 7, respectively. The average TAC_{ABTS} values for extracts of unfermented and fermented *C. maculata* were 2301 and 1517 µmoles TE/g extract ($P < 0.0001$; Table 7), respectively, with an intra-batch variation (%RSD) of 5 and 8 %, respectively. The TAC_{ABTS} of individual batches decreased between 25 and 41 % (Table 8).

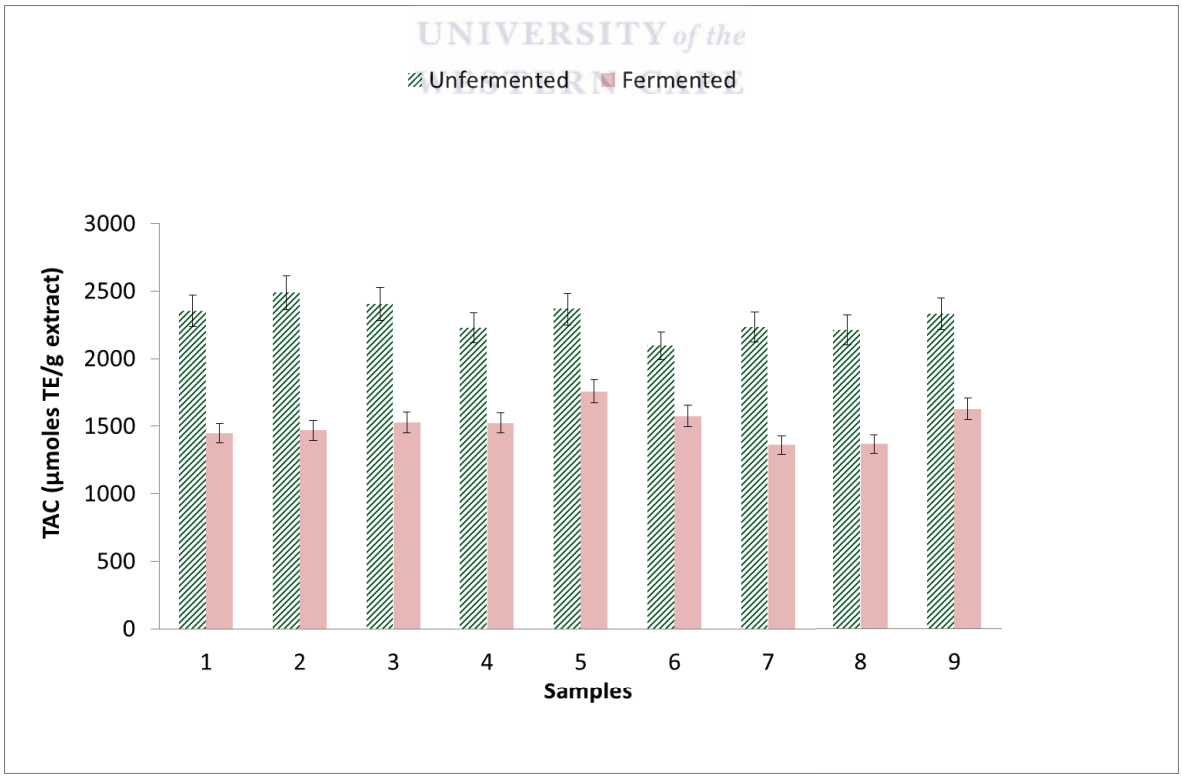

Figure 23. $ABTS^{\bullet+}$ scavenging activity of aqueous extracts of unfermented and fermented *C. maculata* (n = 9). Total antioxidant capacity (TAC) is expressed as µmoles Trolox equivalents (TE)/g extract.

5.3.3 ORAC Assay

For the ORAC assay, the fluorescence intensity was measured at 530 nm after excitation at 485 nm. The net AUC *versus* Trolox concentration was linear over a concentration range of 3 to 30 µM (in a reaction mixture) with a R^2 value of 0.9992 (Addendum; Figure A4). In this case the analysis was performed at final soluble solid concentrations of 0.6 and 0.8 µg/mL for the unfermented and fermented extracts of *C. maculata* in the reaction mixtures, respectively. Data presented in Figure 24 showed that samples 3 and 8 showed the highest TAC_{ORAC} values and samples 6 and 1 showed the lowest TAC_{ORAC} values for the unfermented and fermented extracts, respectively. TAC_{ORAC} values for unfermented *C. maculata* extract, ranged from 5349 to 7337 µmoles/g extract. Lowest and highest TAC_{ORAC} values for fermented extract were 4075 and 4679 µmoles/g extract, respectively. The TAC_{ORAC} values of different batches differed with an intra-batch variation (%RSD) of 9 % and 5 % for the unfermented and fermented batches, respectively. The average TAC_{ORAC} values for unfermented *C. maculata* extracts were significantly ($P < 0.0001$) higher than that of the fermented *C. maculata* (Table 7), decreasing by 35 %, while TAC_{ORAC} values of individual batches decreased between 20 and 41 % (Table 8).

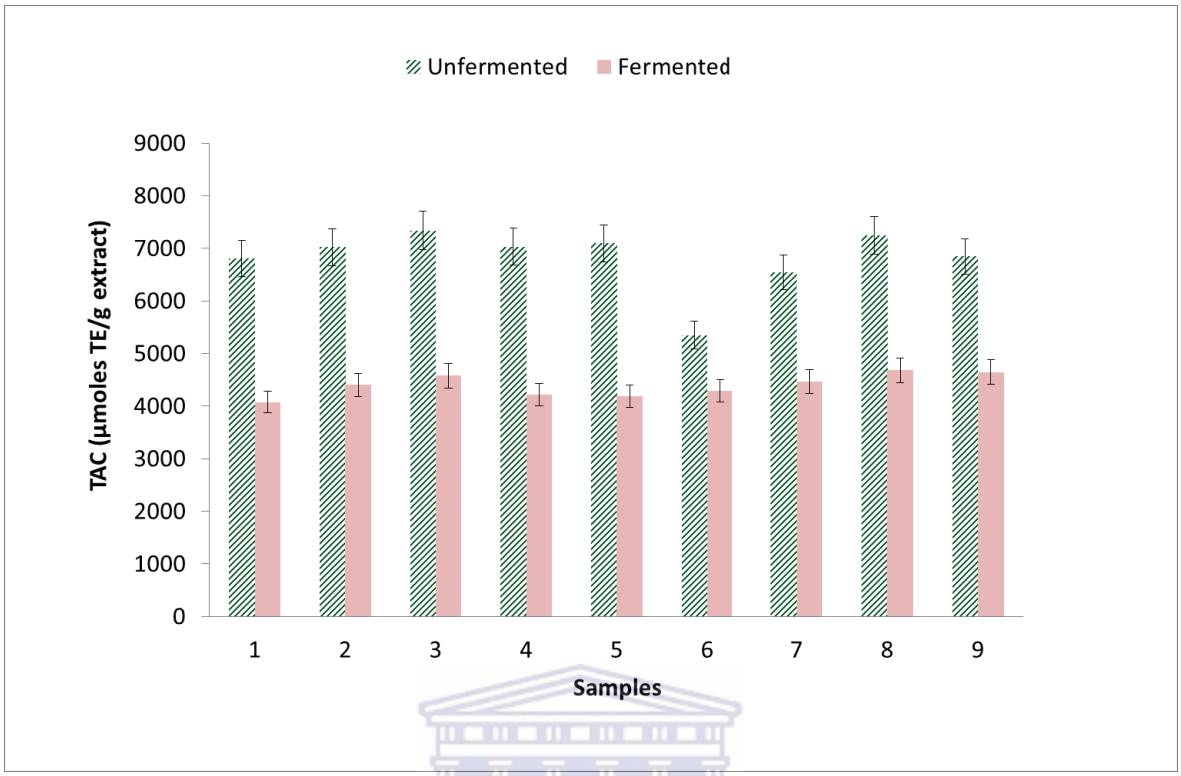

Figure 24. ORACscavenging activity of aqueous extracts of unfermented and fermented *C. maculata* (n = 9). Total antioxidant capacity (TAC) is expressed as µmoles Trolox equivalents (TE)/g extract.

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5.3.4 FRAP Assay

The calibration curve of Trolox with concentrations ranging from 50 to 500 µM (in the reaction mixture) versus absorbance measured at 593 nm showed a linear relationship for ferric reduction with increasing Trolox concentrations (R^2 = 0.9998; Addendum; Figure A5). The FRAP assay was performed at concentrations of 15 and 20 µg/mL of the unfermented and fermented extracts in the reaction mixture, respectively. As indicated in Figure 25, all the extracts (n = 18) showed substantial total antioxidant capacity with the FRAP assay. Samples 3 and 8 showed the highest TAC_{FRAP} values and samples 4 and 6 showed the lowest TAC_{FRAP} values for the unfermented and fermented extracts, respectively.

TAC_{FRAP} values for unfermented *C. maculata* extract, ranged from 1013 to 1220 µmoles/g extract, and for fermented extracts from 520 to 677 µmoles/g extract. An intra-batch variation (%RSD) of 6 % was observed for the unfermented extracts, which increased to 8 % after fermentation. The average TAC_{FRAP} values were significantly higher ($P < 0.0001$) for unfermented extracts than that of fermented extracts. Fermentation decreased the average TAC_{FRAP} value by 43 % (Table 8), with TAC_{FRAP} values of individual batches decreasing between 36 and 52 % (Table 8).

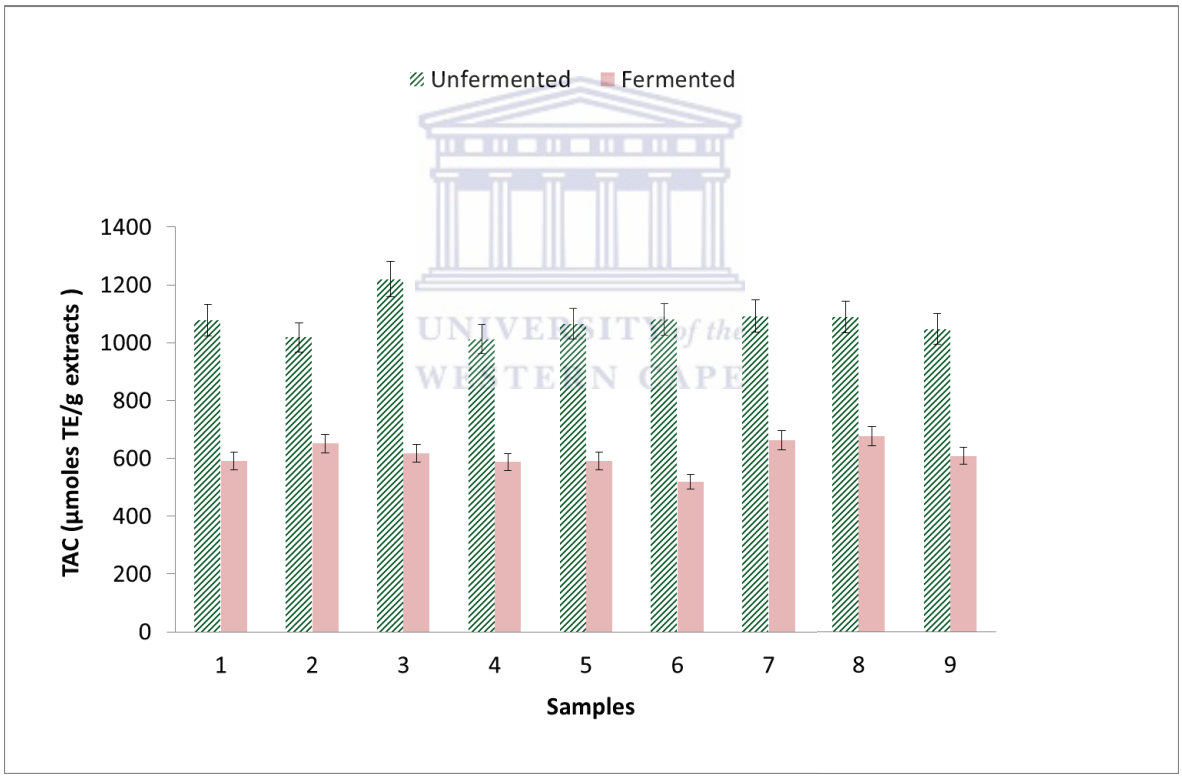

Figure 25. Ferric reducing ability of aqueous extracts of unfermented and fermented *C. maculata* (n = 9) determined by FRAP method. Total antioxidant capacity (TAC) is expressed as µmoles Trolox equivalents (TE) /g extract.

5.3.5 Identification of antioxidants using on-line HPLC DPPH•, ABTS•+ and FRAP assays

The on-line HPLC DPPH•, ABTS•+ and FRAP assays were used to identify major phenolic compounds with antioxidant activity (indicated by the biograms) without the need for isolation of compounds prior to the analysis. Based on the results obtained from the micro-plate antioxidant assays, the samples that showed the highest TAC values for these assays were selected to evaluate relative radical scavenging activity of individual compounds present in aqueous extracts of *C. maculata.* The same amount of extract was injected for the unfermented and fermented *C. maculata* extracts (10 µL of a 6 mg/mL stock solution) to allow direct comparison. In all of the on-line HPLC antioxidant assays except for FRAP, ascorbic acid were included in the reaction mixture for stability of the phenolic compounds during analysis, while Trolox™ was used in all cases for standardisation purposes. The selected samples for analysis (from the unfermented batch) included sample 5 (U5; DPPH•), sample 2 (U2; ABTS•+) and sample 3 (U3; FRAP). Results are depicted in Figures 26A, 27A and 28A. A number of peaks were observed on the UV-HPLC chromatograms of these samples. However, only the major compounds (mangiferin and isomangiferin) showed noticeable scavenging of DPPH•, ABTS•+, depicted by negative peaks as a result of decolourisation, and reduction of FRAP, depicted as positive peaks as a result of colour formation (as indicated on the biograms). Hesperidin (compound 5) and iriflophenone-3-*C*-β-D-glucoside (compound 1) showed no detectable antioxidant activity in all 3 assays used. It is clear from the biograms

for $ABTS^{\bullet+}$ scavenging and iron reduction that an ill-defined area for the retention time window (RTW) from ca 9 to 14 minutes ($RTW_{9\text{-}14}$) is present. This activity peak on the biogram was more pronounced for the FRAP biogram than the $ABTS^{\bullet+}$ biogram. The $DPPH^{\bullet}$ biogram showed very little scavenging activity for this retention time window.

The counterpart samples (i.e. the fermented sample, F5, F2 and F3) were also subjected to HPLC-(bio)chemical detection (Figures 26B, 27B, and 28B) for qualitative comparison. The peak areas for active compounds on the biograms were lower than that observed for the unfermented samples, as could be expected from their lower content in the fermented samples. The peak area for $RTW_{9\text{-}14min}$ was also smaller for the fermented samples on both the $ABTS^{\bullet+}$ and FRAP biograms (Figure 29).

Figures 26C, 27C and 28C show similar UV chromatograms and biograms for other samples, irrespective of relative sample activity (as per micro-plate assays) or fermentation state. No additional peaks or areas of antioxidant activity were observed. Given these observations further analysis of the data was carried out for all samples analysed by on-line HPLC-DAD-(bio)chemical detection to gain more insight.

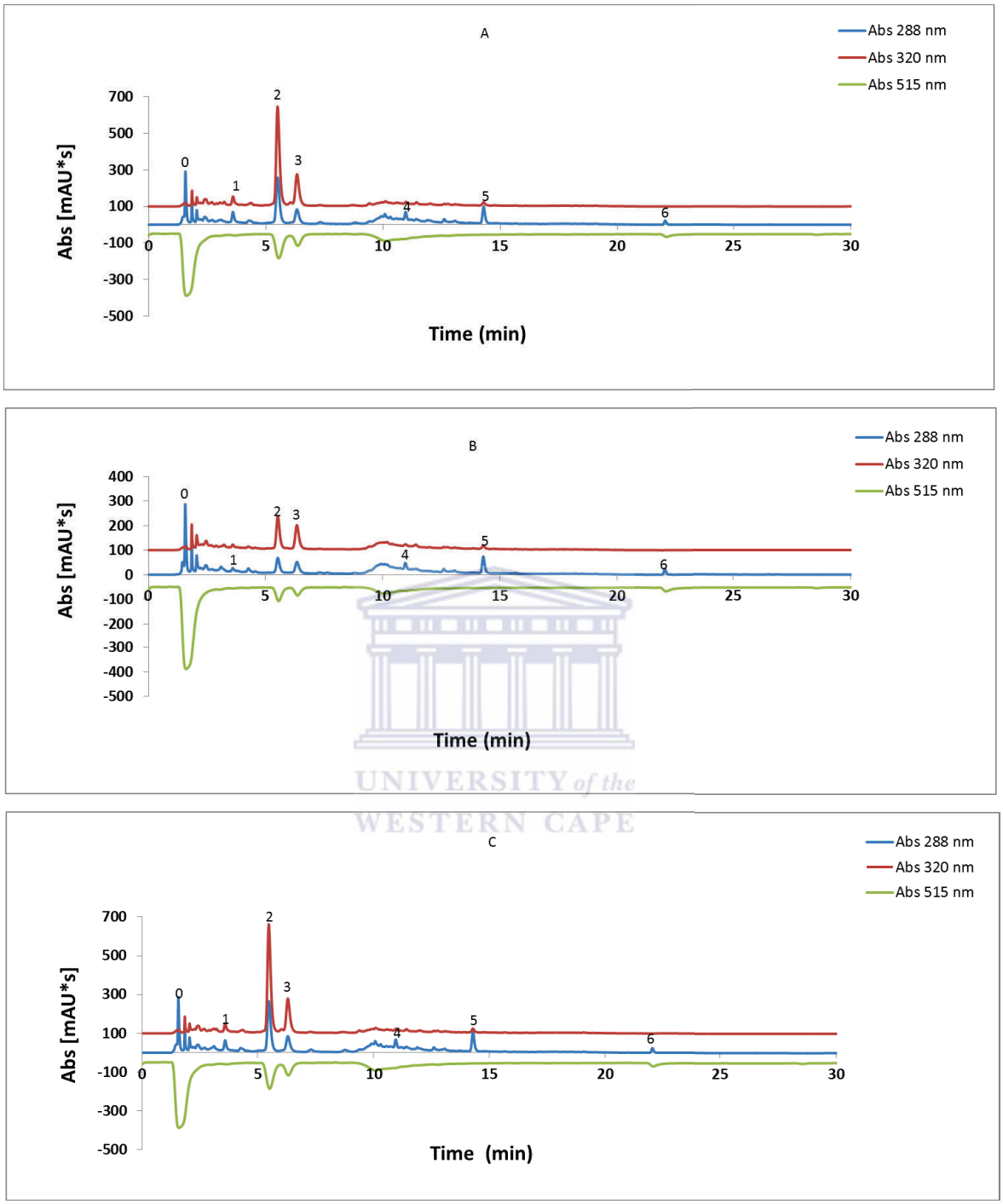

Figure 26. Combined UV chromatograms and DPPH• on-line biograms of (A) the most active unfermented sample (i.e. U5), (B) its fermented counterpart (i.e. F5) and (C) the least active unfermented sample (U2) of aqueous extracts of *C. maculata.* UV absorbance for detection of the major phenolic compounds was measured at 288 and 320 nm. Scavenging of DPPH• was measured at 515 nm, showing a negative peaks in the biogram. (**0**: ascorbic acid (antioxidant stabilizer in extract solution), **1**: iriflophenone-3-*C*-β-D-glucoside, **2**: mangiferin, **3**: isomangiferin, **4**: eriocitrin, **5**: hesperidin, **6**: Trolox (internal standard).

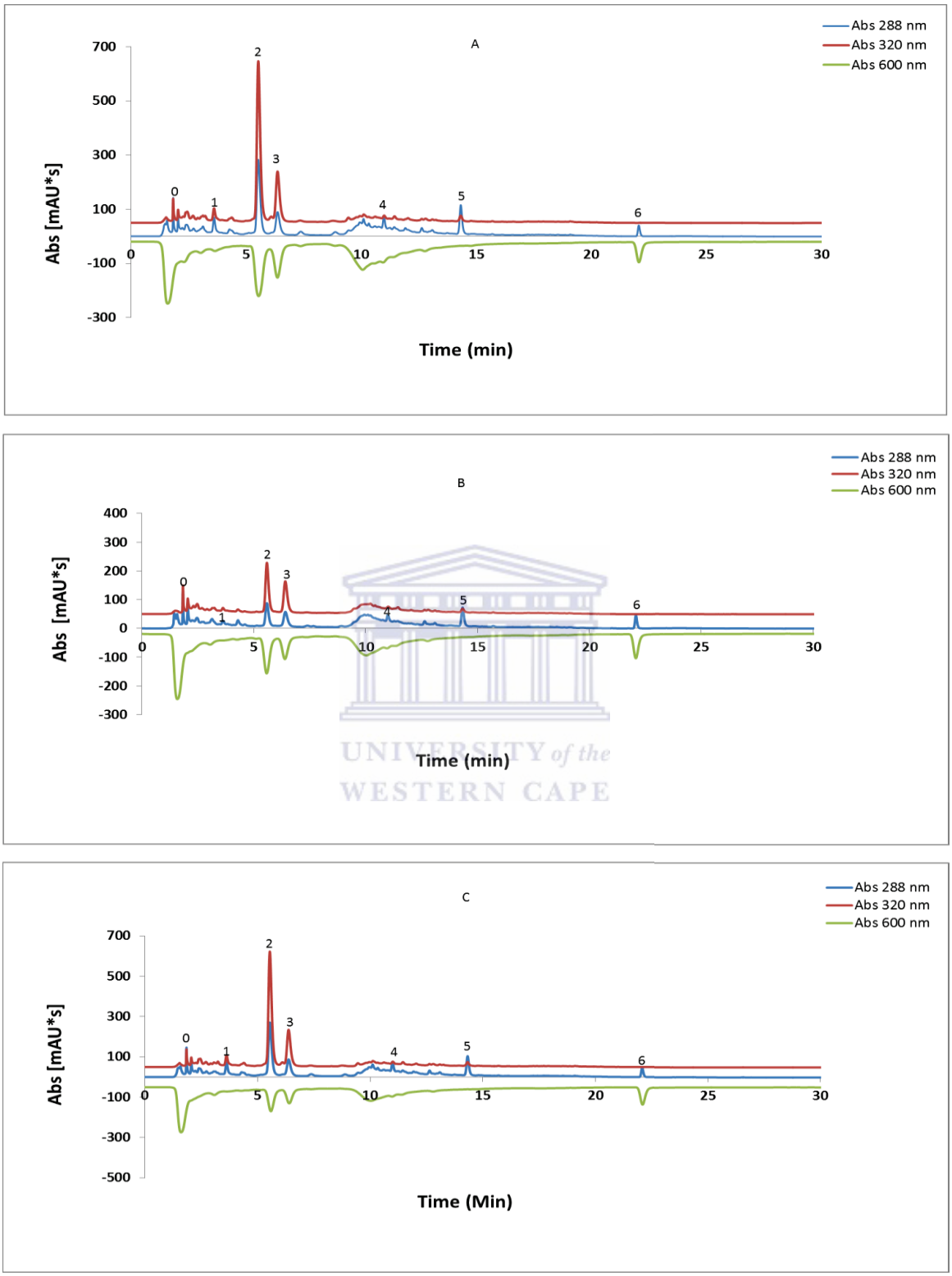

Figure 27. Combined UV chromatograms and ABTS$^{\bullet+}$ on-line biograms of (A) the most active unfermented sample (i.e. U2), (B) its fermented counterpart (i.e. F2) and (C) the most active fermented sample (F5) of aqueous extracts of *C. maculata*. UV absorbance for detection of the major phenolic compounds was measured at 288 and 320 nm. Scavenging of ABTS$^{\bullet+}$ was measured at 600 nm, showing negative peaks in the biogram. (**0**: ascorbic acid (antioxidant stabilizer in extract solution), **1**: iriflophenone-3-*C*- β-D-glucoside, **2**: mangiferin, **3**: isomangiferin, **4**: eriocitrin, **5**: hesperidin, **6**: Trolox (internal standard).

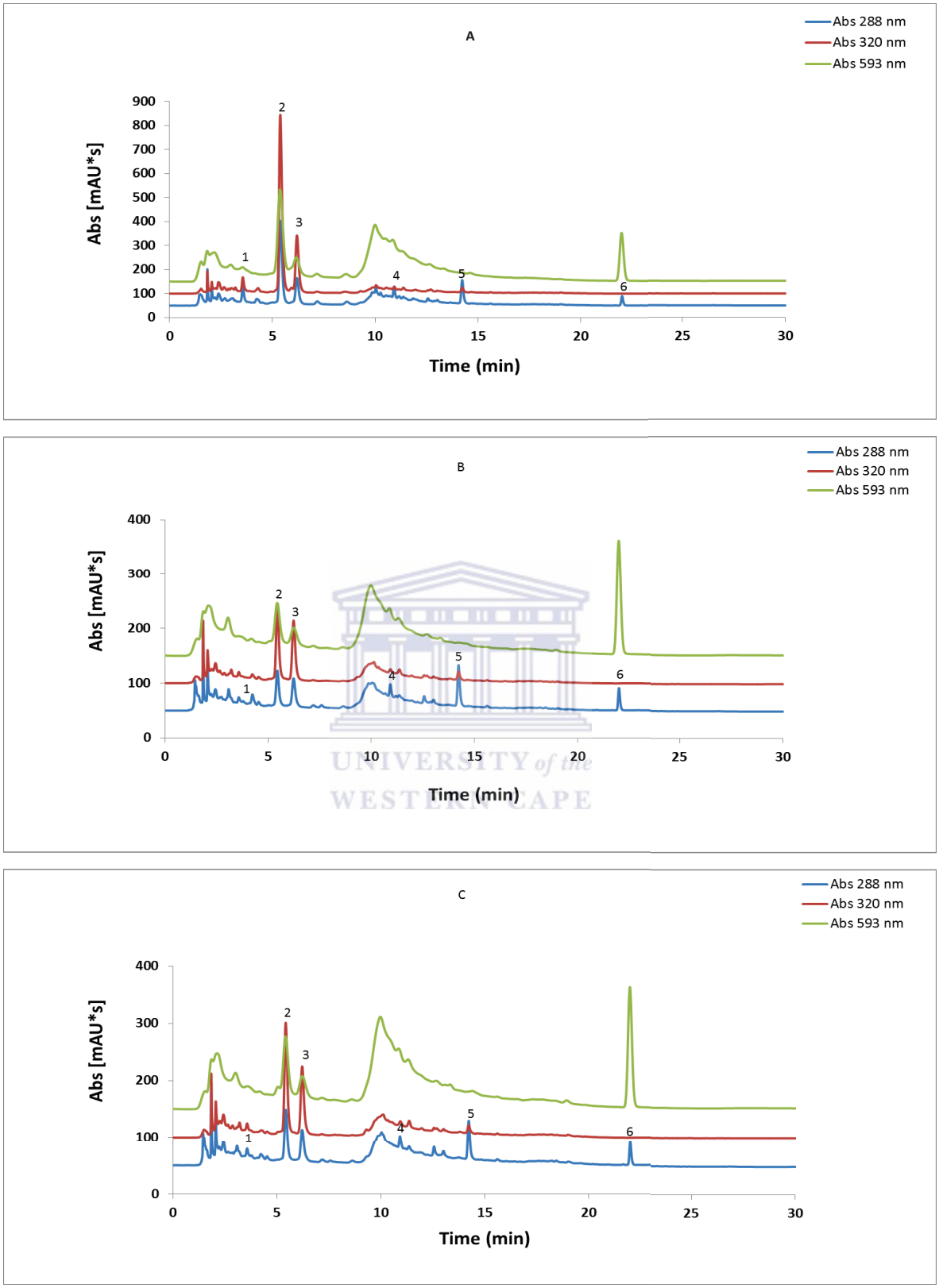

Figure 28. Combined UV chromatograms and FRAP on-line biograms of (A) the most active unfermented sample (i.e. U3), (B) its fermented counterpart (i.e. F3) and (C) the most active fermented sample (F9) of aqueous extracts of *C. maculata*. UV absorbance for detection of the major phenolic compounds was measured at 288 and 320 nm. Reduction of FRAP was measured at 593 nm, showing positive peaks as the UV chromatograms. **1**: iriflophenone-3-*C*- β-D-glucoside, **2**: mangiferin, **3**: isomangiferin, **4**: eriocitrin, **5**: hesperidin, **6**: Trolox (internal standard).

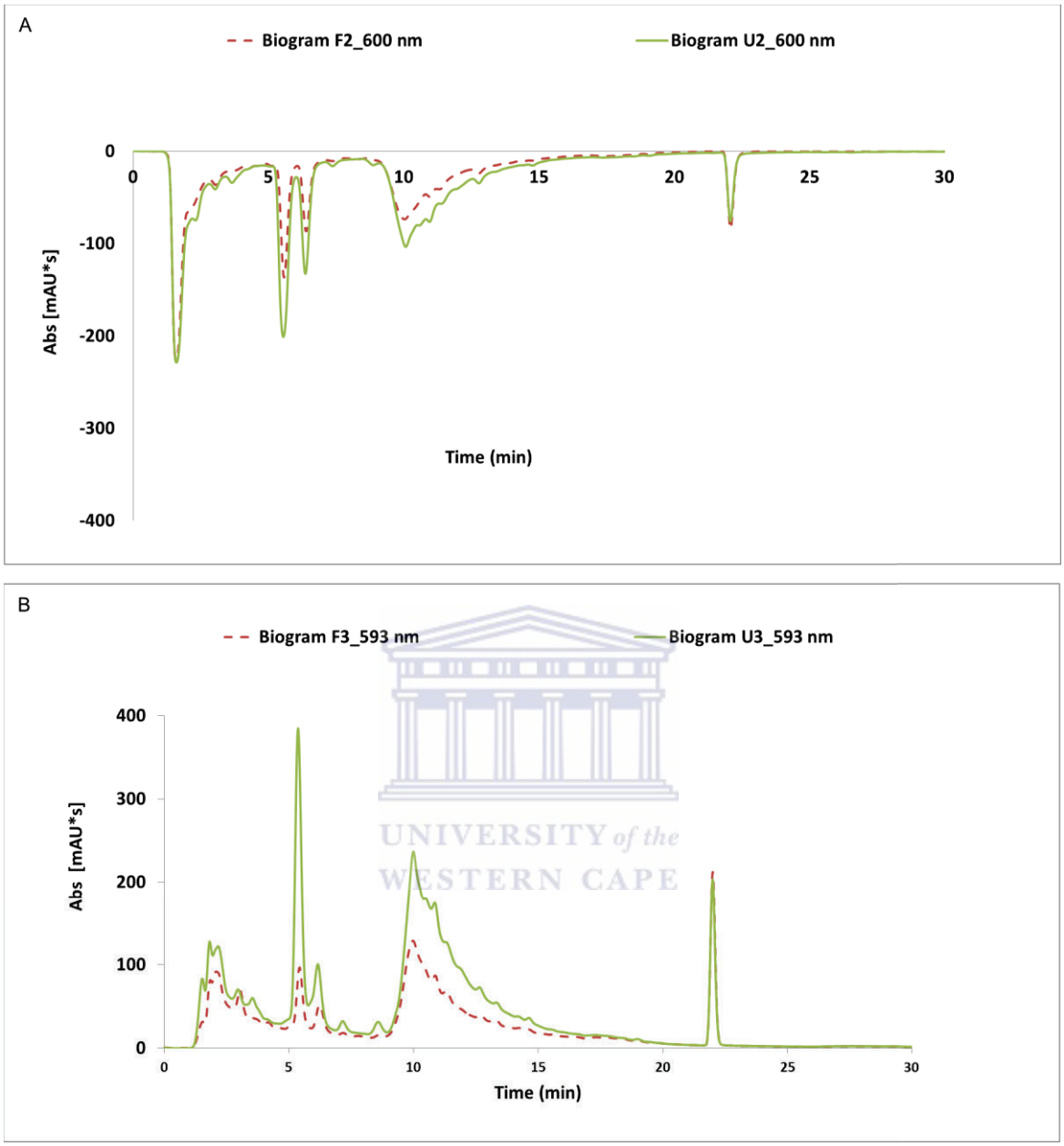

Figure 29. Biograms showing the difference in peak areas of the most active samples per micro-plate assays and the polymeric humps observed between $RTW_{9\text{-}14min}$ as detected with ABTS (A) and FRAP (B) on-line assays.

UV peak areas of the two most active compounds (mangiferin and isomangiferin) and their respective DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and FRAP biogram peaks, given by the most active and least active samples (based on the antioxidant micro-plate assays) were determined and the ratio between the UV peak area and biogram

peak area of the compound for each of the on-line antioxidant assays, calculated. The data are summarised in Tables 10, 11 and 12.

The peak ratios for mangiferin, based on the on-line DPPH$^{\bullet}$ assay, were higher for the unfermented samples, varying between 1.95 and 2.12. While the peak ratios for mangiferin given by the fermented samples were lower, varying from 1.18 to 1.36. For the peak ratios of isomangiferin, the opposite trend in terms of effect of the fermentation state of the extract was observed. In the latter case the peak ratios for the unfermented samples varied between 1.45 to 1.56, while higher ratios, varying from 1.63 to 1.75, were observed for the fermented samples. In summary, the peak ratios of mangiferin, obtained for unfermented samples, were higher than that of isomangiferin ($R_{mangiferin} > R_{isomangiferin}$), while the opposite was observed for the fermented samples ($R_{isomangiferin} > R_{mangiferin}$) (Table 10).

Table 10. Peak ratio of content and antioxidant activity of xanthones as determined by on-line HPLC DAD-DPPH• assay.

Relative activity	Treatment	Sample	Detection	Mangiferin Area [mAU*s]	Isomangiferin Area [mAU*s]
Most active	Unfermented	C. mac U5	UV[a]	5685.87	1951.35
			Biogram[b]	2888.97	1251.07
			Ratio	**1.97**	**1.56**
	Fermented counterpart	C. mac F5	UV[a]	1344.24	1104.36
			Biogram[b]	1141.99	675.88
			Ratio	**1.18**	**1.63**
Most active	Fermented	C. mac F9	UV[a]	1842.08	1254.24
			Biogram[b]	1352.30	728.91
			Ratio	**1.36**	**1.72**
	Unfermented counterpart	C. mac U9	UV[a]	5719.83	2029.94
			Biogram[b]	2937.74	1392.00
			Ratio	**1.95**	**1.46**
Least active	Unfermented	C. mac U2	UV[a]	5723.63	1974.77
			Biogram[b]	2927.75	1358.70
			Ratio	**1.95**	**1.45**
	Fermented counterpart	C. mac F2	UV[a]	1694.63	1247.23
			Biogram[b]	1283.20	711.45
			Ratio	**1.32**	**1.75**
Least active	Fermented	C. mac F3	UV[a]	1212.73	1036.12
			Biogram[b]	972.43	606.13
			Ratio	**1.25**	**1.71**
	Unfermented counterpart	C. mac U3	UV[a]	7034.21	2458.94
			Biogram[b]	3312.74	1650.24
			Ratio	**2.12**	**1.49**

[a] Absorbance determined with diode array detector (DAD) at 320 nm. [b] Absorbance determined with variable wavelength detector at 515 nm

The peak ratios for mangiferin and isomangiferin, based on the on-line ABTS$^{\bullet+}$ assay, were higher for the unfermented samples, varying between 1.21 and 1.27 for mangiferin and 0.71 and 0.73 for isomangiferin. Their respective ratios for the fermented samples varied between 0.59 to 0.65 and 0.62 to 0.66. The

unfermented samples gave higher ratios for mangiferin than for isomangiferin, while for fermented samples similar ratios were observed (Table 11).

Table 11. Peak ratio of content and antioxidant activity of xanthones as determined by on-line HPLC DAD-ABTS$^{\bullet+}$ assay.

Relative activity	Treatment	Sample	Detection	Mangiferin Area [mAU*s]	Isomangiferin Area [mAU*s]
Most active	Unfermented	C. mac U2	UV[a]	6270.98	2187.17
			Biogram[b]	5027.94	3076.90
			Ratio	**1.25**	**0.71**
	Fermented counterpart	C. mac F2	UV[a]	1824.59	1333.39
			Biogram[b]	2809.00	2023.48
			Ratio	**0.65**	**0.66**
Most active	Fermented	C. mac F5	UV[a]	1432.44	1173.91
			Biogram[b]	2415.81	1821.38
			Ratio	**0.59**	**0.64**
	Unfermented counterpart	C. mac U5	UV[a]	6105.71	2171.53
			Biogram[b]	4817.32	2982.51
			Ratio	**1.27**	**0.73**
Least active	Unfermented	C. mac U8	No data available		
Least active	Fermented counterpart	C. mac F8	No data available		
Least active	Fermented	C. mac F7	UV[a]	1823.70	1232.71
			Biogram[b]	2824.19	1985.60
			Ratio	**0.65**	**0.62**
	Unfermented counterpart	C. mac U7	UV[a]	5867.06	2094.83
			Biogram[b]	4837.94	2930.36
			Ratio	**1.21**	**0.71**

[a] Absorbance determined with diode array detector (DAD) at 320 nm. [b] Absorbance determined with variable wavelength detector at 600 nm

The peak ratios for mangiferin obtained in the on-line FRAP assay ranged from 1.27 to 1.45 for unfermented samples, while for fermented samples the ratios

varied from 1.30 to 1.38. The respective peak ratios for isomangiferin varied from 2.13 to 2.34 and 2.07 to 2.31. It is clear that fermentation had little effect on the peak ratios of both compounds. Overall lower ratios were obtained for mangiferin than for isomangiferin (Table 12).

Table 12. Peak ratio of content and antioxidant activity of xanthones as determined by HPLC on-line DAD-BCD FRAP assay

Relative activity	Treatment	Sample	Detection	Mangiferin Area [mAU*s]	Isomangiferin Area [mAU*s]
Most active	Unfermented	C. mac U3	UV[a]	7777.92	2720.37
			Biogram[b]	5853.40	1215.95
			Ratio	**1.33**	**2.24**
	Fermented counterpart	C. mac F3	UV[a]	1489.73	1260.97
			Biogram[b]	1107.10	547.05
			Ratio	**1.35**	**2.31**
Most active	Fermented	C. mac F9	UV[a]	2066.67	1396.62
			Biogram[b]	1492.65	675.61
			Ratio	**1.38**	**2.07**
	Unfermented counterpart	C. mac U9	UV[a]	6430.44	2245.52
			Biogram[b]	5052.25	961.25
			Ratio	**1.27**	**2.34**
Least active	Unfermented	C. mac U4	UV[a]	5452.65	2031.94
			Biogram[b]	4209.65	951.84
			Ratio	**1.30**	**2.13**
	Fermented counterpart	C. mac F4	UV[a]	1031.37	967.80
			Biogram[b]	768.50	439.42
			Ratio	**1.34**	**2.20**
Least active	Fermented	C. mac F6	UV[a]	1184.31	1043.48
			Biogram[b]	913.73	472.25
			Ratio	**1.30**	**2.21**
	Unfermented counterpart	C. mac U6	UV[a]	7184.45	2474.00
			Biogram[b]	4969.03	1087.00
			Ratio	**1.45**	**2.28**

[a] Absorbance determined with diode array detector (DAD) at 320 nm. [b] Absorbance determined with variable wavelength detector at 593 nm

Given that the concentration of these compounds was much higher in the unfermented samples than fermented samples the linearity of the response of the on-line DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays was investigated by determining the response for a series of standards over their concentration ranges expected in the samples. Calibration curves, prepared for DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activity *versus* mangiferin content, showed that while the UV response for content was linear in both cases the colorimetric response for radical scavenging (measured at 515 and 600 nm) was not linear (Figures 30 and 31), resulting in higher peak ratios at higher mangiferin contents (Table 13). In the case of isomangiferin the response to DPPH$^{\bullet}$ (Figure 30) and ABTS$^{\bullet+}$ (Figure 31) was near linear with increasing concentration, resulting in little variation of the peak ratio for both assays (Table 13).

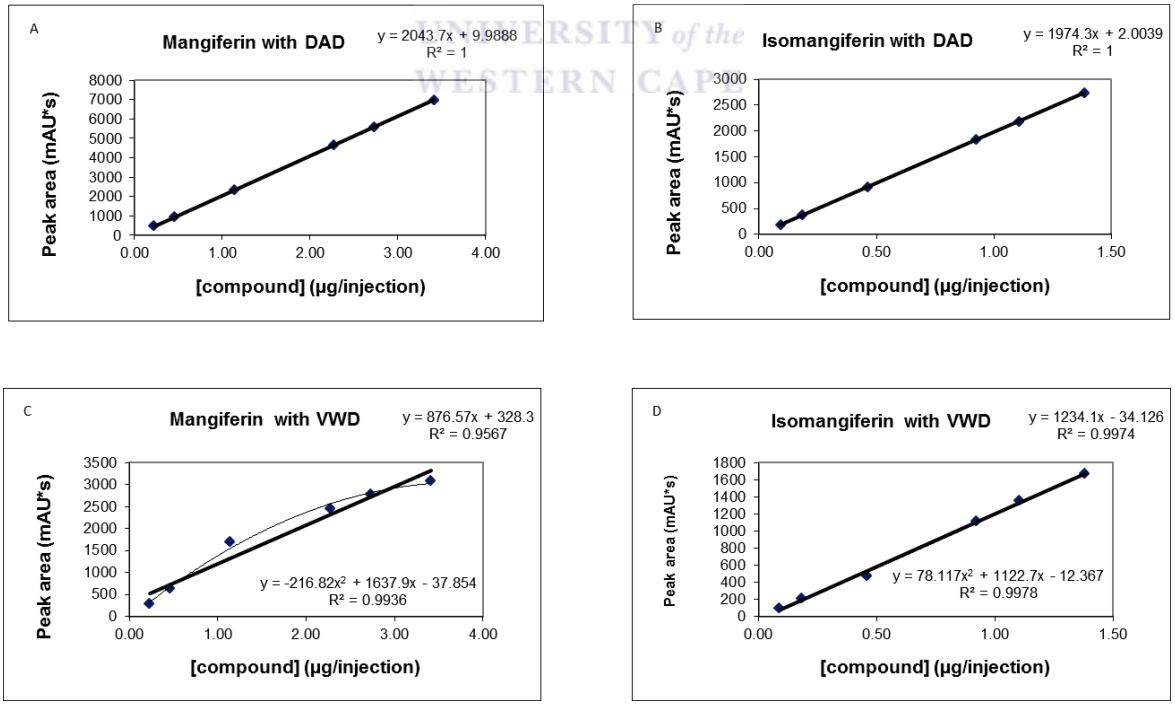

Figure 30. Calibration curves of DPPH$^{\bullet}$ scavenging activity versus mangiferin or isomangiferin content. A and B show linear regression fits of the UV response of the compounds as detected by diode-array-detector at 320 nm, while C and D show polynomial regression fits of the colorimetric response for radical scavenging measured by variable wavelength detector (VWD) at 515 nm.

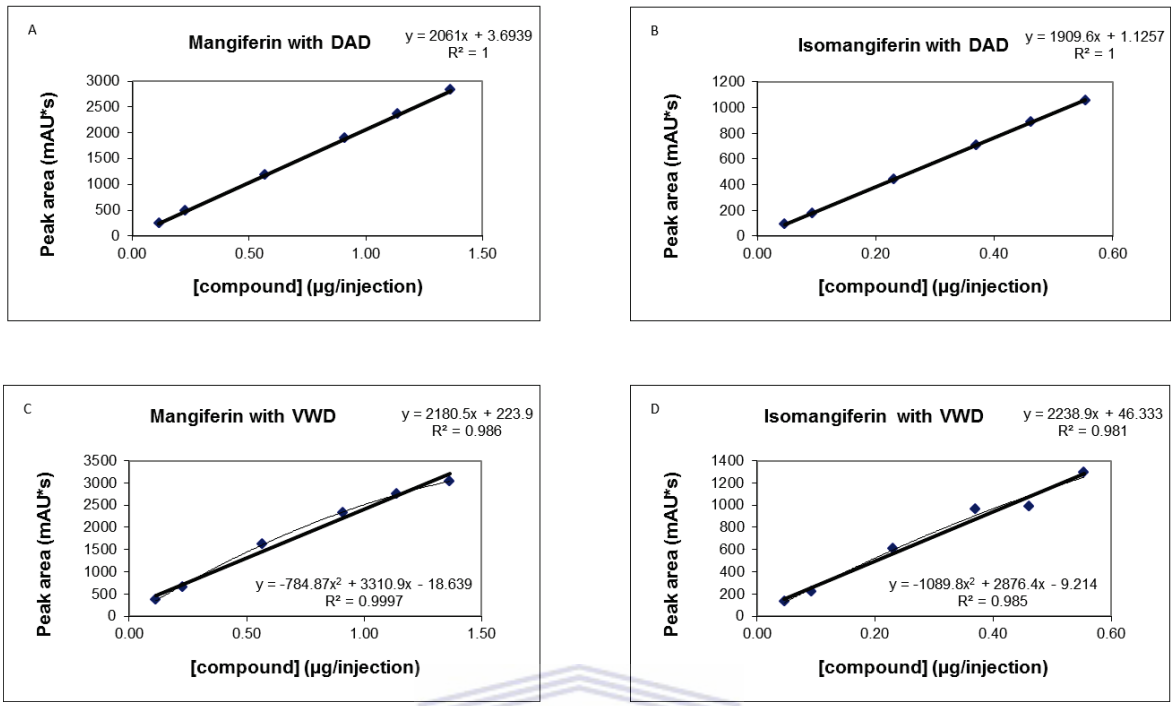

Figure 31. Calibration curves of ABTS$^{\bullet+}$ scavenging activity versus mangiferin or isomangiferin content. A and B show linear regression fits of the UV response of the compounds as detected by diode-array-detector at 320 nm, while C and D show polynomial regression fits of the colorimetric response for radical scavenging measured by variable wavelength detector at 600 nm.

Table 13. Peak ratio of content and antioxidant activity of pure compounds (standards) as determined by on-line HPLC-DAD DPPH$^{\bullet}$ andABTS$^{\bullet+}$ assays

Compound	µg/µL Injected	UV/Biogram ratio	
		DPPH$^{\bullet}$	ABTS$^{\bullet+}$
Mangiferin	0.2273	1.66125	0.65288
	0.4547	1.48965	0.71021
	1.1367	1.37169	0.72270
	2.2733	1.88966	0.80575
	2.7280	2.01353	0.85407
	3.4100	2.26432	0.92940
Isomangiferin	0.0923	1.84950	0.69417
	0.1847	1.71599	0.79461
	0.4616	1.93946	0.71693
	0.9233	1.63881	0.73532
	1.1079	1.61349	0.89706
	1.3849	1.64027	0.81886

5.4 Correlation of phenolic composition and antioxidant activity as per micro-plate assay format

The total polyphenol content (TPC) of freeze-dried aqueous *C. maculata* extracts correlated well with their antioxidant capacity measured with the synthetic radicals, (DPPH$^{\bullet}$, $R^2 = 0.824$; $P < 0.0001$) and (ABTS$^{\bullet+}$, $R^2 = 0.8253$; $P < 0.0001$). A very good correlation was also obtained for the antioxidant capacity measuring the ability of the extracts to reduce iron as measured in the FRAP assay ($R^2 = 0.9064$; $P < 0.0001$). A weaker relationship was observed for the scavenging of the peroxyl radical as measured in the ORAC assay ($R^2 = 7952$; $P < 0.0001$). Results are presented in Figure 32. The use of unfermented and fermented samples ensured variation in TPC values. This contributed to observing correlations between TPC and antioxidant capacity of the extracts.

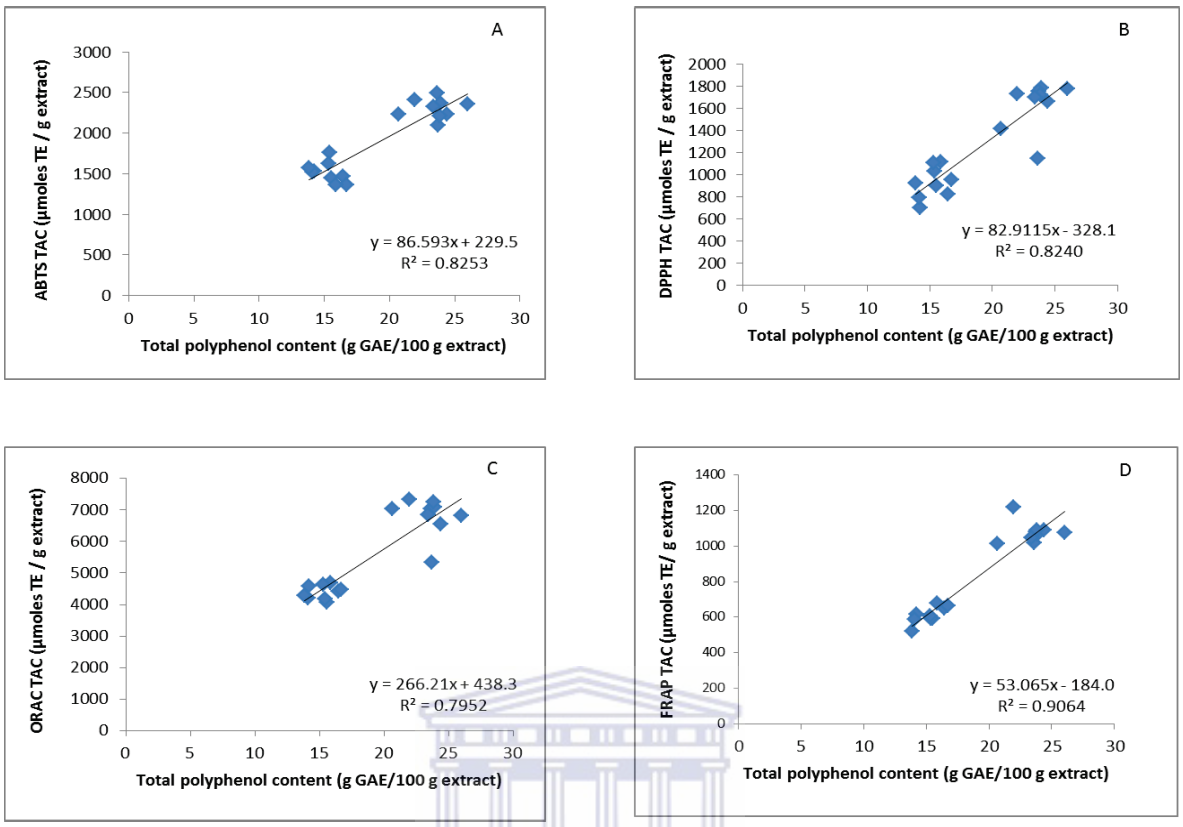

Figure 32. Correlation of the total polyphenols content with total antioxidant capacity of aqueous extracts of *C. maculata* evaluated by ABTS$^{\bullet+}$ (A), DPPH$^{\bullet}$ (B), FRAP (C) and ORAC (D) assays.

The ill-defined polymeric compounds, presented by the "hump", showed a negative, significant correlation ($P < 0.0001$) with the TAC as determined in the different assays (Figure 33). R^2 values varied between 0.7052 and 0.9165, with a relative order of FRAP > ORAC > ABTS$^{\bullet+}$ > DPPH$^{\bullet}$.

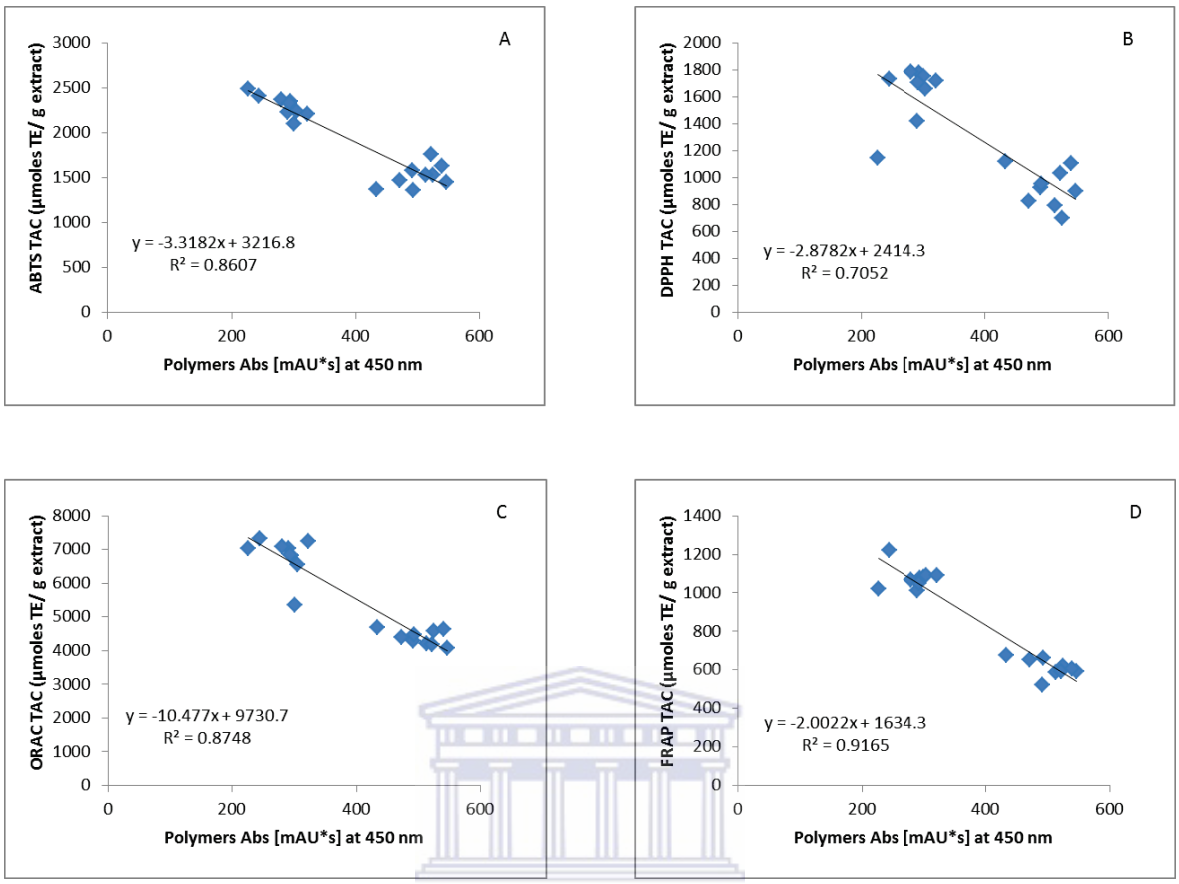

Figure 33. Correlation of polymeric compounds with the total antioxidant capacity (TAC) as evaluated by ABTS$^{\bullet+}$ (A), DPPH$^{\bullet}$ (B), ORAC (C) and FRAP (D) assays.

A correlation analysis of individual phenolic compounds present in *C. maculata* extracts with antioxidant activity was also performed. In all four antioxidant assays used (ABTS$^{\bullet+}$, DPPH$^{\bullet}$, ORAC and FRAP), the individual compounds correlated positively and significantly ($P < 0.0001$) with the total antioxidant capacity (Figures 34 to 38). R^2 values varied between 0.641 for eriocitrin in the DPPH$^{\bullet}$ assay to 0.979 for hesperidin in the FRAP assay. The order of the R^2 values of the different assays were the same for mangiferin, isomangiferin and iriflophenone-3-*C*- β-D-glucoside (FRAP > ABTS$^{\bullet+}$ > ORAC > DPPH$^{\bullet}$). The order of R^2 values for hesperidin was FRAP > ORAC > DPPH$^{\bullet}$ > ABTS$^{\bullet+}$, while the

order for eriocitrin was ABTS$^{\bullet+}$ > FRAP > ORAC > DPPH$^{\bullet}$.

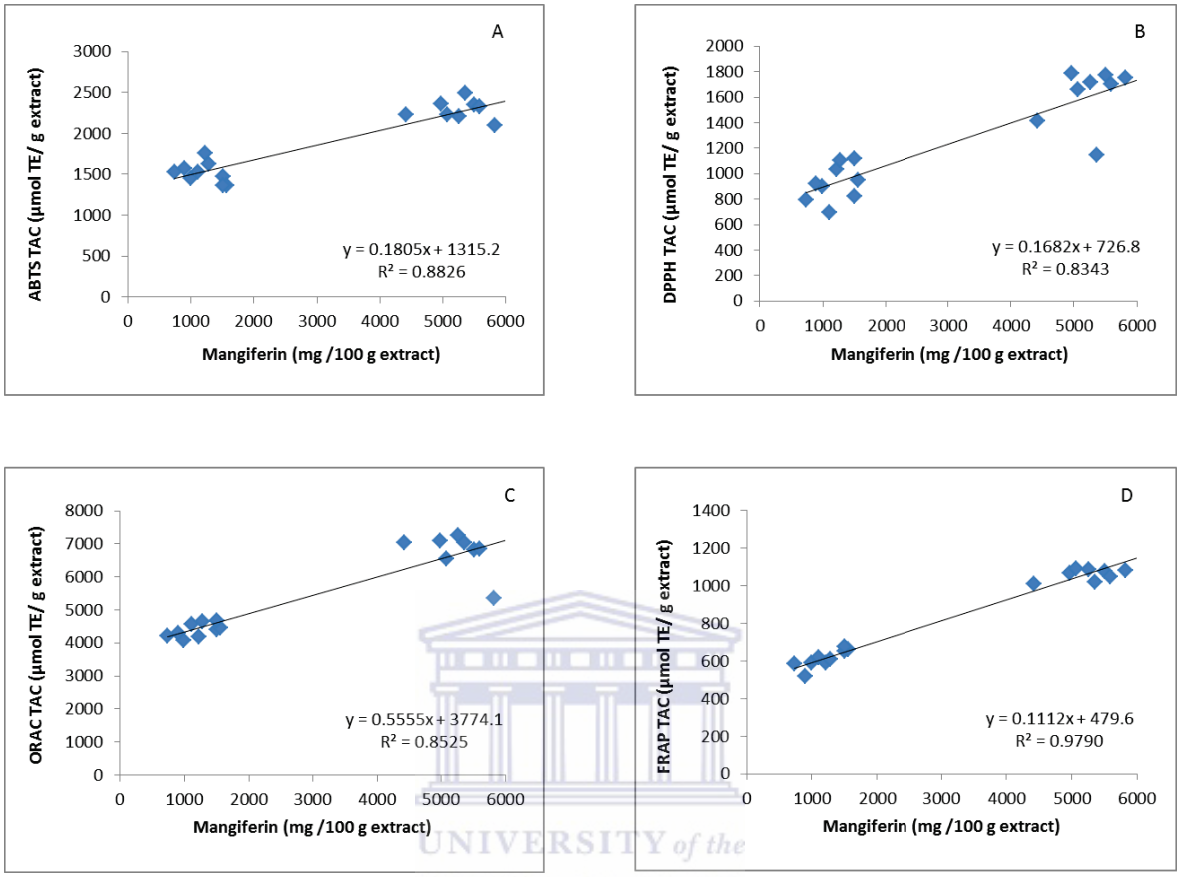

Figure 34. Correlation of mangiferin with total antioxidant capacity (TAC) of the unfermented and fermented extracts of *C. maculata* as evaluated by ABTS$^{\bullet+}$ (A), DPPH$^{\bullet}$ (B), ORAC (C) and FRAP (D) assays.

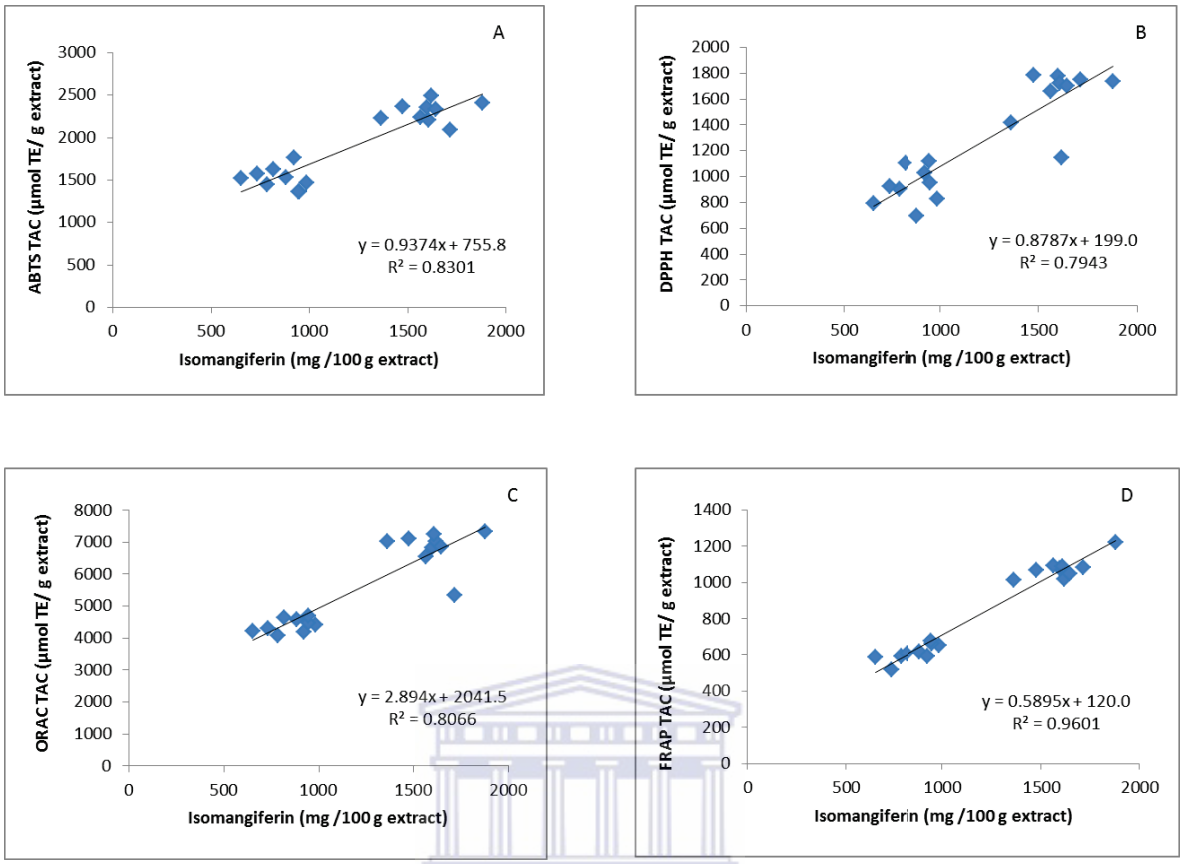

Figure 35. Correlation of isomangiferin with total antioxidant capacity (TAC) of the unfermented and fermented extracts of *C. maculata* as evaluated by ABTS$^{\bullet+}$ (A), DPPH$^{\bullet}$ (B), ORAC (C) and FRAP (D) assays.

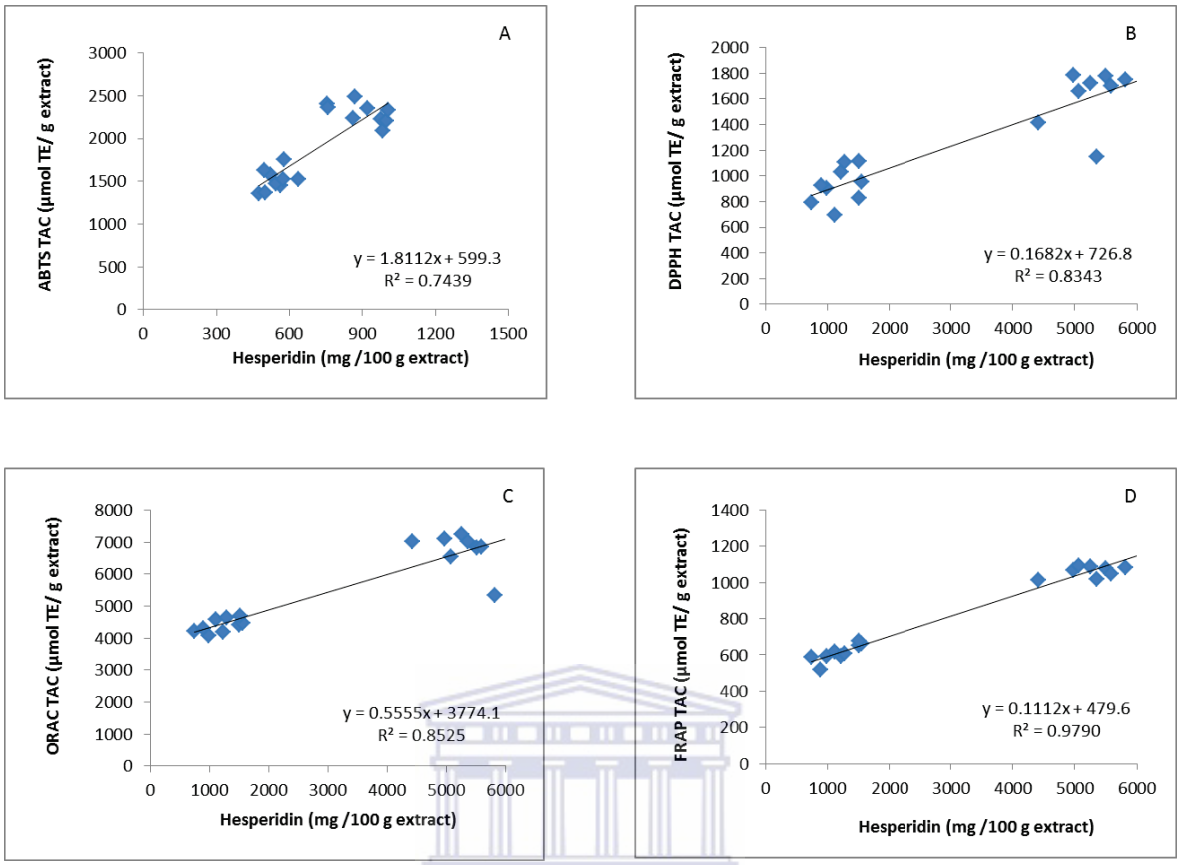

Figure 36. Correlation of hesperidin with total antioxidant capacity (TAC) of the unfermented and fermented extracts of *C. maculata* as evaluated by ABTS$^{\bullet+}$ (A), DPPH$^{\bullet}$ (B), ORAC (C) and FRAP (D) assays.

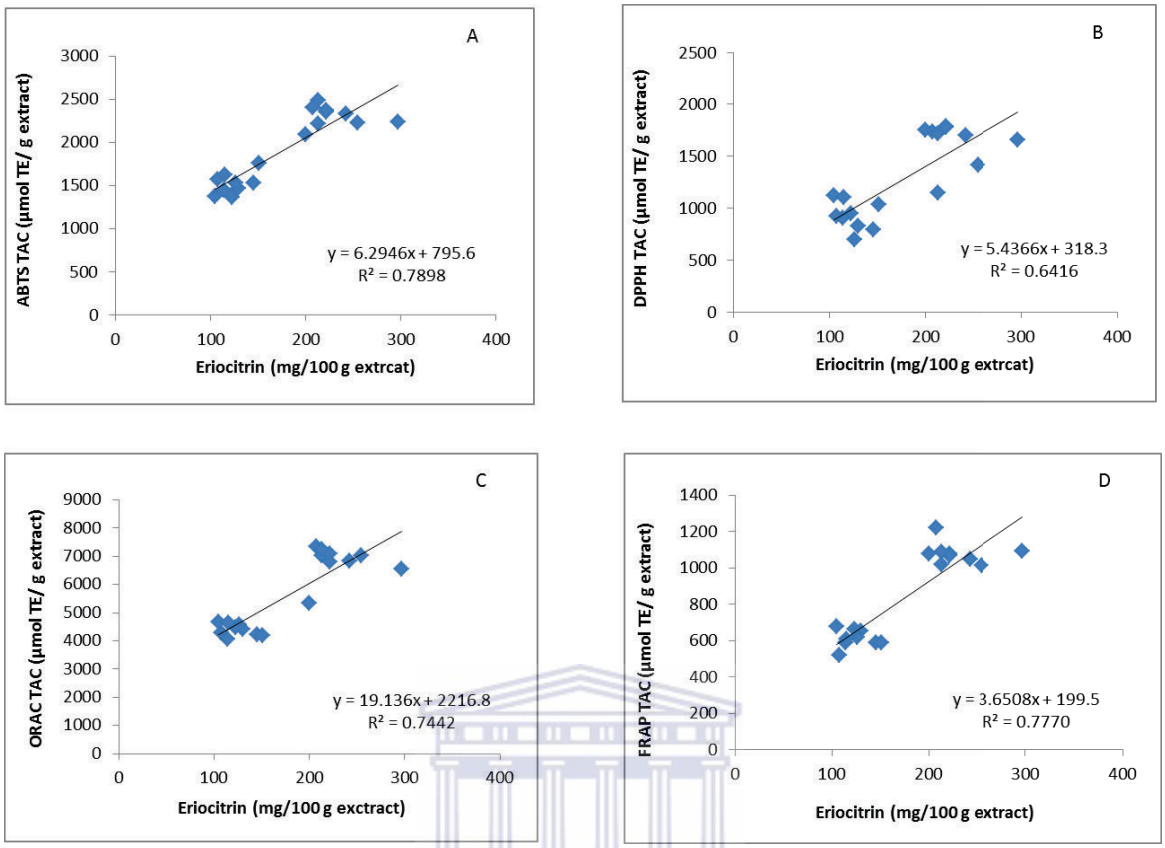

Figure 37. Correlation of eriocitrin with total antioxidant capacity (TAC) of the unfermented and fermented extracts of *C. maculata* as evaluated by ABTS $^{\bullet+}$ (A), DPPH$^{\bullet}$ (B), ORAC (C) and FRAP (D) assays.

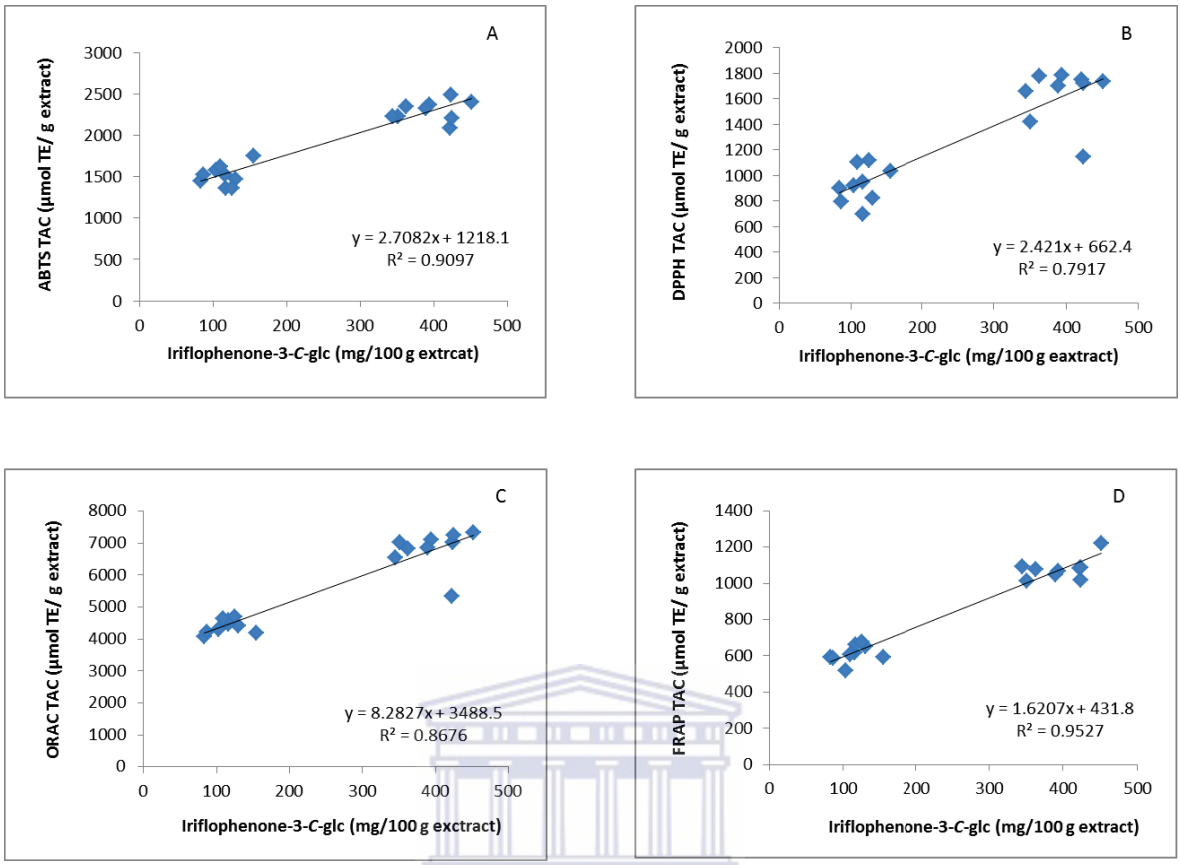

Figure 38. Correlation of iriflophenone-3-*C*-β-D-glucoside with total antioxidant capacity (TAC) of the unfermented and fermented extracts of *C. maculata* as evaluated by $ABTS^{\bullet+}$ (A), $DPPH^{\bullet}$ (B), ORAC (C) and FRAP (D) assays.

5.5 Anti-diabesity potential

The anti-diabesity potential of *C. maculata* was investigated, using inhibition of digestive enzymes, α-glucosidase and pancreatic lipase that are respectively involved in absorption of glucose and triacylglycerols from the intestines. Type II crude porcine pancreatic lipase was selected to test for anti-obesity potential whereas α-glucosidase from *Saccharomyces cerevisiae* and α-glucosidase from rat intestinal acetone powder were used to evaluate anti-diabetic potential of the extracts. These activities were assessed in micro-plate assays. On-line HPLC-

enzyme inhibition assays were attempted in order to identify bioactives in the extracts responsible for enzyme inhibition.

5.5.1 Anti-obesity assays

The anti-obesity potential of *C. maculata* extracts were assessed in terms of their lipase inhibitory effects, using both a colorimetric and fluorimetric assay. Orlistat™, a well-known anti-obesity drug, was used as positive control in both assays.

5.5.1.1 Lipase inhibition with colorimetric detection

For the colorimetric assay (LPC), final concentrations of 200 µg/mL *C. maculata* extract and 25 µg/mL Orlistat™ were used. The activity shown by the extracts was significantly ($P < 0.001$) lower than that of Orlistat™ (60 %) (Figure 39), however, the reduction in lipase inhibition with fermentation was not significant ($P > 0.0733$) (Table 14) as the effect was not consistent. In the case of sample 2 the fermented sample was more effective than its unfermented counterpart, while equivalent inhibition was observed for both treatments of sample 9 (Figure 39). The percentage decrease in lipase inhibition by the extracts after fermentation varied from -15 to 41 % (Table 8). As colour interference of the plant extracts with assay measurement was expected to be responsible for these inconsistent results with fermentation, the experiment was also performed using a fluorimetric probe.

Table 14. ANOVA of enzyme inhibition assays: Lipase inhibition (colorimetric and fluorescence probes) assays.

	Probe	Controls[a]	Unfermented[a, b]	Fermented[a,b]	P[c] value
LPC	pNPB	61.34[d]	41.87 ± 3.48	36.77 ± 7.14	p < 0.0733
LPF	MUB	83.97[d]	56.57 ± 2.30	51.03 ± 3.78	p < 0.0177

[a] Values expressed as % inhibition of enzyme at final concentration (200 µg/mL: LPC and 480 µg/mL: LPF) of extract; [b] Data are mean ± SD where n = 9. [c] P values indicate the significance between unfermented and fermented extracts (without considering the control) [d] Signifies that the control differ significantly (P < 0.005) from the extracts when considered.

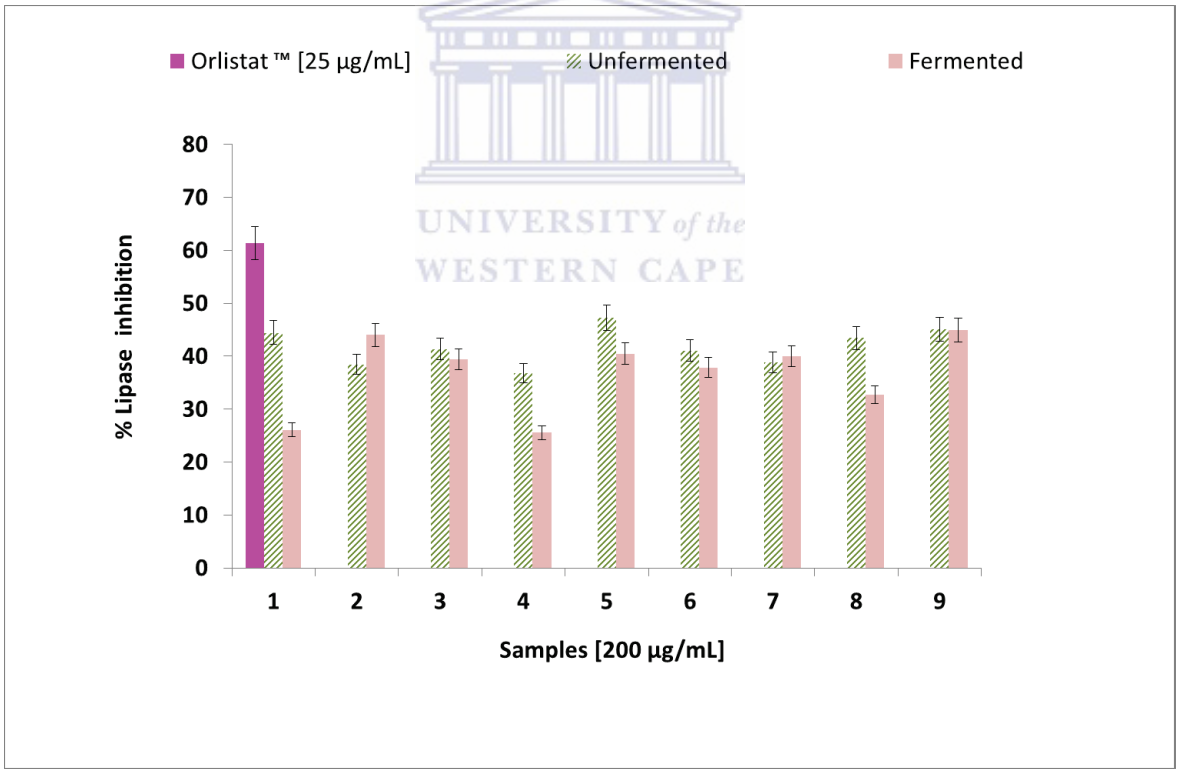

Figure 39. Lipase inhibitory activity of aqueous extracts of unfermented or fermented *C. maculata* (n = 9) at 200 µg/mL in the reaction mixture in comparison with Orlistat™ (control; 25 µg/mL), using a colorimetric probe, para-nitrophenyl butyrate.

5.5.1.2 Lipase inhibition with fluorimetric detection

For this assay higher concentrations (480 µg/mL) of individual extracts were required to obtain measurable lipase inhibitory effects (Figure 40). The percentage inhibition given by Orlistat™ was 84 % at a final concentration 25 µg/mL. This activity was significantly higher than those obtained for the extracts of unfermented and fermented plant material (57 and 51 %, respectively) (Table 14). The percentage inhibition ranged from 53 to 60 and 45 to 52 before and after fermentation, respectively. Even though the same concentration of the control (Orlistat™) was used in both lipase assays, Orlistat™ showed higher lipase inhibitory effect in the fluorimetric assay than with the colorimetric assay (84 % and 61 %, respectively) (Table 14).

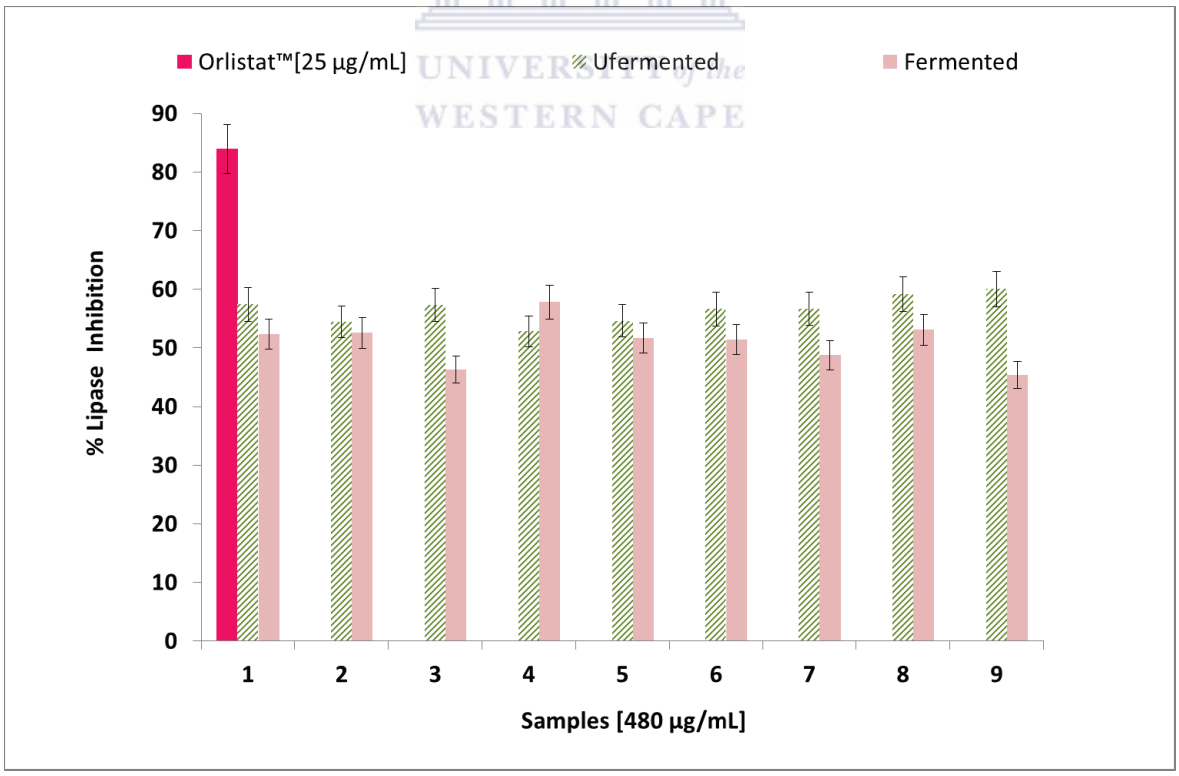

Figure 40. Lipase inhibitory activity of aqueous extracts ofunfermented or fermented *C. maculata* (n = 9) *at* 480 µg/mL in reaction mixture in comparison with Orlistat™ (25 µg/mL), using a fluorimetric probe, 4-methylumbelliferyl butyrate.

5.5.2 Anti-diabetic assays

The anti-diabetic potential of *C. maculata* extracts was assessed in terms of ability to inhibit α-glucosidase. In this case colorimetric and fluorimetric assays, as well as two sources of the enzyme, i.e. yeast (*S. cerevisiae)* and mammalian (rat), were employed. The flavanol, (+)-catechin, and Acarbose™, a drug, were used as positive controls.

5.5.2.1 α-Glucosidase inhibition with colorimetric detection

For the colorimetric assay, unfermented and fermented extracts of *C. maculata* were analysed at a final concentration of 200 µg/mL and (+)-catechin (17 µg/mL) was used as a positive control. The unfermented extracts showed higher α-glucosidase inhibition than the fermented extracts (37 % and 20 %, respectively) (Table 10). The most active of the individual extracts was sample 3, giving 54 and 45 % inhibition before and after fermentation, respectively (Figure 41). The activity shown by the extracts was significantly ($p < 0.0050$) lower than that of (+)-catechin (87 %) (Table 15). Generally, fermentation reduced the α-glucosidase inhibitory effect of the extracts, but individual extracts were not affected to the same degree by fermentation (Figure 41). In the case of sample 6, fermentation resulted in an apparent “increase” in the enzyme activity as indicated by the negative value, while for sample 4 fermentation increased inhibition of the enzyme.

Table 15. ANOVA of enzyme inhibition assays: α-Glucosidase inhibition (colorimetric, fluorimetric assays with yeast or rat intestine acetone powder enzymes) assays.

	Probe	Controls[a]	Unfermented[a,b]	Fermented[a,b]	P[c] value
AGLC	pNPG	87.23[d]	37.40 ± 8.55	20.10 ± 16.33	p < 0.0050
AGCF	4-MUG	55.24[d]	34.31 ± 1.84	29.37 ± 4.53	p < 0.0078
RATG	4-MUG	41.44[d]	60.00 ± 1.47	52.91 ± 1.89	p < 0.0001

[a] Values expressed as % inhibition of enzyme at final concentration (200 µg/mL: AGCL and 500 µg/mL: AGCF and RATG) of extract; [b] Data are mean ± SD where n = 9. [c] P values indicate the significance between unfermented and fermented extracts of *C. maculata* (without considering the controls: (+)-catechin or Acarbose™), [d]Signifies that the control differ significantly (P < 0.005) from the extracts when considered.

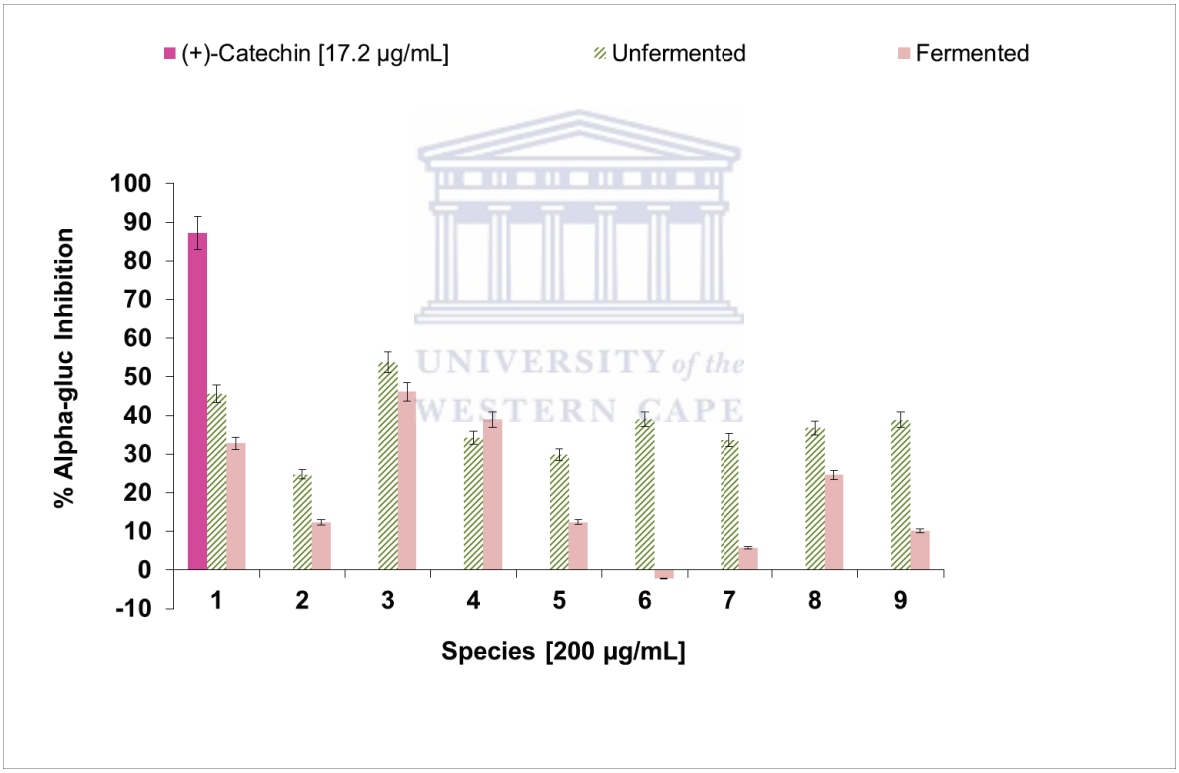

Figure 41. α-Glucosidase (yeast) inhibitory activity of aqueous extracts ofunfermented or fermented *C. maculata* (n = 9) *at* 200 µg/ml in reaction mixture in comparison with (+)-catechin (17 µg/ml), using a colorimetric probe, 4-nitrophenyl α-D-glucopyranoside.

The experiment was also performed using a fluorimetric probe to eliminate interference of colour in the extract.

5.5.2.2 α-Glucosidase inhibition with fluorimetric detection

The freeze-dried extracts were analyzed at a final concentration of 500 µg/mL, while 200 µg/mL was used for (+)-catechin (positive control). A significantly higher ($P < 0.0078$) α-glucosidase inhibitory activity was observed for extracts of unfermented *C. maculata* compared to the fermented batch (34 and 29 %, respectively). However, the activity shown by the extracts was significantly ($P < 0.0050$) lower than that of (+)-catechin (55 %). Unfermented sample 3 (38 %) and fermented sample 6 (36 %) showed the highest activity for α–glucosidase inhibition of these two batches of extracts, while samples 4 and 7 (32 and 21 %) were the least active extracts of the unfermented and fermented batch of samples, respectively (Figure 42). In the case of sample 6 the unfermented and fermented extracts showed the same inhibitory activity.

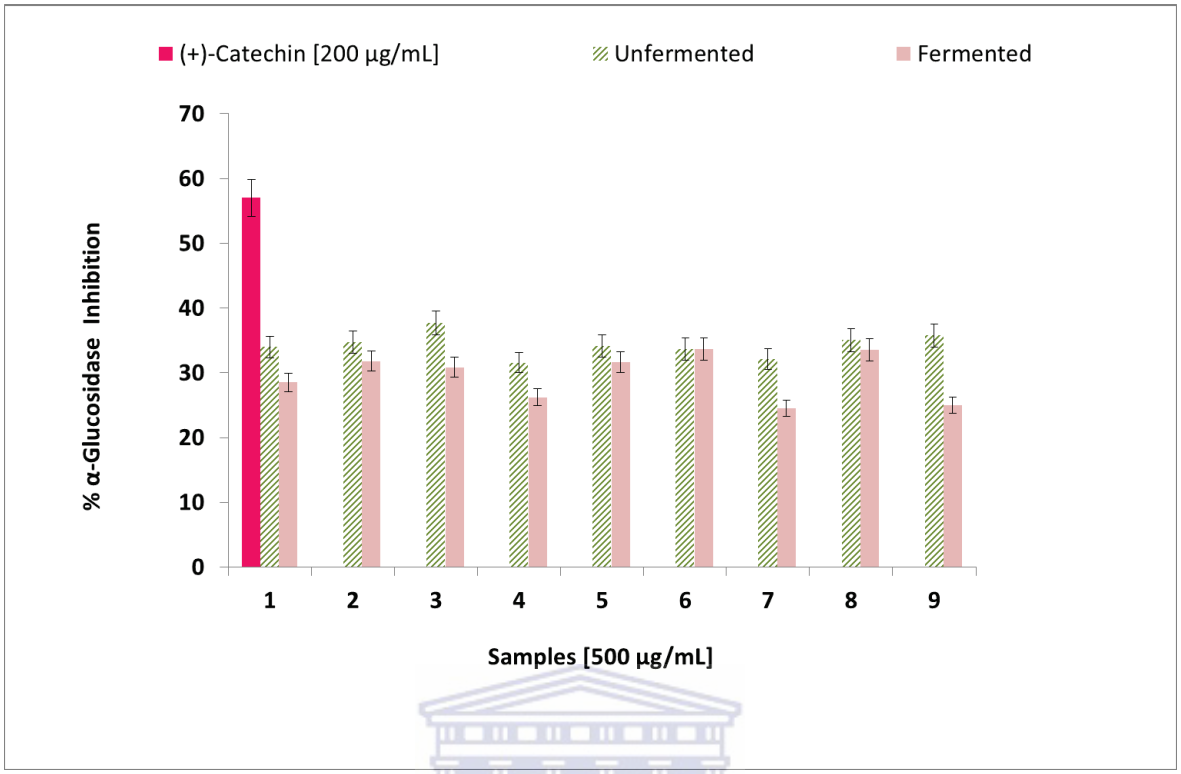

Figure 42. α-Glucosidase (yeast) inhibitory activity of aqueous extracts of unfermented or fermented *C. maculata* (n = 9) at 500 µg/ml in reaction mixture in comparison with (+)-catechin (200 µg/ml), using a fluorimetric probe, 4-methylumbelliferyl-α-D-glucopyranoside.

5.5.2.3 Rat α-glucosidase inhibition with fluorimetric detection

A mammalian enzyme (rat intestinal acetone powder) was also used to evaluate the anti-diabetes activity of aqueous extracts of *C. maculata* as the substrate specificity of α-glucosidase differs greatly depending on the source **(Kimura *et al.*, 2004)**. The same concentrations as used in the colorimetric assay were required to obtain a measurable inhibition of the enzyme. Acarbose™ (202 µg/mL) was used as a positive control. Results are presented in Figure 43 and Table 15. The inhibition of α-glucosidase by unfermented extracts (60 %) was significantly higher ($P < 0.0001$) than that of fermented extracts (53 %)

(Table 15). An average of 12 % decrease in α-glucosidase inhibition was observed after fermentation (Table 8). Sample 8 was deemed the most active in case of both unfermented and fermented extracts (62 and 58 %, respectively), whereas lowest inhibition was observed for sample 4 of the unfermented and fermented extracts (58 and 50 %, respectively). An intra-batch variation (%RSD) of 3 % was observed for the unfermented extracts, which increased to 4 % after fermentation. All plant extracts, at the concentration tested (500 µg/mL) showed a significantly higher inhibitory activity than Acarbose™ (41 %).

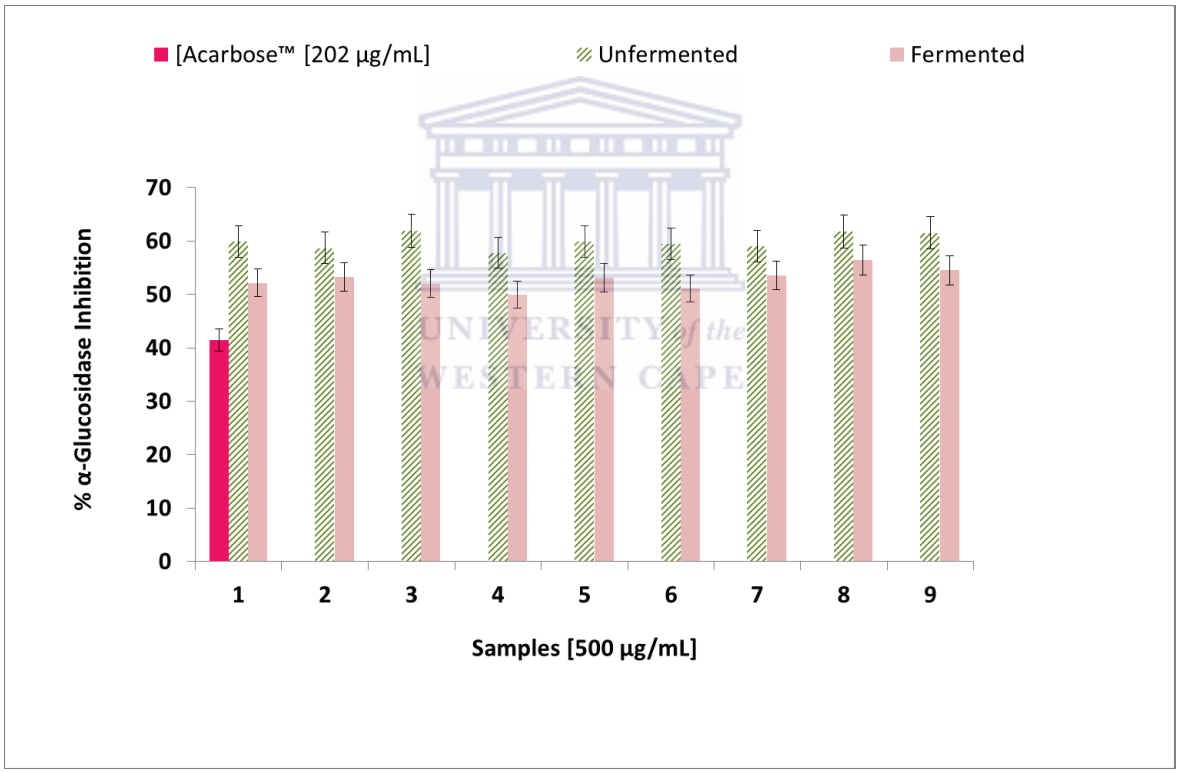

Figure 43. α-Glucosidase inhibitory activity of aqueous extracts of *C. maculata* (n = 9 fermented or unfermented samples fermented and unfermented samples) *at* 500 µg/ml in reaction mixture, in comparison with Acarbose™ (202 µg/ml), using a fluorimetric probe (4-methylumbelliferyl-α-D-glucopyranoside) and rat intestinal acetone powder (source of enzyme).

5.5.3 Identification of bioactives (on-line HPLC lipase and α-glucosidase inhibition)

In the attempt to identify enzyme inhibitors from the *C. maculata* extracts, the on-line HPLC-lipase and α-glucosidase inhibition assays were performed. The most active samples (as per micro-plate assay results) were selected for these analyses, however, no detectable activity was obtained from the extracts for both enzyme assays. A typical on-line HPLC-lipase inhibition biogram of unfermented *C. maculata* extract, together with the UV-Vis chromatogram used for detection of the compounds, is depicted in Figure 44. The 320 nm chromatogram is slightly off-set for illustration purposes. The lipase inhibition biogram, determined with fluorescence detection, showed no activity (depicted as a flat line). Similar results were obtained with the HPLC-α-glucosidase inhibition assay (data not shown).

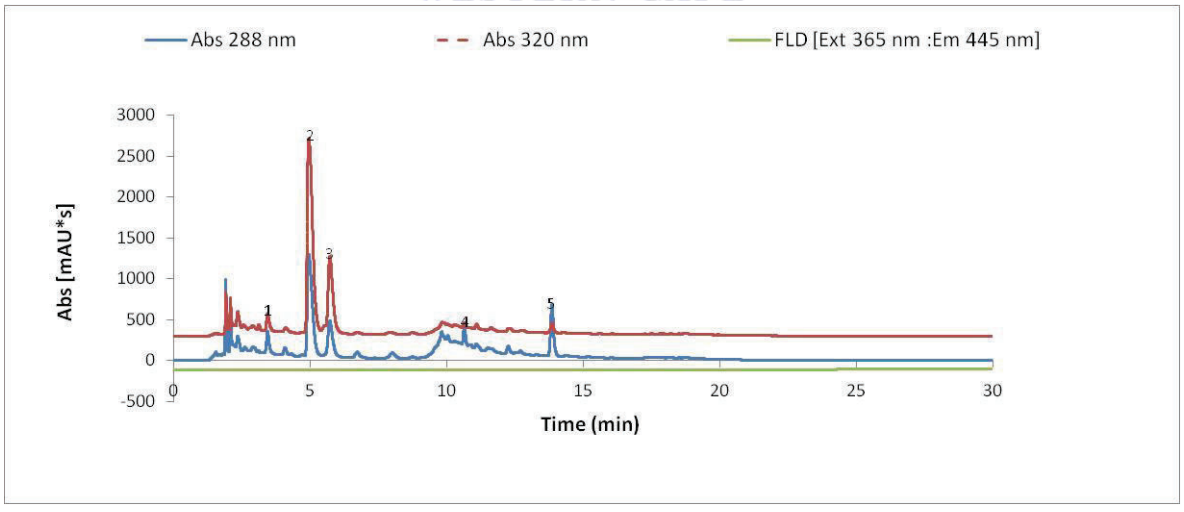

Figure 44. Combined UV chromatograms and lipase on-line biogram of the most active unfermented sample (i.e. U5) of aqueous extracts of *C. maculata.* UV absorbance for detection of the major phenolic compounds was measured at 288 and 320 nm. Inhibition of lipase was measured with a fluorescence detector after excitation at 365 nm and emission at 445 nm. The biogram shows no negative peaks to identify activity. The UV peaks are: **1**: iriflophenone-3-*C*- β-D-glucoside, **2**: mangiferin, **3**: isomangiferin, **4**: eriocitrin, **5**: hesperidin.

5.6 Correlation of phenolic composition and anti-diabesity activity

The correlation between phenolic composition (TPC, individual phenolic compounds and polymers) and enzyme inhibition was investigated and the results are depicted in Figures 45 to 48. Significant, positive correlations were obtained between the enzyme inhibition parameters and composition ($P < 0.001$), except for the polymers, showing a significant, negative correlation ($P < 0.001$). Based on the categories of **Taylor (1990)** the correlation coefficients ($r = R^2$) varied from low or weak to very high (Table 16). Lipase inhibition correlated the best with the hesperidin content of the extracts ($R^2 = 0.5432$), in comparison with eriocitrin gave the weakest correlation ($R^2 = 0.4245$). The correlation thus varied from moderate to high according to the categories of **Taylor (1990)**. Inhibition of yeast α-glucosidase correlated the best with the polymers ($R^2 = 0.4420$) and the weakest in comparison with eriocitrin ($R^2 = 0.2157$). In the case of rat α-glucosidase generally higher correlations ($R^2 > 0.6047$), were observed than for yeast α-glucosidase. Isomangiferin and eriocitrin gave the best ($R^2 = 0.9048$) and weakest ($R^2 = 0.6047$) correlation with the inhibition of rat α-glucosidase, respectively, thus varying from moderate to strong.

Table 16. Correlation between phenolic constituents and enzyme inhibition parameters

Variables	LPF	AGCF	RATG	P values
TPC	0.4644	0.2877	0.8400	P < 0.001
Eriocitrin	0.4238	0.2157	0.6047	P < 0.001
Mangiferin	0.4722	0.3892	0.8972	P < 0.001
Isomangiferin	0.6600	0.4112	0.9048	P < 0.001
Hesperidin	0.4356	0.2981	0.6394	P < 0.001
Iriflophenone-3-C- β-Dglucoside	0.4530	0.4286	0.8733	P < 0.001
Polymers	0.4545	0.4420	0.9006	P < 0.001

R^2 values for individual variables versus inhibition of obesity and diabetes enzyme(s) and significance of the correlation.

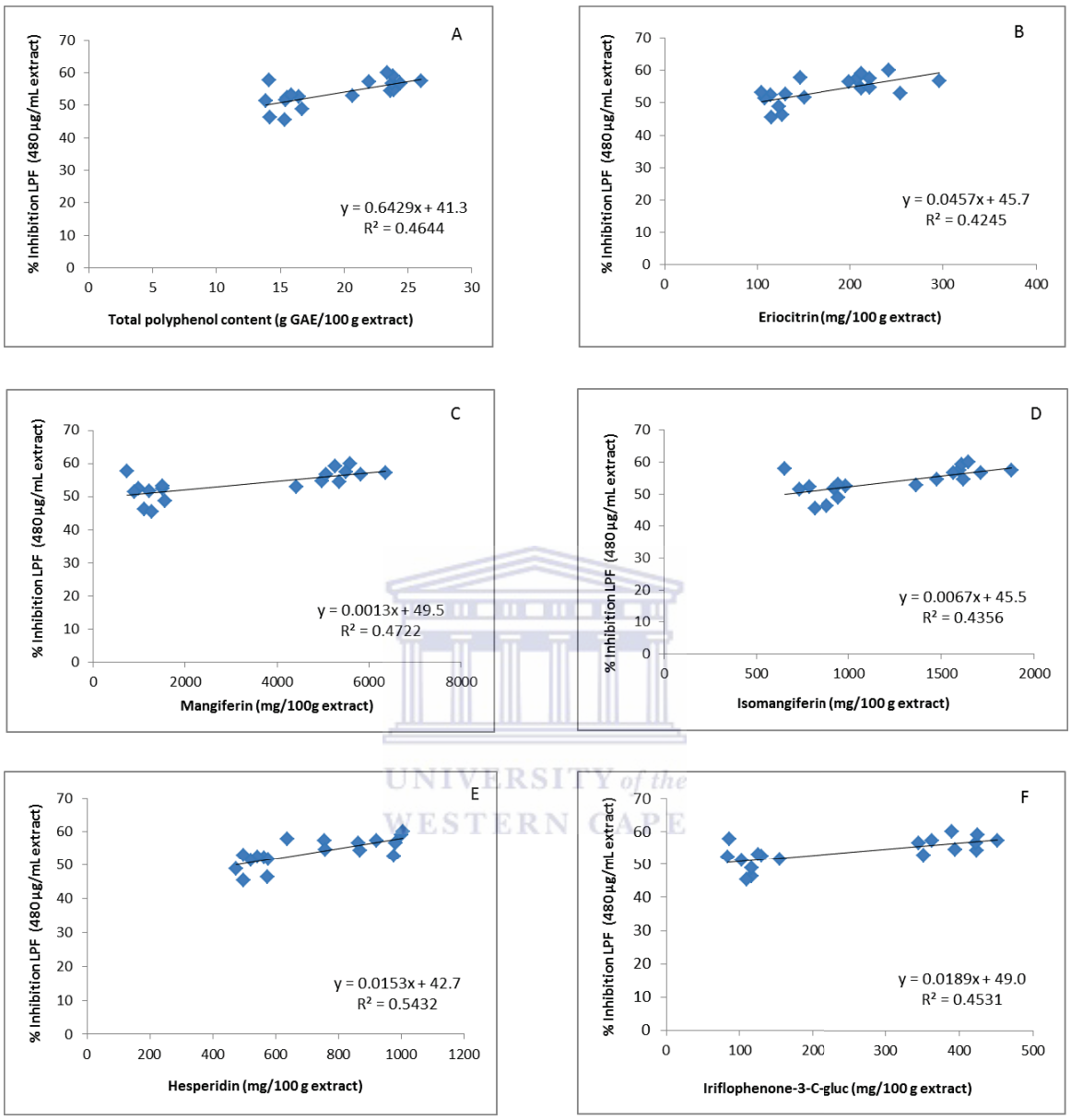

Figure 45. Correlation of TPC and individual compounds with lipase inhibitory activity of unfermented and fermented *C. maculata* extracts (480 µg/mL), determined using fluorimetric probe, 4-Methylumbelliferyl butyrate.

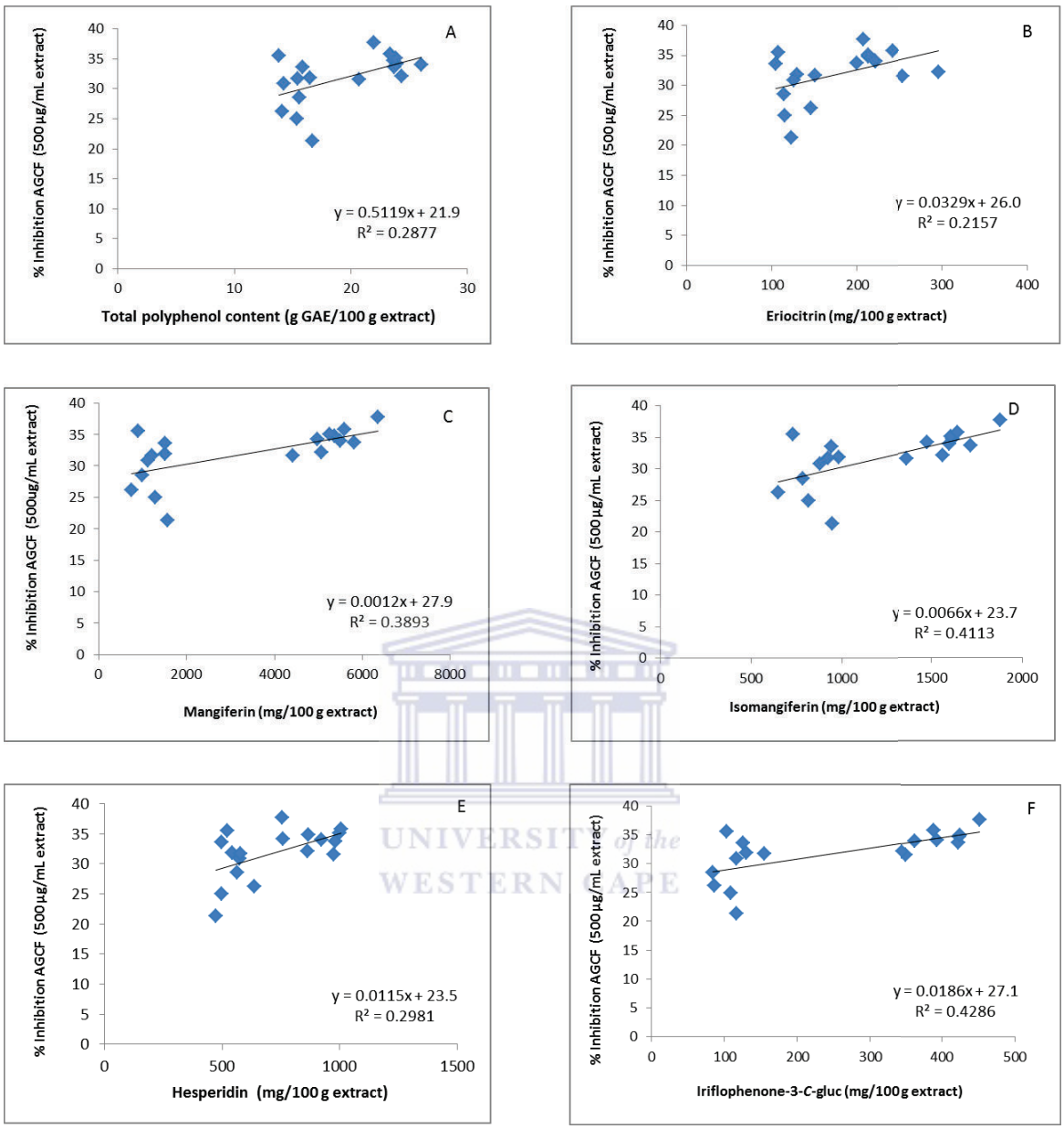

Figure 46. Correlation of TPC and individual compounds with yeastα-glucosidase inhibitory activity of unfermented and fermented *C. maculata* extracts (500 µg/mL), determined using fluorimetric probe.

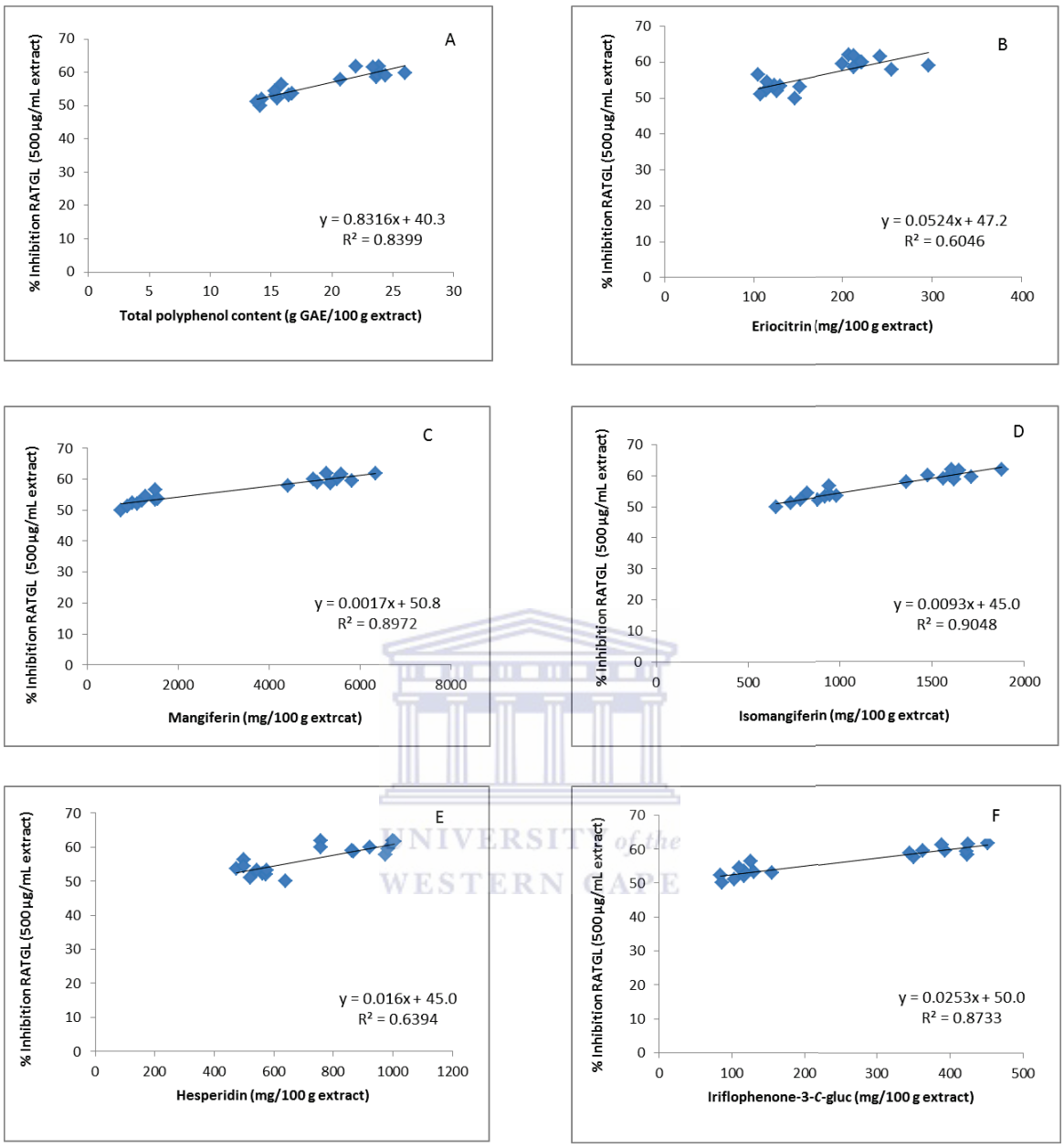

Figure 47. Correlation of TPC and individual compounds with α-glucosidase inhibitory activity of unfermented and fermented *C. maculata* extracts (500 µg/mL), determined using fluorimetric probe and rat intestinal acetone powder α-glucosidase.

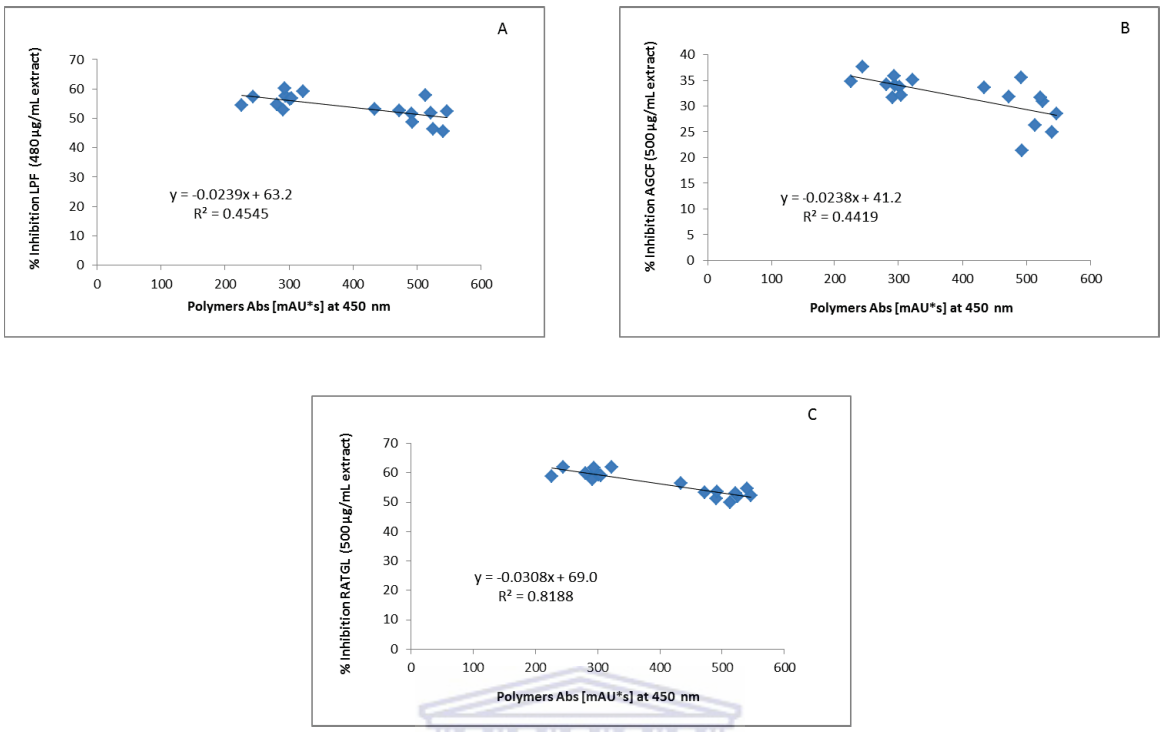

Figure 48. Correlation of polymeric compounds with the anti-diabesity activity as evaluated by lipase (LPF), α-glucosidase (AGCF and RATGL) inhibition assays.

5.7 Principal component analysis (PCA) of freeze-dried aqueous extracts of unfermented and fermented *C. maculata*

PCA was performed to obtain the association between the samples and various parameters (composition and activity), depicted by the scores and loading plots (Figures 49 to 51). The score plot shows how samples relate to each other based on their phenolic composition and bio(activities). Samples with similar profiles lie close to each other in the map while samples with different profiles lie far apart. The information on the reason for samples clustering together is reveald by the loading plot. In this manner it is possible to visually assess similarities and differences between samples and determine whether samples can be grouped, based on all parameters **(Ringner, 2008)**. Figure 49 clearly shows a separation

between unfermented and fermented samples with PC1 and PC2 explaining 83.70 and 5.01 % of the variation, respectively. The polymer content associated with the fermented samples, while all other parameters associated with unfermented samples. PCA was also performed on the two groups of samples, i.e. unfermented (Figure 50) and fermented (Figure 51), to again, identify the most “bioactive” sample(s) of each group. Sample 3 of the unfermented extracts best associated with α-glucosidase inhibition (yeast) as depicted in the biplot (Figure 50). Furthermore, the best association was observed between sample 3 and FRAP, isomangiferin, mangiferin and iriflophenone-3-*C*-β-D-glucoside values. For lipase inhibition the best association with samples 8 and 9 were observed (Figure 50). When the fermented samples are considered, sample 8 with high TPC and mangiferin content is identified as being the most “bioactive” in terms of rat α-glucosidase inhibition. Fermented sample 8 showed good association with FRAP and ORAC values and sample 7 best associated with the ORAC values (Figure 51). It should be noted that not all the variance are explained by PC1 and PC2 as combined they explain only ca. 60 % of the variance.

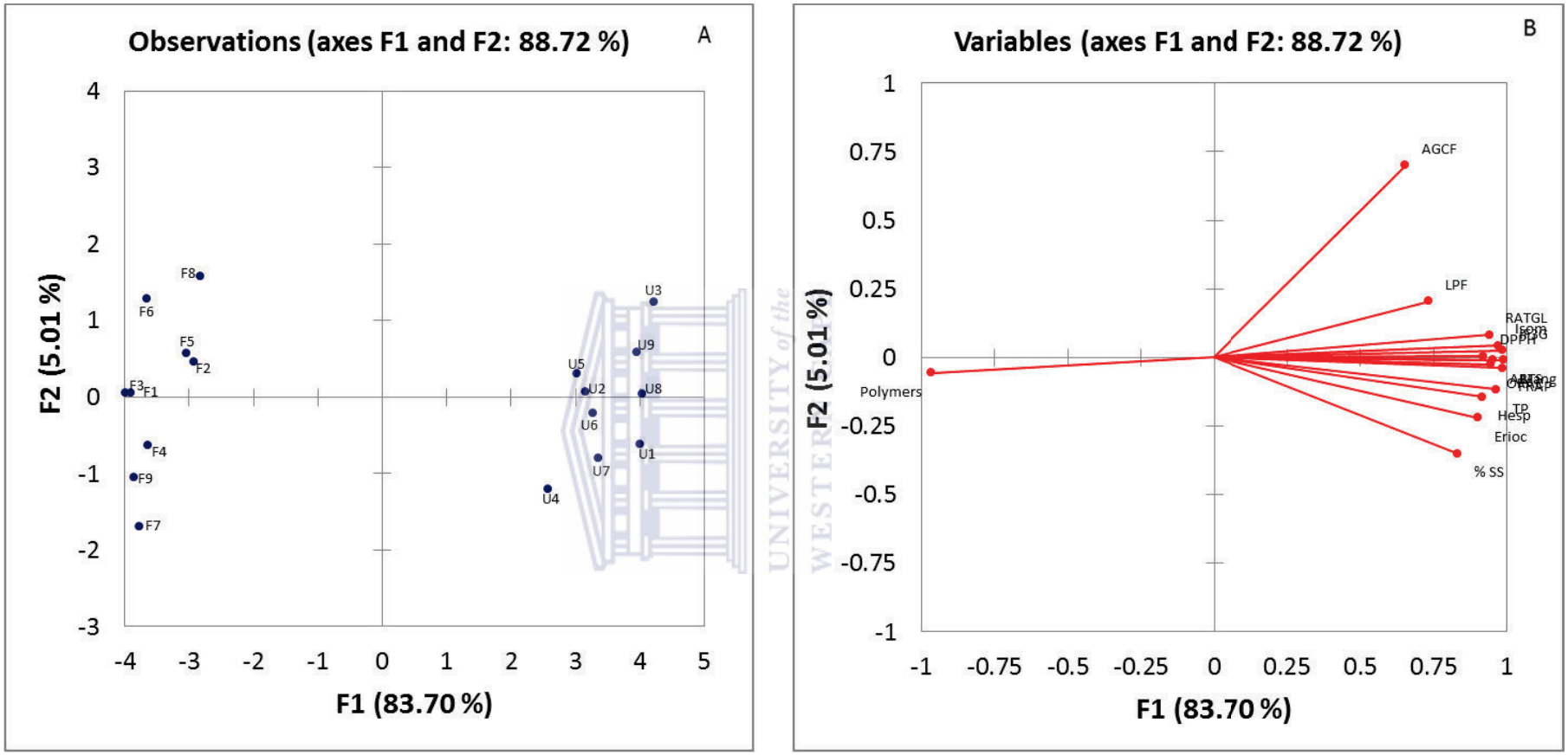

Figure 49. **A**. PCA scores plot showing the position of the 9 freeze-dried extracts unfermented (U) and fermented (F) *C. maculata* . **B**. PCA loading plot showing the position of the % SS (soluble solids), TPC (total polyphenol content), Hesp (hesperidin), Erioc (eriocitrin), IF3G (iriflophenone-3-C-glucoside), Mang (mangiferin) and Isom (isomangiferin) including different antioxidant assays (ABTS$^{\bullet+}$, DPPH$^{\bullet}$, ORAC and FRAP), the enzyme assays with fluorimetric detection (LPF: lipase inhibition, AGCF: α-glucosidase inhibition with yeast enzyme and RATGL: α-glucosidase inhibition with mammalian enzyme) and Polymers (polymeric compounds).

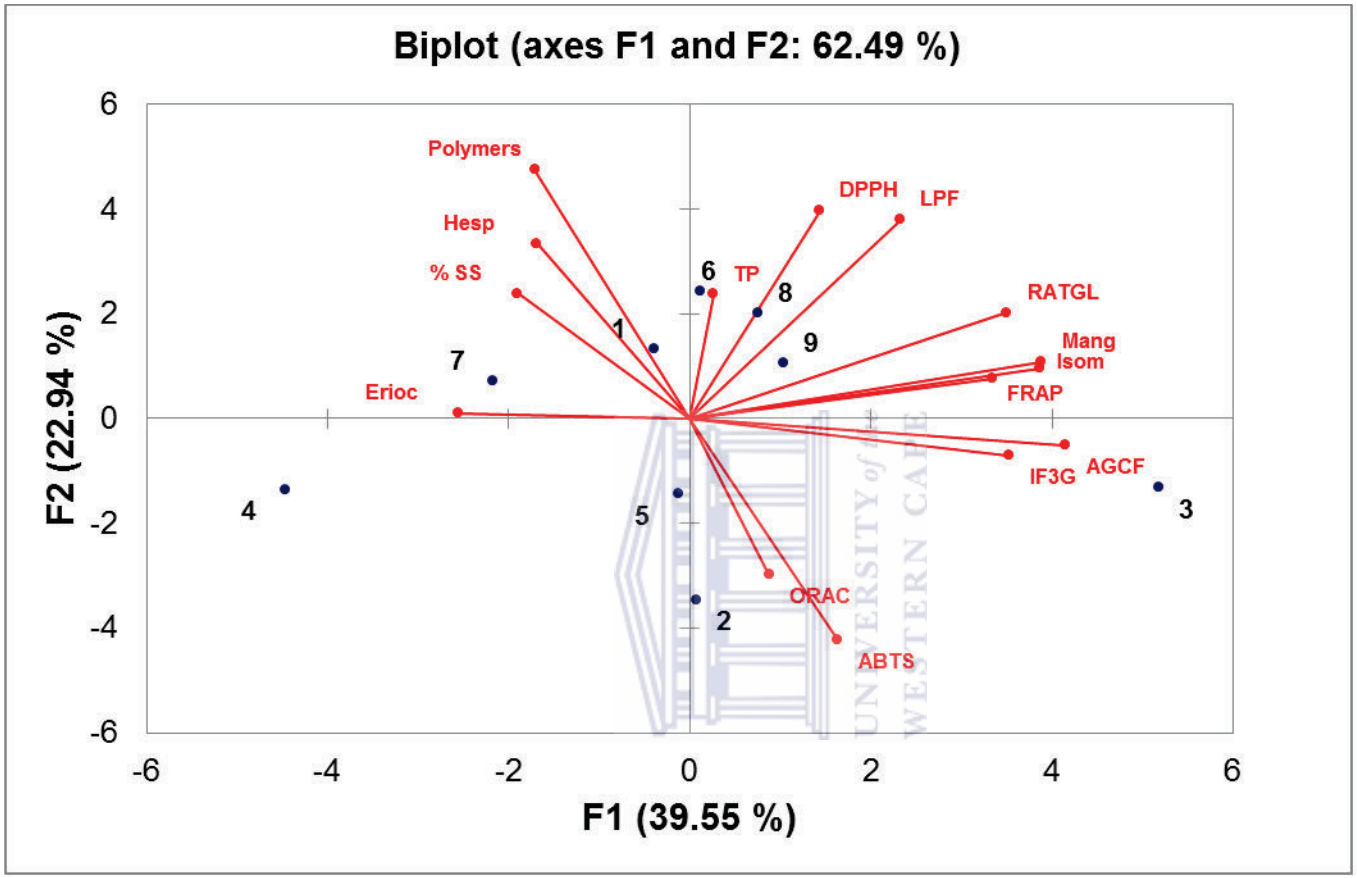

Figure 50. PCA biplot with scores (samples) and loadings (parameters) for the unfermented extracts of *C. maculata* (n = 9). The parameters are: % SS (soluble solids), TPC (total polyphenol content), Hesp (hesperidin), Erioc (eriocitrin), IF3G (iriflophenone-3-*C*-glucoside), Mang (mangiferin) and Isom (isomangiferin) including different antioxidant assays ($ABTS^{\bullet+}$, $DPPH^{\bullet}$, ORAC and FRAP), the enzyme assys with fluorimetric probe (LPF: lipase inhibition, AGCF: α-glucosidase inhibition with yeast enzyme and RATGL: α-glucosidase inhibition with mammalian enzyme) and Polymers (polymeric compounds).

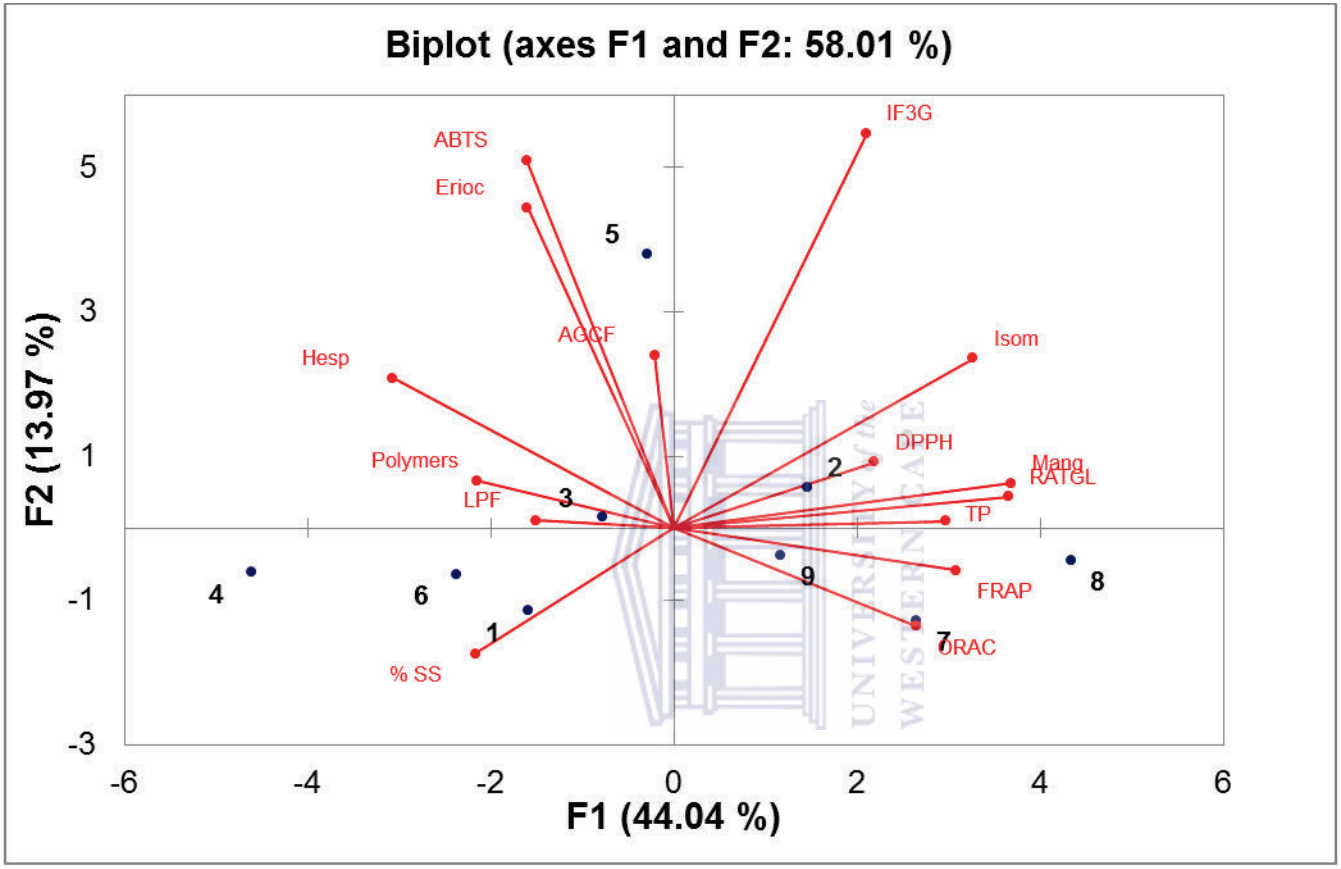

Figure 51. PCA biplot with scores (samples) and loadings (parameters) for the fermented extracts of *C. maculata* (n = 9). The parameters are: % SS (soluble solids), TPC (total polyphenol content), Hesp (hesperidin), Erioc (eriocitrin), IF3G (iriflophenone-3-*C*-glucoside), Mang (mangiferin) and Isom (isomangiferin) including different antioxidant assays ($ABTS^{\bullet+}$, $DPPH^{\bullet}$, ORAC and FRAP), the enzyme assys with fluorimetric probe (LPF: lipase inhibition, AGCF: α-glucosidase inhibition with yeast enzyme and RATGL: α-glucosidase inhibition with mammalian enzyme) and Polymers (polymeric compounds).

5.8 Effect of fermentation on soluble solids content and phenolic composition

Two matrices, illustrating unfermented and fermented samples with the highest and lowest soluble solids content, TPC and contents of individual compounds, were compiled to give an overview of sample variability (Figure 52). From these matrices it is clear that no single sample had the highest or lowest content of all parameters. Furthermore, unfermented samples with highest content of a parameter did not give the fermented counterparts with highest parameters. The same observation is true for samples with the lowest activity. The extent to which fermentation decreased the soluble solids and phenolic content was thus not the same for all samples.

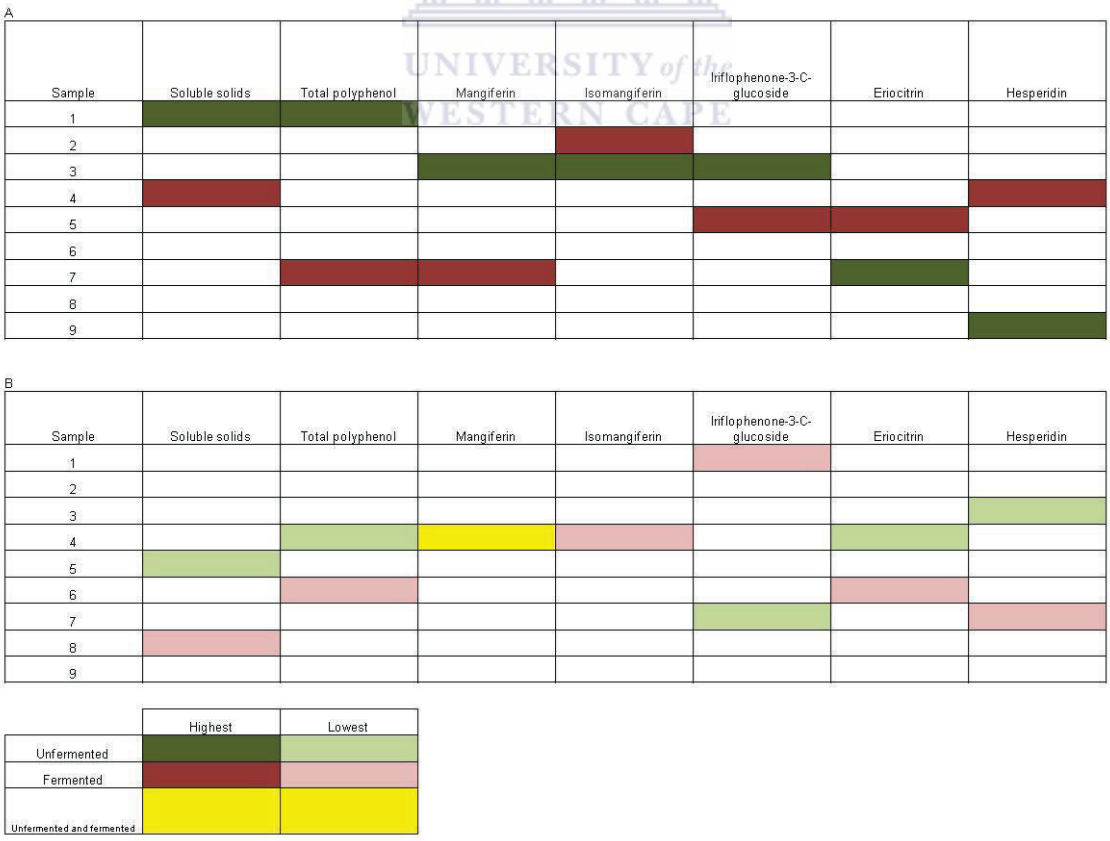

Figure 52. Matrices showing samples with the highest (A) and lowest (B) values for soluble solids, TPC (total polyphenol content), mangiferin, isomangiferin, iriflophenone-3-*C*-β-D-glucoside, eriocitrin and hesperidin.

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Chapter 6

Discussion and Conclusions

Several *Cyclopia* species have been used traditionally for the preparation of the herbal tea known as honeybush tea **(Joubert *et al*., 2008a)**. Its re-discovery in the 1990s partly as result of consumer interest in herbal teas and antioxidants lead to commercialisation of several species, namely *C. intermedia*, *C. subternata* and *C. genistoides* **(Joubert *et al*., 2011)**. Due to its health benefiting properties and its peculiar growth requirements, the demand of honeybush tea exceeds production, thus raising interest in commercial development of other species such as *C. maculata*. Although the unfermented (green) tea has also entered the herbal tea market **(Joubert *et al*., 2008b)**, the success of this herbal tea still relies on the fermented form, because of its unique sweet flavour and taste **(Joubert *et al*., 2011)**.

The current study follows investigations undertaken by a small number of researchers studying the potential health properties of *C. maculata* **(Hubbe, 2000; Dudhia *et al*., 2013; Pheiffer *et al*., 2013; Schulze, 2013; Chellan *et al*., 2014)**. **Hubbe (2000)** evaluated the antioxidant potential of aqueous extracts of several *Cyclopia* spp., including *C. maculat*a for future development of an antioxidant nutraceutical product. **Dudhia *et al*. (2013)** and **Pheiffer *et al*. (2013)** showed that *C. maculata* has potential as an anti-obesity nutraceutical, while the

ability of *C. maculata* to attenuate streptozotocin-induced diabetes and β-cell toxicity was demonstrated by **Chellan *et al*. (2014)**.

Analysis of the aqueous extracts of *C. maculata* showed the presence of high levels of mangiferin, a xanthone known for its antioxidant properties **(Dar *et al*., 2005)**, glucose-lowering effects **(Muruganandan *et al*., 2005)** and the ability to inhibit α-glucosidase **(Phoboo *et al*., 2012)** and pancreatic lipase **(Moreno *et al*., 2006)**, two intestinal enzymes relevant to carbohydrate and lipid metabolism **(Hanhineva *et al*., 2012; Birari and Bhutani, 2007)**.

Within this context, further investigation of the antioxidant and anti-diabesity potential of *C. maculata* was deemed relevant. The present study focused on the effect of fermentation on the antioxidant and anti-diabesity potential of *C. maculata* as assessed in non-cell based assays. Hot water extracts, prepared according to a simulated industrial process, was used, considering its relevance for the food and supplement industries **(De Beer *et al*., 2012)**. Additionally, traditional consumption of honeybush is in the form of a cup of tea, prepared by brewing the fermented plant material for extended periods at low heat **(Du Toit *et al*., 1998)**. Modern preparation of a cup of tea entails infusing one teabag for 5 min in freshly boiled water **(Joubert *et al*., 2008a)**. As part of the evaluation of *C. maculata* as a potential raw material for the production of a standardized antioxidant nutraceutical with anti-diabesity properties, several batches of the plant material were harvested to gain insight into the variation in composition and

bioactivity of the different batches, as well as their behaviour during fermentation. The plant material was expected to vary as it represents wild genotypes, even though all the batches were harvested from a single population in the wild due to limited availability.

In the current study, fermentation decreased the extract yield, total polyphenols content (TPC) and individual polyphenol contents of the extracts, while the polymer content increased. Previous studies on *Cyclopia* species have also shown that fermentation decreased extract yield **(Joubert *et al*., 2008b)** and the soluble solids content of the extracts **(Hubbe, 2000)** and infusion is also affected by the process **(Du Toit and Joubert, 1999; Theron, 2012)**.The soluble fraction is represented by the dried extract (after filtration to remove plant material). The polymers were present at low concentrations, given the low absorbance values observed (Figure 3b). The low polymer values could be a result of low solubility. **Du Toit *et al.* (1998)** showed that fermentation was accompanied by changes in CIELab colour parameters of the plant material, indicative of browning that was attributed to formation of brown polymers. They also found that as fermentation progressed, the infusion colour became lighter, postulated to be the result of the formation of less soluble polymers. The present study demonstrated that the total polyphenol content of the hot water extract decreased by 35 % with fermentation, while **Hubbe (2000)** reported a decrease of 47 %, which could be explained by the different fermentation conditions used (90 °C/16 h *vs* 70 °C/60 h, respectively).

Generally, depending on processing conditions and the type of plant material, different mechanisms are proposed to explain the reduction of TPC and individual polyphenol content observed **(Maillard and Berset, 1995)**. Enzymatic oxidation is initiated when the cellular structure of plant material is damaged. However, high temperatures employed in the present study should inactivate the enzymes as demonstrated for green tea (*Camellia sinensis*) production **(Tounekti *et al*., 2013)**. In the presence of oxygen, chemical oxidation and other degradation reactions will take place **(Shingai *et al*., 2011)**. The high temperatures used during fermentation of *C. maculata* would facilitate these changes, i.e. during fermentation it could be expected that polyphenols are subjected to chemical modifications. These could include polymerisation and complexation with other molecules leading to decreased solubility **(Bonvehi and Coll, 1997; Aikpokpodion and Dongo, 2010)**. Moreover, **Maillard and Berset (1995)** proposed three hypotheses when investigating kilning of barley that could also explain the changes caused by honeybush fermentation: release of bound phenolic compounds; partial degradation of lignin which could lead to the release of phenolic acid derivatives; and/or the thermal degradation of the phenolic compounds. Other investigations on the effect of heat on polyphenols showed that the type of compounds and temperature plays an important role in the extent of change **(Nwanguma and Eze, 1996; Larrauri *et al*., 1997; Ebun and Santosh, 2011)**.

Joubert *et al*. (2008b), using the same fermentation conditions as **Hubbe (2000)**, found that fermentation reduced the TPC of hot water extracts of *C. intermedia, C. subternata, C. sessiliflora* and *C. genistoides* between 23 % and 46 % with *C. genistoides* being the least affected. This indicates that *Cyclopia* species are not affected to the same degree by fermentation. Furthermore, it is noteworthy that **Hubbe (2000)** observed a 33 % reduction of TPC of *C. genistoides* using the same fermentation conditions as **Joubert *et al*. (2008b)**. These findings suggest that other factors apart from fermentation conditions could play a role. This could also be associated with the varying contents of individual phenolic compounds present in the different *Cyclopia* species **(Joubert *et al*., 2008b)** or within a specific species and their susceptibility to thermal degradation. Both the present study and **Joubert *et al*. (2008b)** showed that individual phenolic compounds are also not affected to the same extent by fermentation, i.e. mangiferin was shown to be highly susceptible to thermal degradation, while hesperidin was less affected. A study by **Neelakandan and Kyu (2009)** showed that mangiferin (in a dry form) is stable, as a significant weight loss was only observed at temperatures above 250 °C. However, **Chen *et al*. (2008)** reported that mangiferin in aqueous solution is unstable at T > 100 °C over time. The fermentation temperature used in this study was 90 °C and the plant material was kept moist (>60 % moisture content) throughout the fermentation period of 16 h. These conditions would thus be conducive to thermal degradation of mangiferin as observed for *C. maculata*. It is noteworthy that isomangiferin, regardless of its structural similarity with mangiferin, was

more resistant to thermal degradation, showing 47 % degradation compared to 77 % of mangiferin. Both compounds have the same aglycone, i.e. 1,3,6,7-tetrahydroxy xanthone, but differ in the position of the sugar. Isomangiferin is the 4-*C*-glucoside regio-isomer of mangiferin. One could speculate that the position of sugar play a protective role against oxidation under these conditions and that their behaviour under oxidative conditions, such as during antioxidant activity determination, would provide some insight. However, **Hubbe (2000)** reported similar DPPH$^{\bullet}$ scavenging activity for mangiferin and isomangiferin, while **Malherbe *et al*. (2014)** found isomangiferin to be slightly less reactive towards DPPH$^{\bullet}$ in an on-line HPLC-BCD assay. However, in the on-line ABTS$^{\bullet+}$ and ORAC assays, the reverse were found **(Malherbe *et al*., 2014)**. Given these findings, the relative antioxidant activity of mangiferin and isomangiferin does not explain their susceptibility to degradation under oxidative conditions. This conclusion is supported by the behaviour of the benzophenone, iriflophenone-3-*C*-β-D-glucoside, given its high level of thermal degradation (71 %) and relative weak antioxidant activity in the on-line assays **(Malherbe *et al*., 2014)**. No data on the thermal stability of iriflophenone-3-*C*- β-D-glucoside could be found in literature, but benzophenone decomposes on heating to produce toxic agents to react with strong oxidants **(No and No, herbpedia.wdfiles.com)**. From these results it is evident that the antioxidant activity of a compound does not give an indication of its susceptibility to thermal degradation under oxidative conditions.

Antioxidant activity of the extracts as affected by fermentation was investigated, using a battery of assays to take into account the overall concentrations and

compositions of diverse antioxidants **(Tabart *et al*., 2009)**. A reduction in total antioxidant capacity (TAC) values for all assays employed was detected with fermentation. This is not surprising, as previously discussed, fermentation caused a significant reduction in TPC of the extracts as well as the individual polyphenols. Good correlations between TPC, contents of individual compounds and the different antioxidant assays were observed. The decrease in content, chemical modifications such as polymerisation and oxidative degradation would explain the reduction in TAC. Polymerisation of polyphenols entails formation of bonds at phenolic hydroxyl groups, which would be accompanied by loss of antioxidant activity. The negative correlation between polymer content and TAC values supports this. Chemical modification of flavonoids which take place during fermentation in the presence of oxygen would also include oxidation of the hydroxyl groups on the A-ring or the breaking of the C2=C3 bond on the B-ring of flavonoids, structural features for antioxidant activity **(Nijveldt *et al*., 2001; Woodman *et al*., 2005; Xiao *et al*., 2013a, b)**. The flavanones, hesperidin and eriocitrin, have also been shown to be less affected by fermentation than mangiferin. Therefore, the type of compound could also influence the susceptibility of the compound to thermal degradation.

The decrease in antioxidant capacity of the extracts with fermentation as shown with the $DPPH^{\bullet}$, $ABTS^{\bullet+}$, ORAC and FRAP assays was in agreement with trends observed in previous studies **(Hubbe, 2000; Joubert *et al*., 2008b)**. **Hubbe (2000)** employed the DPPH and superoxide anion radical scavenging assays, while **Joubert *et al*. (2008b)** employed the FRAP and $ABTS^{\bullet+}$ scavenging

assays. In the present study the same reference antioxidant, Trolox, was used for all assays so that TAC values could be compared. TAC values of the different assays increased in the order FRAP < DPPH$^{\bullet}$ < ABTS$^{\bullet+}$ < ORAC for both the unfermented and fermented extracts. From the on-line biograms it is clear that constituents are present in the extracts that reacted in the FRAP assay, but not in the other assays. The difference between the TAC values of the different assays is explained by their mechanisms of action **(Prior *et al*., 2005)**. The FRAP assay gives an indication of the ability of the extracts to reduce iron, whereas DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays measure hydrogen donating ability. The ORAC is based on the inhibition of peroxyl-radical-induced-oxidation **(Ou *et al*., 2001)**. Other factors that would affect radical scavenging are the solvent and pH **(Sharma and Bhat, 2009)**. Solvent polarity significantly influences the antioxidant activity. Alcoholic solvents have been reported to have a great effect on the methods that involve hydrogen transfer mechanism and polar antioxidants such as polyphenols **(Litescu *et al*., 2014)**.

Considering the antioxidant activity of individual compounds, previous studies have shown that their reactivity depends on the radical. The flavanone, hesperidin, showed low to no antioxidant activity in comparison to mangiferin when evaluated in DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and FRAPassays **(Hubbe, 2000; Joubert *et al*., 2008b)**. However, this compound behaved as a better antioxidant in the FRAP assay than it did in the ABTS$^{\bullet+}$ assay **(Joubert *et al*., 2008b)**. **Tabart *et al*. (2009)** also showed that hesperidin had no activity in the DPPH$^{\bullet}$ and little

activity in ABTS$^{\bullet+}$ assays, but high activity in the ORAC assay. Eriocitrin showed similar antioxidant capacity in the ABTS$^{\bullet+}$ and FRAP assays and was more active than hesperidin in both assays, and more active than mangiferin in the FRAP assay **(Joubert *et al*., 2008b)**. The low antioxidant activity observed for hesperidin could be attributed to glycosylation of the compound, as this has been reported to decrease antioxidant activity **(Williamson *et al*., 1999; Londono-Londono *et al*., 2010)**. **Malherbe *et al*. (2014)** showed that the benzophenone, iriflophenone-3-*C*-β-D-glucoside, presented no scavenging ability against DPPH$^{\bullet}$, but scavenged ABTS$^{\bullet+}$ and peroxyl radicals generated in the ORAC assay. In the ABTS$^{\bullet+}$ assay, iriflophenone-3-*C*-β-D-glucoside was a weaker antioxidant than mangiferin and isomangiferin ($TEAC_{ABTS}$ of 1.04, 1.67 and 1.82, respectively). Similarly, it was a weaker antioxidant than isomangiferin in the ORAC assay ($TEAC_{ORAC}$ of 3.61 and 4.14, respectively). However, the antioxidant capacity of iriflophenone-3-*C*-β-D-glucoside was equivalent to that of mangiferin ($TEAC_{ORAC}$ 3.69). Moreover, isomangiferin showed a slightly lower antioxidant capacity than mangiferin against DPPH$^{\bullet}$, but higher capacity against ABTS$^{\bullet+}$ and ORAC.

In the present study the antioxidant capacity of the individual compounds was not determined, but the analysis of the extracts by the on-line HPLC assays (FRAP, DPPH$^{\bullet}$ and ABTS$^{\bullet+}$) provide some indication of the activity of the compounds. Hesperidin showed no detectable activity (unlike the xanthones). This apparent lack of activity is partially attributed to the difference in reaction times between the micro-plate and on-line HPLC assays, as times vary from as short as

0.6 minutes in the on-line assays to 120 minutes in the micro-plate DPPH• assay. The short reaction time of the on-line assays thus limits the time for the potential antioxidant (i.e hesperidin) to react with the radical, as on-line antioxidant assays are suitable for quick reacting antioxidants **(Malherbe *et al*., 2012)**. **Schulze (2013)** using another approach, investigated the use of advanced chromatographic fingerprint analysis to identify compounds that contributed to the prediction of TAC_{DPPH} as determined by micro-plate assay. Compounds identified in this manner were iriflophenone-di-*O*,*C*-hexoside, iriflophenone-3-*C*- β-D-glucoside, mangiferin, isomangiferin, vicenin-2, 3-hydroxy phloretin-3',5'-di-*C*-hexoside, eriocitrin, scolymoside a 7-*O*-rutinoside of luteolin, phloretin-3',5'-di-*C*-β-D-glucoside and hesperidin. **Dudhia *et al*. (2013)** analysed hot water extracts of unfermented *C. maculata* and showed that it contained traces of scolymoside and low levels of phloretin-3',5'-di-*C*-β-D-glucoside (170 mg/100 g extract). As discussed some of these compounds have been shown to be antioxidants. By comparison, radical scavenging activity of scolymoside was found to be better than that of chlorogenic acid **(Wang *et al*., 2003)**. Vicenin-2 has been reported to have moderate radical scavenging ability against DPPH• (IC_{50} of 90.5 µM), but a significantly weaker antioxidant capacity than quercetin (IC_{50} of 7.2 µM) **(Velezo *et al*., 2009**).The dihydrochalcone, phloretin-3',5'-di-*C*-β-D-glucoside, due to the similarity in structure to nothofagin, a compound present in rooibos and reported as a potent antioxidant (IC_{50} = 4.04 µM) **(Snijman *et al*., 2009)**, could also be expected to contribute to the antioxidant activity of *Cyclopia* extracts. The results of the present study

suggest that TAC values would be more suitable as quality control parameters than individual compound contents. If standardization of extracts needs to be performed, a large number of samples would be recommended to gain insight into the extent of expected variation. When interpreting TAC values, the antioxidant assay used must be taken into account as different values are obtained with different assays **(Prior *et al*., 2005)**. A contributing factor to this is the lack of validated protocols and methods dedicated to antioxidant assessment of either, food, beverages biological samples or pharmaceutical formulas. Sample handling is another contributing factor to the variation between these assays **(Litescu *et al*., 2014)**.

The anti-diabesity potential of *C. maculata* extracts was assessed using inhibition of two enzymes important in digestion; α-glucosidase and pancreatic lipase as indication of anti-diabetic and anti-obesity potential, respectively. Micro-plate assays were used in both cases. Inhibition was obtained against the enzymes used in the study, suggesting the use of unfermented *C. maculata* extracts as potential anti-diabesity nutraceuticals. Generally, fermentation reduced the enzyme inhibitory activity of the extracts. Again, samples were not affected to the same degree by fermentation. Colour interference of the fermented extracts (as a result of polymers, known to absorb at 405 nm) **(Cilliers *et al*., 1990; Theron, 2012)** was encountered with the colorimetric assays, thus necessitating the use of fluorescence detection. However, higher concentrations of the extracts were required to obtain a measureable activity when using fluorimetry rather than

colorimetry. It is noteworthy that the increase in sample concentration did not affect the activity of the extract but only increased the accuracy of the results as the fluorimetric assay is reported to be more sensitive than colorimetric assay **(Margesin *et al*., 2002)**. This obeserevation could result from molecular interactions between particular compounds as antagonistic and synergistic effects have been demonstrated for phenolic compounds in complex mixtures **(Palafox-Carlos *et al*., 2012)**.

Mimicking the method used by **Muller *et al*. (2012)** for α-glucosidase inhibitory effects of rooibos extracts, the unfermented and fermented extracts of *C. maculata* were firstly screened for their α-glucosidase inhibitory effects in the micro-plate assay format using yeast α-glucosidase as it is readily available in a pure form **(Hogan *et al*., 2010)**. The extracts were further evaluated using the mammalian enzyme (from rat) for physiological relevance of the analysis **(Hu *et al*., 2013; Misbah *et al*., 2013)** and different results were obtained. At 500 µg/mL, using the yeast and rat α-glucosidase fluorimetric assays, an increase in enzyme inhibitory activity was observed (unfermented: 43 % and fermented: 52 % increase) when analysis was performed using rat α-glucosidase. However, the α-glucosidase inhibitory activity of the extracts was significantly weaker than that of the controls (55 % and 41 % inhibition for (+)-catechin and Acarbose™, respectively, both evaluated at a final concentration of 200 µg/mL). These findings suggest distinct differences in inhibitory potency of active compounds in the extracts towards α-glucosidase

from different sources. This difference in activity by plants extracts have also been observed by other researchers, for example, **Hogan *et al*. (2010)**, observed lower activity for pomace extracts when using rat α-glucosidase than yeast α-glucosidase. Differences in substrate specificity of the different enzyme sources used **(Kimura *et al*., 2004)** could also play a role. These were also in agreement with the findings of **Kim *et al*. (2008a)**, in which they investigated the inhibitory effects of 2,4,6-tribromophenol against two enzymes of different sources (*Saccharomyces cerevisiae* and *Bacillus. stearothermophilus*) and obtained IC_{50} values of 60.3 and 110.4 μM, respectively. In addition to that, **Uddin *et al*. (2012)** also obtained significantly different results (IC_{50} = 89 and 63 μM, respectively) when investigating the α-glucosidase inhibitory activity of pistagremic acid (PA) against yeast and mammalian (rat intestinal) enzymes. They explained this in terms of the molecular binding mode, which revealed hydrogen bonding interactions between PA and amino acids surrounding the catalytic site of the enzymes. This enzyme specificity also contribute to the necessity of using different positive controls (such as (+)-catechin for yeast and Acarbose™ for rat intestinal enzyme) for a valid and accurate comparison **(Uddin *et al*., 2012)**.

In an attempt to identify compounds responsible for the activity of the extracts, the on-line HPLC-BCD assay, as employed by **Muller *et al.* (2012)**, was also used in the present study. Compounds present in the aqueous extracts of *C. maculata,* however, did not show any detectable activity. Pure mangiferin (major

compound), tested in the micro-plate assay, was shown to inhibit rat α-glucosidase, but it had substantially lower activity than Acarbose™ (IC_{50} = 52 mM and 1.8 mM, respectively) **(Phoboo *et al*., 2012)**, which could explain the undetectable activity shown by the mangiferin containing extracts of *C. maculata* as per on-line HPLC assays. The relative concentrations of compounds should be considered. The stock solution injected (10 µL) had a very low mangiferin concentration (360 µg/mL = 0.85 µM). On the other hand, (+)-catechin, added to the extract before injection, also did not show inhibitory activity, whereas **Muller *et al*. (2012)** demonstrated a negative peak for (+)-catechin, i.e. inhibitory activity. Mangiferin was also shown to inhibit the digestive enzymes sucrase and isomaltase (from rat) **(Yoshikawa *et al*., 2001)**. Its derivative, isomangiferin, is also reported to be "potent" α-glucosidase inhibitor at high concentration **(Sellamuthu *et al*., 2009; Girones-Vilaplana *et al*., 2014)**. The inhibitory effect of these xanthones was supported by a strong correlation between their content in the *C. maculata* extracts and the inhibition of rat α-glucosidase (R^2 values 0.9472 and 0.9512, respectively) observed in this study. **Shivaprasad *et al*. (2014)** showed yeast α-glucosidase inhibitory activity of mangiferin to be similar to that of Acarbose™ (IC_{50} = 69.04 µg/mL and 74.95 µg/mL, respectively), while **Dineshkhumar *et al*. (2010)** had previously shown this compound to be 50 % more effective (IC_{50} = 41.88 µg/mL) than the standard Acarbose™ (IC_{50} = 83.33 µg/mL) using the same source of enzyme. It is however, noteworthy that this drug has been reported to be a weak inhibitor of yeastα-glucosidase **(Uddin *et al*., 2012)**. Hesperidin, a major compound in the *C.*

maculata extracts was shown to have weak α-glucosidase inhibitory activity **(Shen *et al*., 2012)**, which would explain the moderate correlation (R^2 = 0.7996) obtained in this study. One of the phenolic compounds detected in the extracts, but not quantified due to very low levels, is the flavone aglycone, luteolin. Potent α-glucosidase inhibitory activity has been reported for this compound at 5 mg/mL (17 μM) **(Kim *et al*., 2000; Nicolle *et al*., 2011)**. Vicenin-2 (apigenin-6,8-di-*C*-β-D-glucoside) has also been reported to inhibit not only yeast α-glucosidase but also protein tyrosine phosphatase 1B and rat lens aldose reductase **(Islam *et al*., 2014)**. These findings suggest this compound to have multiple target-oriented therapeutic qualities for the treatment of diabetes and diabetes-associated complications, such as inflammation and oxidative stress. **Kim *et al*. (2008)** suggested that the potential of α-glucosidase inhibitors is related to their ability to mimick the pyranosyl moiety of glucose (α-D-glucose). Moreover, the presence of these compounds in *C. maculata* extracts could explain the α-glucosidase inhibition observed in this study using the micro-plate assays. The difference in anti-diabetic properties of compounds is owed to the structure-activity relationship with the enzyme due to enzyme specificity.

Structural configurations of the phenolic compounds have been reported to play a major role in binding of the compound to the active site of α-glucosidase. Many of the structural features necessary for antioxidant activity were also shown to be important for enzyme inhibition. Hydroxylation has a major impact on α-glucosidase inhibitory activity. On the other hand, hydrogenation of C2-C3

double bond and glycosylation as observed for hesperidin may result in weak activity **(Xiao *et al*., 2013 a, b)**. Slight modifications in the chemical structures may have an impact on the activity, for example, **Chen *et al.* (2013)**, demonstrated different IC_{50} values (244 and 266 µM) for α-glucosidase inhibitory activity of vitexin and its regio-isomer isovitexin, respectively. The difference in position of the sugars could be responsible for the lower α-glucosidase inhibitory activity of isovitexin. This could also be explanatory to the reported difference in α-glucosidase inhibitory potency of mangiferin and isomangiferin.

As observed for all other parameters measured in the current study, fermentation resulted in a significant decrease in lipase inhibitory activity of *C. maculata* extracts. Due to thermal susceptibility or resistance of individual compounds present in the extracts, variability in the lipase inhibitory effect of individual extracts was observed. Both unfermented and fermented extracts showed inhibition of lipase when assessed using the colorimetric or fluorimetric probes. In an attempt to identify active lipase inhibitors on the on-line HPLC-BCD assay, no detectable activity was observed from the compounds. As the method was still in development when applied other factors than inability of the individual compounds to inhibit the enzyme could have contributed to this apparent inactivity. A number of the phenolic compounds in *C. maculata* extract have been shown to have lipase inhibitory effects in microplate assays by other researchers. Hesperidin showed an IC_{50} value of 32 µg/mL when evaluated for lipase inhibition activity by **Kawaguchi *et al*. (1997)**. **Yoshikawa *et al*. (2002)** showed weak

activity against lipase for a mangiferin concentrated hot water extract of *Salacia reticulata.* Moreover, IC_{50} values of 1750 and 1 µg/mL were reported for lipase inhibition of mangiferin and Orlistat™, respectively **(Shivaprasad *et al*., 2014)**. **Zheng *et al*. (2010)** demonstrated that luteolin exhibited weaker lipase inhibition activity (17 % inhibition) than Orlistat™ (87 % inhibition) at the same concentration (25 µg/mL).

Therefore, the presence of mangiferin and hesperidin as major compounds in the extracts of *C. maculata* could explain the significant lipase inhibitory activity observed as per micro-plate assays. As for α-glucosidase inhibition, the presence and position of the sugar moiety affects lipase activity of the compounds. For example, **Lee *et al*. (2010)** demonstrated that the inhibitory activity of luteolin which does not have a C-glycosyl group on the A-ring of its flavonoid moiety, against pancreatic lipase was much weaker than those of C-glycosylated derivatives on their A-ring.

Previous research showed that crude extracts could be more potent lipase inhibitors than their isolated compounds. For instance, **Bustanji *et al*. (2010)** demonstrated that a methanolic extract from *Rosmarinus officinalis* (rosemary) showed higher lipase inhibitory activity than its phenolic acids, chlorogenic acid and caffeic acid (IC_{50} values of 13.8, 96.5 and 32.6 µg/mL, respectively).

No detailed literature could be found on anti-obesity potential of the newly identified compounds of *C. maculata* (i.e. iriflophenone-di-*O*,*C*-hexoside,

3-hydroxyphloretin-3',5'-di-*C*-hexoside, scolymoside, vicenin-2, and phloretin-3',5'-di-*C*-β-D-glucoside) of *C. maculata* **(Schulze, 2013)** as determined by the ability to inhibit lipase. In the present study iriflophenone-3-*C*-β-D-glucoside and eriocitrin showed moderate correlation (R^2 values of 0.6731 and 0.7370, respectively) with lipase inhibition activity of the extracts. Lipase inhibitory activities of the pure compounds has not yet been evaluated, however, extracts of *C. maculata* and *C. subternata* in which these compounds are present in relatively high levels, have been shown to inhibit adipogenesis in 3T3-L1 pre-adipocytes, suggesting the extracts to possess anti-obesity properties **(Dudhia *et al*., 2013)**. **Zhang et al. (2013)** showed that iriflophenone-3-*C*-β-D-glucoside regulates lipid homeostasis.

A factor that cannot be ruled out when considering the contribution of individual compounds to the activity of a complex mixture such as a plant extract is synergism and antagonism. The effectiveness of inhibition by different individual phenolic components depends on their site of action, their mechanism and their binding affinities, which may either elevate or reduce therapeutic potencies of the active compounds **(Boath *et al*., 2012; Qin *et al*., 2012)**. Therefore, the mechanism of action of active constituents should be well established before making conclusions about the potency of the extracts. Mixtures of phenolic acids were shown to be more effective inhibitors of lipase than single compounds **(Cai *et al*., 2012)**. **Boath *et al*. (2012)** demonstrated that polyphenol-rich berry extracts potentiated the inhibition caused by the drug, Acarbose™. Low doses of

the drugs in combination with phenolic compounds could thus reduce the reported side effects of the commercial anti-diabesity drugs.

In summary, the presence of several compounds in the unfermented extracts of *C. maculata*could explain to some extent the observed lipase and α-glucosidase inhibitory activity of these extracts as per micro-plate assays. The polymers present in the extracts might also contribute to the observed activity as a good correlation was obtained between polymer content and ratα-glucosidase inhibition (R^2 = 0.9049) and a moderate correlation for lipase (R^2 = 0.6742), suggesting the polymers to also exhibit anti-diabesity potential. This finding is in agreement with that of **Nakai *et al.* (2005)**, showing that polymerisation of tea polyphenols enhances pancreatic lipase inhibition. This could be related to the affinity of polyphenols, especially oligomeric polymers to bind proteins **(Kawai *et al.*, 2003)**.

Although varying concentrations of individual compounds were detected in different extracts and the effect of fermentation on the composition of the extract varied, small inter-batch variation (RSD < 5 %) was observed for the enzyme inhibition assays and antioxidant assays. By using principal component analysis (PCA) the most “active” extract(s) as determined by one or more parameters could be identified. Unfermented sample 3 gave the highest association with both α-glucosidase inhibition and the FRAP assay. However, unfermented samples 8

and 9 showed great association with lipase inhibition. Both extracts also showed the best association with DPPH$^{\bullet}$ scavenging activity.

The original plant material used in the current study comprised leaves and stems of *C. maculata,* harvested from different bushes to assess inter-batch variation. The individual compound concentrations as determined in the present study were similar to that determined by **Schulze (2013)**, who analysed a large sample set of *C. maculata* seedling plants raised in pots (n = 40) and plants collected in the wild (n = 10). Interestingly, **Schulze (2013)** also showed that the inter-sample %RSD for individual compound content values were much higher for *C. subternata* samples (from seedling plants) than for *C. maculata* samples.

The phenolic content of aqueous extracts from *C. maculata* was comparable to that of previous studies **(Dudhia *et al*., 2013; Schulze, 2013)** with regards to the individual compounds identified in the current study. The total polyphenol content of the extracts had a mean value (24 %), similar to those observed by **Dudhia *et al*. (2013)** (22 %). The highest mean values of the extracts in the present study were observed for mangiferin (5.4 %), isomangiferin (1.6 %) and hesperidin (0.9 %). Lower mean values were observed for iriflophenone-3-*C*-β-D-glucoside (0.4 %) and eriocitrin (0.2 %). These results were similar to that of **Schulze (2013)**, observing mean values of 5.1, 1.5 and 0.9 % for mangiferin, isomangiferin and hesperidin, respectively for hot water extracts of *C. macul*ata leaves and stems. Similarly, low mean values were also observed for

iriflophenone-3-*C*-β-D-glucoside (0.5 %) and eriocitrin (0.3 %) **(Schulze, 2013)**. Compared to hot water extracts of *C. subternata* **(De Beer *et al*., 2012)** *C. maculata* extracts contains much higher levels of mangiferin, isomangiferin and hesperidin, but lower levels of iriflophenone-3-*C*-β-D-glucoside and eriocitrin. **De Beer *et a*l. (2012)**, analysing *C. subternata* and **Du Preez (2014)**, analysing *C. maculata*, showed that hesperidin occurs predominantly in the stems, while mangiferin, isomangiferin and eriocitrin are predominant in the leaves. **De Beer et al. (2012)** also analysed for iriflophenone-3-*C*-β-D-glucoside, showing higher levels in the leaves than the stems. This indicates that the ratio of leaves to stems will affect the composition of the extract. *Cyclopia maculata*, when not harvested frequently, forms thick stems **(Joubert *et al*., 2011)**. It is noteworthy that, although *C. maculata* extracts contain higher levels of the major antioxidants (i.e. xanthones) than *C. subternata*, it showed low TAC values than the latter species as evaluated by DPPH, ORAC and FRAP assays **(De Beer *et al*., 2012)**. The lower TPC values of the unfermented *C. maculata* extracts compared to those of *C. subternata* extracts would support this in view of the correlation between TAC and TPC, demonstrated in the present study.

Irrespective of these quantitative differences in composition and antioxidant activity between *C. maculata* and *C. subternata*, **Dudhia *et al*. (2013)** reported similar anti-obesity properties as assessed in 3T3-L1 pre-adipocytes for hot water extracts of these species. In their study, both unfermented andfermented *C. maculata* displayed anti-adipogenic properties, suggesting that fermentation

does not affect the ability to inhibit fat and tryglyceride accumulation. These findings thus suggest that *C. maculata* is a promising anti-diabesity nutraceutical sincefermented plant extracts are preferred due to their sensory properties. Moreover, this potential should be evaluated in a complex physiological system before a conclusion can be made with regards to the relative potency of the extracts.In comparison with other species, less variation in the individual phenolic content of *C. maculata* extracts has been demonstrated in the current study. This could be advantageous with regard to the production of a standardised honeybush extract by industry.

Recommendations

The overall aim of the study was to evaluate antioxidant and anti-diabesity potential of *C. maculata* using *in vitro* non-cell based screening assays.

From the results obtained in this study, the following conclusions may be drawn:

- *Cyclopia maculata*, like other commercial *Cyclopia* species, is rich in polyphenols, some of which possess a number health benefiting properties, thus supporting its potential as a health beverage or nutraceutical.
- Based on multiple biological activities of compounds, such asvicenin 2, mangiferin, isomangiferin, hesperidin and iriflophenone-3-*C*-β-D-glucoside, present in *C. maculata,* this plant might be a useful source of compounds that could provide a lead compound for the development of multiple target-oriented therapeutic modalities for the treatment of diabetes and diabetes-associated complications.

- Fermentation resulted in a significant reduction of the phenolic content of the extracts, as well as a significant reduction of activity in all parameters evaluated in this study. This is mainly due to the decrease in content of individual phenolic constituents. Structural modification of active compounds as a result of oxidation may also have affected activity. Therefore, unfermented plant material should preferentially be used for preparation of extracts.
- Different TAC values were obtained for different antioxidant assays. These relate to the different mechanisms of the assays and solvent mediums. Overall the FRAP assay would be the recommended assay for investigation on antioxidant capacity of plant extracts as is showed the best correlation with all quantified phenolic compounds including total polyphenol and polymeric compound contents. However, no single assay can accurately reflect all radical sources or all antioxidants in a mixed or complex system **(Prior *et al*., 2005)**. Therefore, considering the physiologically relevant radical of the ORAC assay, this assay should preferably be included as one of the biological non-cell based assays when evaluating the nutraceutical potential of *C. maculata*.

- The major compounds in *C. maculat*a extracts, i.e. mangiferin, isomangiferin and iriflophenone-3-*C*-β-D-glucoside, could be used as chemical markers to obtain a standardized antioxidant nutraceutical. Their anti-diabesity activity, however, needs to be further explored before they can be used as chemical markers for a nutraceutical product aimed at the anti-diabesity market.

- A combination of the measured parameters could be useful in product standardisation by providing a basis for specifying minimum levels.
- Total polyphenols and total antioxidant activities are generally used by industry to standardise honeybush extracts **(Joubert *et al*., 2011)**, however, complex physiological systems are also to be considered in future to account for synergistic and antagonistic effects of these complex mixtures.
- Orlistat™ and Acarbose™ (the only clinically available anti-diabesity drugs) have been associated with adverse gastrointestinal side effects. Plant extracts such as *C. maculata* extracts are a promising solution to this problem. However, toxicology studies are recommended.

Based on these conclusions, the following recommendations can be made:

- The use of selected plants, propagated on large scale, will aid product standardisation.
- A larger number of samples should in future be analysed for lipase or α-glusosidase inhibitroty potential to set representative baseline data for selected parameters used for standardization of a *C. maculata* nutraceutical.
- For future research, an improved HPLC method such as that recently developed by Schulze (2014) should be employed for better separation of the phenolic constituents of the extracts and to gain greater insight into the phenolic composition of *C. maculata*. This could also facilitate improved on-line monitoring of biological activities.
- Further research on the anti-diabesity potential of *C. maculata* is recommended, with special focus on the combination of the clinical anti-

diabesity drugs and bioactive compounds, tested in appropriate complex physiological systems.

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Addendum

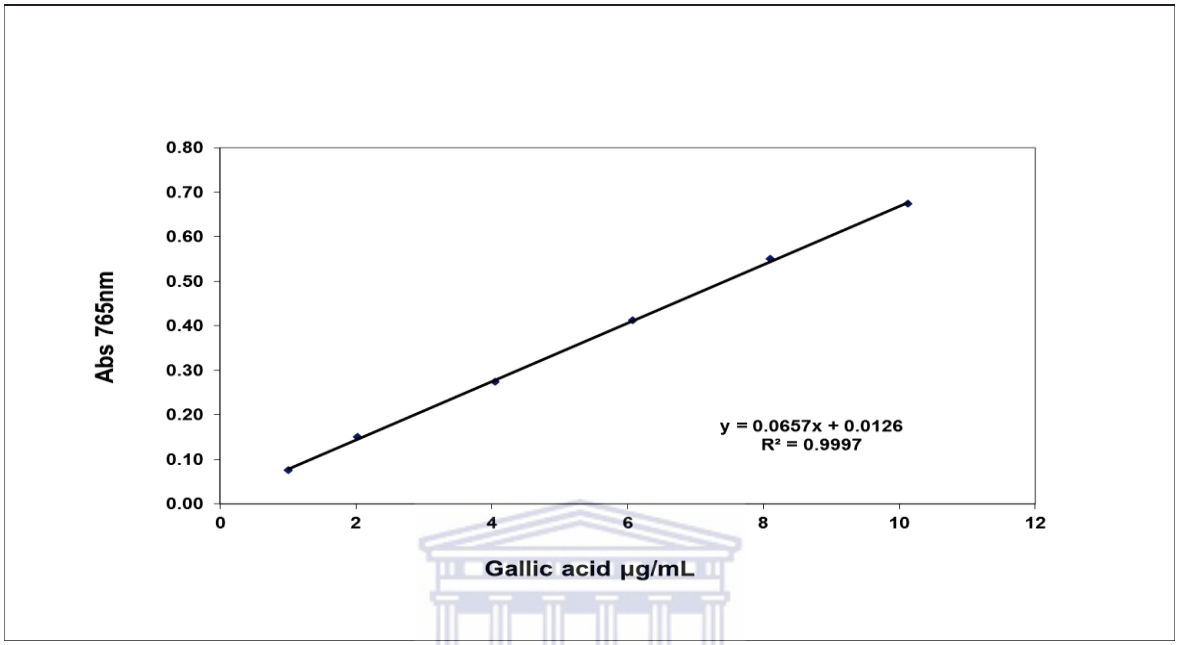

Figure A1. Standard curve of gallic acid concentration (µg/mL) in reaction mixture *versus* absorbance at 765 nm in theFolin–Ciocalteau Reagent (FCR) method.

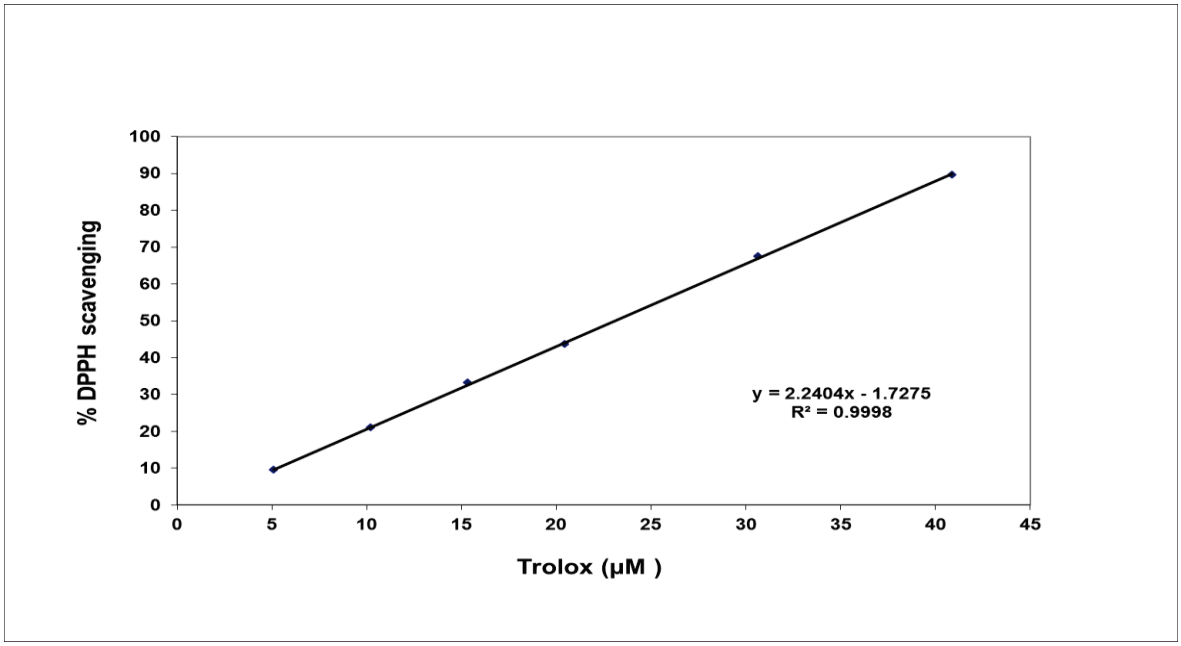

Figure A2. Standard curve of Trolox concentration (µM) in reaction mixture *versus*percentage scavenging of DPPH• measured at 515 nm in the DPPH• scavenging method.

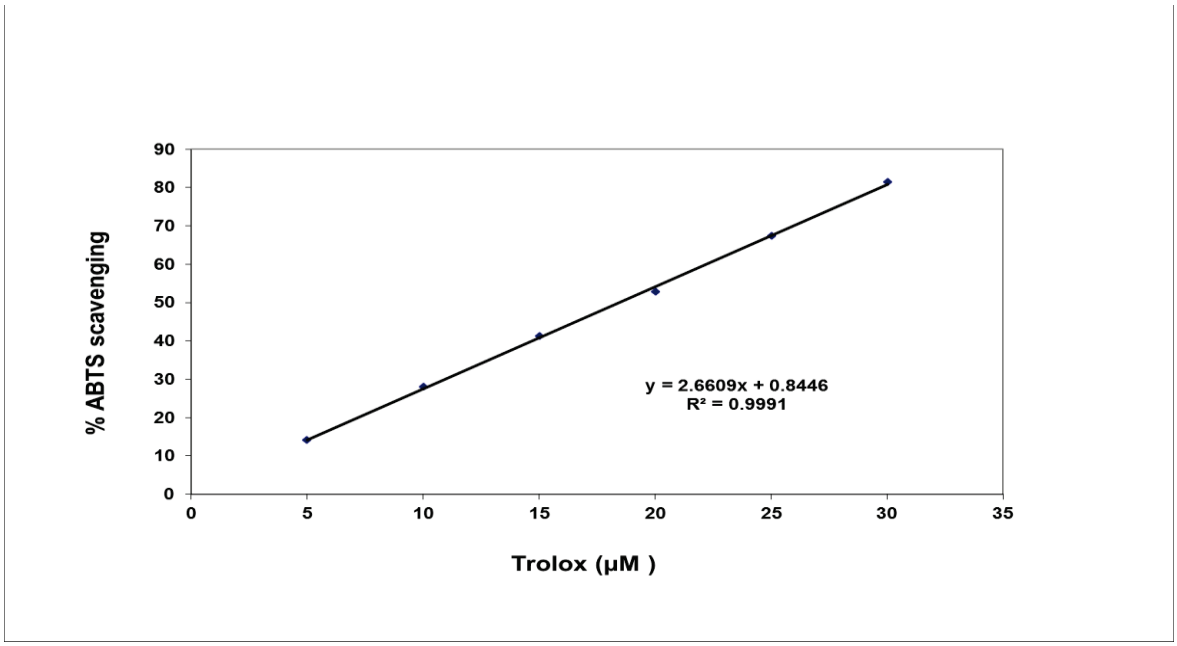

Figure A3. Standard curve of Trolox concentration (µM) in reaction mixture *versus* percentage scavenging of $ABTS^{\bullet+}$ measured at 734 nm in the $ABTS^{\bullet+}$ method.

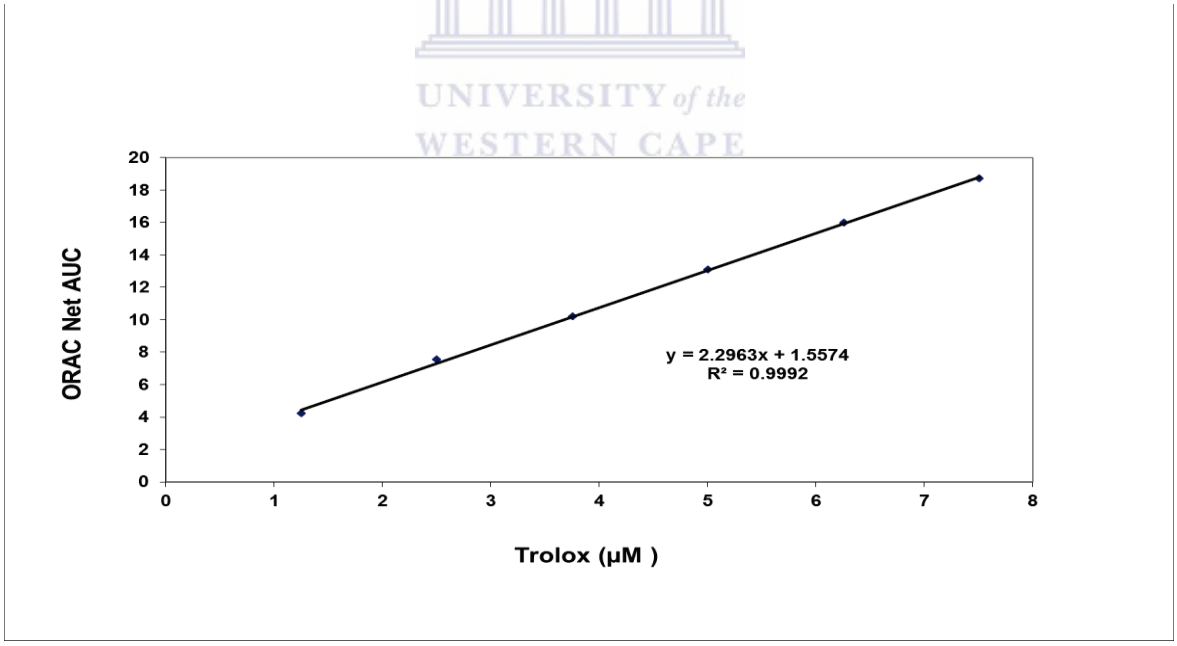

Figure A4. Standard curve of Trolox concentration (µM) in reaction mixture *versus* fluorescence intensity (Net AUC) measured at 485/20 nm Excitation and 530/25 nm emissionin the ORAC method.

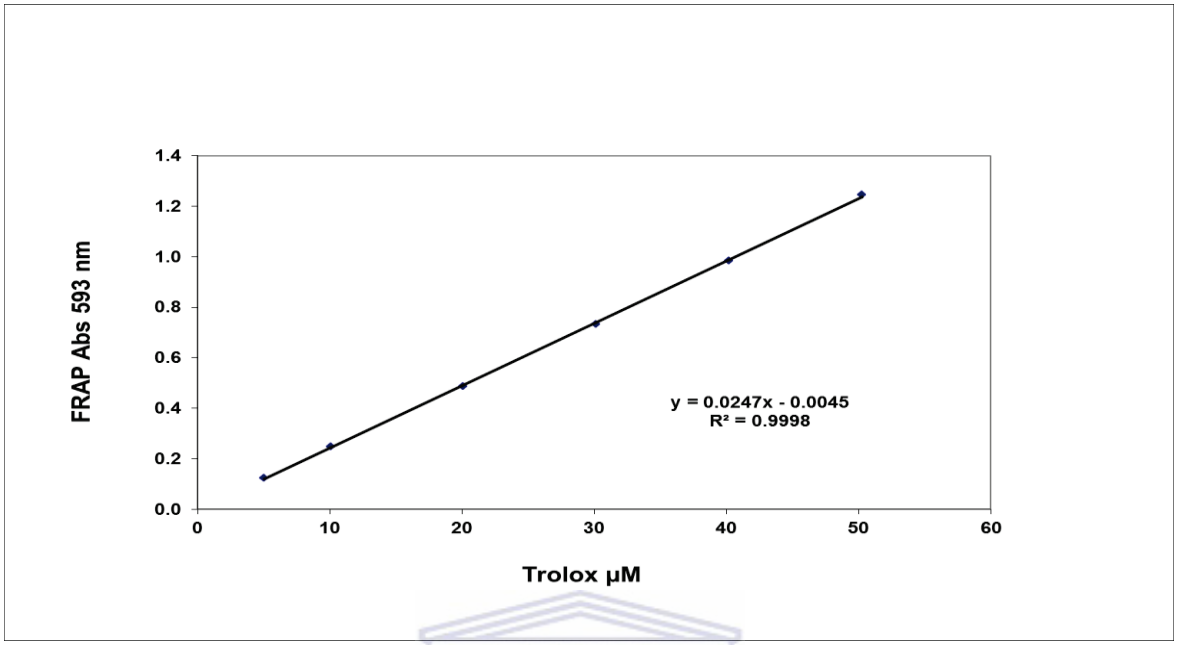

Figure A5. Standard curve of Trolox concentration (µM) in reaction mixture *versus*decrease in absorbance at 593 nm in the FRAP method.