UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

In vitro toxicity of weight loss supplements, conjugated linoleic acid, Levo-carnitine and hydroxycitric acid

By

Bonisiwe Georginah Chiloane

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

Master of Science

in the

Faculty of Health Sciences

Department of Anatomy
University of Pretoria

2019

In vitro toxicity of weight loss supplements, conjugated linoleic acid, Levo-carnitine and hydroxycitric acid

By

Bonisiwe Georginah Chiloane

SUPERVISOR: Prof HM Oberholzer

CO-SUPERVISORS: Dr JC Serem

Prof MJ Bester

DEGREE: MSc (ANATOMY with specialisation in Human Cell Biology)

Abstract

South Africa is experiencing a speedy epidemiologic transition with an alarming increase in obesity and associated disease. The appeal of over-the-counter dietary supplements as a "magic bullet" for weight loss entices many patients who desire to lose weight. The aim of this study was to provide evidence regarding the effect of three common weight loss dietary supplements or ingredients, and these are conjugated linoleic acid, L-carnitine and hydroxycitric acid at the daily recommended dosage. The antioxidant activity (chemical and cellular), toxicity (reactive oxygen species induction, cellular viability, erythrocyte haemolysis), effects on lipid accumulation (differentiated and differentiating adipocytes) and blood coagulation was determined using ephedrine as a weight loss control.

The chemical and cellular oxidative/antioxidant effects of ephedrine, conjugated linoleic acid, L-carnitine and hydroxycitric acid were determined at low (0,75, 1, 2 and 4 µg/mL) and high (25, 50 and 250 µg/mL) concentrations with the oxygen radical absorption capacity assay. The cellular antioxidant effects of ephedrine, conjugated linoleic acid, L-carnitine and hydroxycitric acid were determined at low (7,5 – 42,5 µg/mL) and high (250 – 2500 µg/mL) concentrations with cellular 2',7'-dichlorofluorescein diacetate assay. The cytotoxicity and haemolytic activity were determined in murine fibroblasts (L929), undifferentiated and differentiated murine fibroblasts (3T3-L1 cells) and human erythrocytes using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and haemolysis assays, respectively. The effects on lipid accumulation in differentiated 3T3-L1 adipocytes were evaluated with the Oil Red O assay at concentrations of 10 and 100 µg/mL. In addition, the effects of 10 and 100 µg/mL of each

weight loss compound on erythrocyte morphology and fibrin networks were examined using scanning electron microscopy.

Neither L-carnitine nor hydroxycitric acid had antioxidant activity, however, only hydroxycitric acid at 500 – 2500 µg/mL protected 3T3-L1 preadipocytes against oxidative damage. Both did not induce oxidative stress. In contrast, conjugated linoleic acid was found to have antioxidant activity at 25 – 250 µg/mL, however this translated into oxidative damage or pro-oxidant effect in 3T3-L1 preadipocytes. Of concern is that conjugated linoleic acid is marketed as a product with antioxidant properties and this effect was not observed using cellular models. No antioxidant or oxidative effects were observed for ephedrine and conjugated linoleic acid, however at 500 µg/mL both weight loss compounds were cytotoxic. All compounds at 10 and 100 µg/mL did not alter lipid levels or reduce lipid accumulation in differentiated adipocytes. Ephedrine, conjugated linoleic acid, L-carnitine and hydroxycitric acid at increasing concentrations following 3, 24 and 48 hour exposure did not cause human erythrocyte haemolysis. Exposure of human whole blood to the weight loss compounds for 30 minutes, did not cause changes to erythrocyte morphology and the structure of the fibrin network that formed. Findings were that ephedrine, conjugated linoleic acid, L-carnitine and hydroxycitric acid does not adversely affect blood haemostasis.

High concentrations of each weight loss compound were used and does not necessary represent blood levels following absorption, which would be lower. It can therefore be concluded that in healthy individuals, these weight loss compounds will not adversely affect cellular function although conjugated linoleic acid and ephedrine were cytotoxic at high concentrations. Future studies should focus on the effects of these compounds on different cellular pathways and the effects on blood should be evaluated in obese patients, where these over-the-counter weight loss compounds may have a beneficial ability to reduce oxidative stress and improve blood haemostasis.

Declaration

I, Bonisiwe Georginah Chiloane hereby declare that this research dissertation is my own work and has not been presented by me for any degree at this or of any other University.

Signed:  .....

Date: 01/08/2019

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

University of Pretoria

South Africa

Acknowledgements

First and foremost, I would like to give thanks to God Almighty for his grace for sustaining me throughout this course. Without his mercy and kindness this research would have not been a success.

My sincere thanks goes to **Prof HM Oberholzer**, my supervisor for her expertise in identifying this research and funding the project. Her support helped me all the time during the writing of this dissertation.

I have great pleasure in acknowledging my gratitude to **Dr June Serem**, my co-supervisor for her time, for her patience, motivation and expert guidance especially during research and writing of this dissertation. She made this research project possible.

Besides my supervisor and co-supervisor, I would also like to thank **Prof. MJ Bester**, my co-supervisor for identifying this research project. Her insightful comments and expertise improve this study.

Last but not the least, I would like to offer my sincere regards and gratitude to my family and friends for their support, encouragement and giving me time to work on this research project.

Table of Contents

Chapter 1: Introduction 1

Chapter 2: Literature review 3

2.1 Introduction 3

2.2 Obesity 5

2.3 Conjugated linoleic acid 17

2.4 L-carnitine 23

2.5 Hydroxycitric acid 24

2.6 Ephedrine 27

2.7 Aim and objectives 28

Chapter 3: Chemical and cellular antioxidant activity and cytotoxic effects of ephedrine, CLA, L-carnitine and HCA 30

3.1 Introduction 30

3.3 Methods 32

3.4 Results 36

3.5 Discussion 43

3.6 Conclusion 52

Chapter 4: Effects of ephedrine, CLA, L-carnitine and HCA on accumulated lipid droplets and adipocyte differentiation in differentiated 3T3-L1 cells 53

4.1 Introduction 53

4.2 Materials 54

4.3 Methods 55

4.4 Results 57

4.5 Discussion 61

4.6 Conclusion 71

Chapter 5: The effect of ephedrine, CLA, L-carnitine and HCA on erythrocytes and whole blood 72

5.1 Introduction 72

5.2 Materials 73

5.3 Methods 74

5.4 Results 76

5.5 Discussion 88
5.6 Conclusion 92
Chapter 6: Concluding discussion 94
Chapter 7: References 97
Appendix 119
Ethical clearance 119
Consent form 120

List of Tables

Table 2.1:	WHO classification of obesity……	3
Table 3.1	Dosage recommendations of SOLAL™ weight loss products ……	33
Table 4.1:	Differentiation medium……	55
Table 4.2	Differentiation of 3T3-L1 adipocytes……	56

List of Figures

Figure 2.1:	The coagulation cascade……	14
Figure 2.2:	Obesity-associated impairment in haemostasis……	15
Figure 2.3:	CLA isomers……	18
Figure 2.4:	Summary of anti-obesity mechanisms of CLA……	22
Figure 2.5:	Structure of L-carnitine……....	23
Figure 2.6:	Structure of (-)-hydroxycitric acid……	25
Figure 2.7:	Ephedrine-type alkaloids isomers……	27
Figure 3.1:	Oxygen radical absorbance capacity of CLA, L-carnitine, HCA and ephedrine……	36
Figure 3.2:	The effect of CLA, L-carnitine, HCA and ephedrine on ROS production in undifferentiated 3T3-L1 cells……	37
Figure 3.3:	The protective effect of CLA, L-carnitine, HCA and ephedrine on AAPH induced intracellular ROS production in undifferentiated 3T3-L1 cells…….	38
Figure 3.4:	*In vitro* cytotoxicity activity of CLA, L-carnitine, HCA and ephedrine against L929 cells…….	39
Figure 3.5:	*In vitro* cytotoxicity activity of CLA, L-carnitine, HCA and ephedrine against AAPH-induced cytotoxicity in L929 cells…….	40
Figure 3.6:	*In vitro* cytotoxicity activity of CLA, L-carnitine, HCA and ephedrine against 3T3-L1 cells….	42
Figure 3.7:	*In vitro* cytotoxicity activity of CLA, L-carnitine, HCA and ephedrine against AAPH-induced cytotoxicity in 3T3-L1 cells…….	43
Figure 4.1:	Phase contrast microscope images of ORO stained 3T3-L1 adipocytes…….	58
Figure 4.2:	Spectrophotometric quantification of lipid content using ORO staining method in 3T3-L1 adipocytes…….	59
Figure 4.3:	*In vitro* cytotoxic activity of CLA, L-carnitine, HCA and ephedrine against differentiated 3T3-L1 adipocytes…….	60
Figure 4.4	Lipid formation inhibition by ORO staining method in 3T3-L1 adipocytes……	61
Figure 5.1:	Haemolytic activity of CLA, L-carnitine, HCA and ephedrine on human erythrocytes…….	77
Figure 5.2:	Scanning electron micrographs of erythrocytes…….	79-83
Figure 5.3:	Scanning electron micrographs combination of erythrocytes and fibrin network…….	85-87

List of Abbreviations and Chemical Formulae

3T3	3-Day transfer inoculum 3 ×10^5 cells
8-epi-PGF$_{2\alpha}$	8-Epi-prostaglandin-F$_{2\alpha}$
%	Percentage
°C	Degrees centigrade
µL	Microlitres
µM	Micromolar
µmol/L	Micromole per litre
AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
ACC	Acetyl-CoA carboxylase
ADRP	Adipocyte differentiation-related protein
ANOVA	Analysis of variance
AO/PI	Acridine orange/ propidium iodide
aP2	Adipocyte fatty acid binding protein
APTT	Activated partial thromboplastin time
ATP	Adenosine triphosphate
AUC	Area under curve
BALB/c	Bagg Albino mice
Bcl2	B-cell lymphoma 2
BFM	Body fat mass
BME-UV1	Primary bovine mammary epithelial cells
BMI	Body mass index
BMR	Basal metabolic rate
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
C/EBPs	CAAT/ enhancer binding proteins
CIDEC	Cell death-inducing DFFA-like effector
CLA	Conjugated linoleic acid
cm^2	Centimetres squared
CO_2	Carbon dioxide
CVD	Cardiovascular disease
DCF	Dichlorofluorescein
DCFH-DA	2',7'-Dichlorofluorescein diacetate
ddH_2O	Double distilled water

dddH_2O	Deionised double distilled water
DEX	Dexamethasone
DMSO	Dimethyl sulphoxide
DMEM	Dulbecco's modified essential medium
DNA	Deoxyribonucleic acid
DPPH	1,1-Diphenyl-2-picryl-hydrazyl
DSHEA	Dietary Supplement Health and Education Act
ECACC	European Collection of Cell Culture
Em	Emission wavelength
EtOH	Ethanol
ESR	Electron spin resonance
Ex	Excitation wavelength
FA	Formaldehyde
FABP4	Fatty acid binding protein
FAS	Fatty acid synthase
FCS	Foetal calf serum
FDA	Food and Drug Administration
FTO	Fat mass and obesity associated
GA	Glutaraldehyde
GLUT4	Glucose transporter 4
GI	Glycaemic index
GPDH	Glycerol-3-phosphate dehydrogenase
GPx	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HAT	Hydrogen atom transfer
Hb	Hemoglobulin
HDL	High-density lipoprotein
HDMS	Hexamethyldisilazane
HepG2	Human hepatoma cancer cells
HSL	Hormone-sensitive lipase
IBMX	Isobutyl methylxanthine
iNOX	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinases
kg/m^2	Kilogram per metre square
KCl	Potassium chloride

L929	Mouse fibroblast cell line
LDL	Low density lipoprotein
LP-CLA	*Lactobacillus plantarum*- conjugated linoleic acid
LPL	Lipoprotein lipase
MCF7	Human breast cancer cells
MDA	Malondialdehyde
MDA-MB-231	Human breast cancer cell line
mg/d	Milligram per day
min	Minutes
MJ/day	Megajoules per day
ml	Millimetres
MS	Metabolic syndrome
MSCs	Mesenchymal stem cells
MSHs	Melanocyte-stimulating hormone
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
$NaHPO_4$	Sodium hydrogen phosphate
Na_2HPO_4	Disodium hydrogen phosphate
$Na_2HPO_4 \cdot 2H_2O$	Disodium phosphate dibasic dehydrate
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NaOH	Sodium hydroxide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nm	Nanometres
$NO^{\cdot}$	Nitric oxide species
NO	Nitric oxide
NOX	NADPH oxidase
NYP	Neuropeptide Y
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PKA	Protein kinase
PPAR δ/γ	Peroxisome proliferator-activated receptors delta/gamma
RBCs	Red blood cells
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

SCD 1 & 2	Stearoyl-CoA desaturase 1 & 2
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy (Microscopy method, Chapter 5)
SEM	Standard error of mean (Statistical analysis, Chapter 3, 4 &5)
SES	Socio-economic status
SH-SY5Y	Neuroblastoma cells
SOD	Superoxide dismutase
SRI	Serotonin reuptake inhibitors
T2DM	Type 2 diabetes mellitus
TE	Trolox equivalent
TEM	Transmission electron microscope
TF	Tissue factor
THP-1	Human monocytic cells
TNFα	Tumour necrosis factor alpha
t-PA	Tissue-type plasminogen activator
TUNEL	Terminal deoxynucleotidyltransferase-mediated d-UTP nick-end labelling
UCPs	Uncoupling proteins
$O_2^{-\cdot}$	Superoxide ion
$OH^{\cdot}$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
ORAC	Oxygen radical absorption capacity
ORO	Oil Red O
OTC	Over-the-counter
VLDL	Very low density lipoprotein
vWF	Von Willebrand factor
WAT	White adipose tissue
WHO	World Health Organisation
WHR	Waist/hip ratio

Chapter 1: Introduction

Obesity and its associated non-communicable diseases such as type 2 diabetes (T2DM), hypertension, cardiovascular disease (CVD), dyslipidaemia, chronic kidney disease as well as certain cancers are a serious public health concern (Blokhin & Lentz, 2013; Feng *et al*., 2016). Factors contributing to obesity are genetic factors, changes in metabolism, diet, lack of physical activity, socio-economic status (SES) and lifestyle (Feng *et al*., 2016). Challenges regarding lack of compliance to first-line weight loss treatment i.e. lifestyle and behavioural modification such as calorie reduction and increased exercise have led to alternative interventions including pharmacological therapy, surgery and the use of weight loss dietary supplements (Rios-Hoyo & Gutierrez-Salmean, 2016). Currently, Orlistat is the only prescribed anti-obesity drug available and it is linked to serious side effects such as hypertension, dry mouth, constipation, headache and insomnia (Weigle, 2003). In 2010, the U.S Food and Drug Administration (FDA) withdrew a previously prescribed drug, sibutramine from the market due to its related negative cardiovascular side effects (Feng *et al.*, 2016). Weight loss surgery is considered for individuals with a body mass index (BMI) of ≥ above 35 kg/m^2 (Lawlor & Chaturvedi, 2016). Therefore, over-the-counter (OTC) weight loss dietary supplements appeal to many people as alternatives to traditional therapy as these products are marketed as “magic bullets” for weight loss with the perception of low toxicity profiles and easy accessibility to the general population (Saper *et al*., 2004; Rios-Hoyo & Gutierrez-Salmean, 2016). There are still insufficient double-blinded, randomized clinical studies that have been conducted to evaluate the clinical effects of dietary supplements (Turk *et al*., 2009). Although preclinical and small clinical scenarios have reported certain effects of dietary ingredients, most of these studies still have insufficient evidence regarding the purity grade of the supplement, the patient’s lifestyle (diet and physical activities) and health-related conditions (such as nutritional status or concomitant diseases), food-drug interactions, overdose and potential adverse effects (Rios-Hoyo & Gutierrez-Salmean, 2016).

It remains a great challenge to assess the clinical effect of dietary supplements as the regulations of dietary supplements are not as strict as those of prescribed drugs. Supplements may be viewed as food in some countries such as the United States of America (USA) whereas in other jurisdictions these products are considered to be food supplements, complementary medicines, prescription medicines or in some instances controlled substances (Egras *et al*., 2010; Rios-Hoyo & Gutierrez-Salmean, 2016; Dwyer *et al*., 2018). Moreover, scientific challenges and regulatory systems that relate to the use of dietary supplements differs from country to country. This makes it difficult for regulatory scientists to protect consumers from

harm and to ensure that consumers make informed decisions regarding these products. Another challenge with dietary supplement health products is that different opinions and viewpoints exist that are often very controversial and polarized (Dwyer *et al*., 2018).

Dietary supplements are widely available as OTC or via online shopping sites, making them easily accessible to the general population with limited control and clinical evidence to support their efficacy and safety (Rios-Hoyo & Gutierrez-Salmean, 2016). As obesity is associated with co-morbidities such as T2DM, hypertension, dyslipidemia and CVD the use of unregulated OTC products may aggravate these pre-existing conditions. Therefore, it is essential, using laboratory-based testing, to determine the effects of the active ingredients present in these products prior to evaluation in animal studies and later in a clinical setting.

In this study, the *in vitro* cytotoxicity of three commonly used weight loss dietary supplements or ingredients namely CLA, L-carnitine and HCA were assessed. Furthermore, the effects of these molecules on reactive oxygen species (ROS) production and its associated toxicity; the effects on the coagulation system as well as lipid accumulation in adipocytes were investigated by using ephedrine as a weight loss control.

Chapter 2: Literature review

2.1 Introduction

Obesity is a nutritional disorder characterized by excessive accumulation of fat causing an increase in body weight (O'Connell, 2004; Marseglia *et al*., 2015). Body mass index (BMI) is the method commonly used for classifying if an individual is overweight or obese. BMI is calculated by dividing body weight (in kilograms) by the square of the individual's height (in meters). An individual is classified as obese when their BMI is ≥ 30.0 kg/m^2 irrespective of sex. The World Health Organization (WHO) has identified several categories of BMI and associated risk of comorbidity and is presented in Table 2.1 below (O'Connell, 2004).

Table 2.1: WHO classification of obesity (O'Connell, 2004)

Class	BMI (kg/m^2)	Risk of comorbidity
Underweight	< 18.5	Low
Normal range	18.5 – 24.9	Average
Overweight (grade 1 obesity)	25.0 – 29.9	Mild increase
Obese (grade 2 obesity)	30.0 – 39.9	Moderate/severe
Morbid/severe obesity (grade 3)	≥ 40.0	Very severe

Obesity has become a serious health problem worldwide, affecting more than 1.3 billion adults in both developed and developing countries (Cois & Day, 2015). The southern African region is particularly affected by rising trends. South Africa, a middle-income country, is experiencing a speedy epidemiologic transition with 56% of white men, 49% of black men and 75% of black women being reported to have BMI of ≥ 25.0 kg/m^2. This is of great concern due to the associated health risks related to obesity (Skaal & Pengpid, 2011; Cois & Day, 2015). In 2008, the average BMI of South African males was estimated at 26.9 kg/m^2 compared to the world average of 23.8 kg/m^2 and that of South African females was estimated at 29.5 kg/m^2 compared to world average of 24.1 kg/m^2 (Cois & Day, 2015).

Obesity and its associated complications are costly to society. Obesity-related disease treatment cost approximately $52 billion annually and another $50 billion directed on weight loss treatment yearly with half of this amount directed to weight loss foods and the rest being spent on various weight loss programs, supplements and medications. Consequently, an estimated amount of more than $100 billion is spent annually to prevent the occurrence of obesity and associated disease (O'Connell, 2004).

Obesity is associated with increased risk of diseases, such as T2DM, CVD, dyslipidemia, altered mechanisms of blood pressure responses, chronic kidney disease and cancer as the result of metabolic haemostasis dysregulation (Blokhin & Fentz, 2013). Central to the development of these diseases is increased ROS levels that causes cell and tissue damage (McMurray *et al*., 2016; Furukawa *et al*., 2017). A specific target is the blood where obesity is associated with increased susceptibility for thrombosis, inflammation, decreased fibrinolysis as well as enhanced generation of thrombin and hyperactivity of platelets (Samad & Ruf, 2013).

Studies have shown that reducing daily food intake, caloric restricted diets, exercise, and behavioural therapy can improve weight loss (O'Connell, 2004). Yet, many patients regularly turn to use OCT weight loss dietary supplements, including vitamins and minerals (such as folate and calcium), herbal therapies and botanical agents (such as ephedra and ginkgo biloba) and enzymes (such as lipase and amylase) (Saper *et al*., 2004). Dietary supplements are products intended to supplement one's diet (i.e. to add more nutritional value), as these products consist of one or more dietary ingredients such as vitamins, minerals, amino acids, herbs, metabolites and extracts. Dietary supplements are either in capsules, tablets, gelatin capsules, liquids or powder form and are taken orally (Rios-Hoyo & Gutierrez-Salmean, 2016). These supplements can be purchased in pharmacies, grocery and health food stores or online (Fontanarosa *et al*., 2003; Saper *et al*., 2004). In 2001, the weight loss supplements retail sales were estimated to be more than $1.3 billion (Saper *et al*., 2004).

Patients turn to OTC weight loss products as these products are advertised as a quick and easy way to lose weight with fewer requirements for diet refinement and physical activity. Moreover, these weight loss products are labelled as "natural" remedies and can be obtained without a doctor's prescription and are promoted as also having health benefits in addition to weight loss (Saper *et al*., 2004). Unfortunately, the safety and efficacy of most OTC weight loss supplements and ingredients have not been established and discontinuation usually results in the regain of pre-treatment weight or even the gaining of more weight (O'Connell, 2004). The reason for this is that the FDA, with regards to safety and efficacy, does not require proof from manufacturers before marketing supplements based on the Dietary Supplement Health and Education Act (DSHEA) of 1994 (Saper *et al*., 2004). Also, regulations for dietary supplements differs from that required by the FDA for prescription medications and OTC drugs. The DSHEA has established that substances classified as dietary supplements are not considered as drugs and has subsequently challenged the regulation of these products by FDA (Fontanarosa *et al*., 2003). Another problem with some of the OTC weight loss products is that they may contain hidden ingredients such as sibutramine that can negatively impact on

the health of an individual. In addition, these products may have adverse toxic effects such as increasing ROS and associated risks including increased susceptibility for complication such as thrombosis.

The following sections in this literature review will focus on various factors contributing to obesity as well as the effect of obesity on the coagulation system. In addition, the three weight loss products as well as ephedrine used in this study will be discussed in greater detail and includes structure, function, mechanism of action, safety and efficacy in the treatment of obesity.

2.2 Obesity

2.2.1 Causes of obesity

Obesity is a condition that involves both the increase of the size and number of fat cells and occurs when energy consumption is much larger than energy output. Factors such as diet quality and quantity, SES, environment, genetic input and physiological and psychological status affects the balance between energy input and energy output (Ali & Crowther, 2009). These factors will be discussed further in greater detail.

2.2.1.1 Caloric intake

High fat diets rich in saturated fatty acids are common in developed countries. Body weight is affected by diet, by regulating satiety mechanisms and metabolic efficiency or through secretion and action of insulin (Ali & Crowther, 2009). For this reason, people on a diet that is high in calories may be predisposed to obesity, due to increased, postprandial insulin levels as a result of a high carbohydrate intake that will increase the amount of triglycerides stored in the adipose tissue depots (Hardy *et al*., 2012). High insulin levels induce hunger through depletion of blood glucose, and this results in more food intake, which cause an increase in secretion of insulin. Eventually, this process will result in the promotion of weight gain and chronic hyperinsulinemia (Ali & Crowther, 2009).

The diet of the modern world consists of more fat and less fibre than what is recommended (Ali & Crowther, 2009). A study by Howard *et al*., (2006) showed that fat makes up 37.8% of the total energy intake compared to the < 30.0% recommended level, while fibre intake constituted 8.6 g/1000 kcal per day compared to 14 g/1000 kcal per day recommended intake (39% less fibre). An increase in fatty acids in the diet results in an activation of peroxisome proliferators-activated receptors delta and gamma (PPARδ and PPARγ) promoting adipose synthesis and expansion, resulting in weight gain (Howard *et al*., 2006). Previous literature

has reported a significant correlation between high fat containing diets and the development of obesity (Raatz *et al*., 2017; Hu, 2018).

2.2.1.2 Socio-economic status and education level

Immigration has an influence on obesity in the case where a population that share a common genetic inheritance lives under a new socio-economic and cultural conditions. The prevalence of obesity increases when a population migrates from rural areas to an industrialised environment (Ali & Crowther, 2009). Urbanisation has been associated with an increased risk of becoming overweight or obese in South Africa. Indeed, a higher prevalence of obesity is found among urbanised black South African women (Armstrong *et al*., 2006).

Two independent studies showed that there is a strong correlation between high BMI and low SES when compared to the middle and high socio-economic groups. These two studies further demonstrated that low SES is closely associated with accelerated weight gain during adulthood (Martikainen & Marmot, 1999; Lahmann *et al*., 2000). The relationship between SES and prevalence of obesity could be as the result of low income, limiting the availability of healthier food in the diet (Ali & Crowther, 2009).

Data from a study that was conducted in 1989 (Sobal & Stukard, 1989) showed that in many populations the relationship between level of education and the prevalence of obesity are inversely associated, especially in women. These results support the findings of a study undertaken in Poland involving 588 healthy, occupationally active, age 21 – 62 years with 12 years of education (completed secondary school) participants. In this study an association between the level of education and the prevalence of obesity in married men was high (Lipowicz, 2003). In contrast, a national survey conducted by Puoane *et al*., (2002) in South Africa, revealed that women with 12 years of education or more have higher BMIs when compared with women with 1 – 12 years of education. This could be due to the latter group being more likely to perform higher levels of manual work compared to women that are more educated (Ali & Crowther, 2009).

2.2.1.3 Genetic factors

Genetic factors play a major role in energy balance and therefore in determining BMI. Human obesity phenotypes have been associated with more than 300 genes, markers and chromosomal regions. In humans, about 30 – 70% of the variance in BMI is associated with genetic factors (Ali & Crowther, 2009).

A congenital leptin deficiency reported in 1997 was the first human obesity syndrome that affected a single gene. Leptin inside the arcuate nucleus of the hypothalamus lowers the orexigenic signal expression and promotes the stimulation of anorexigenic signals resulting in a decrease in food intake. Other genes that act in the hypothalamus to regulate appetite have also been identified however, information on the role of these genes in human obesity is limited. Polygenic obesity is the most common form of obesity due to simultaneous mutations occurring in multiple genes. Most of these genes are expressed in the central nervous system and regulate food intake. Fat mass and associated obesity genes are strongly associated with the polygenic form of obesity (Slocum *et al*., 2013). It has been previously shown that the expression of fat mass and obesity associated (FTO) is high in the brain although, the exact function of this gene is not fully understood (McTaggart, 2011).

2.2.1.4 Factors acting early in life, during puberty, pregnancy and aging

In both females and males, rapid weight gain during early life is a major risk factor for obesity later in life. Gestation and early infancy are the critical and sensitive periods for development of obesity in childhood, and adolescence is the critical period for adiposity rebound. A high BMI at age 5 or 7 years and during adiposity rebound is strongly correlated with adult obesity. Rebound that is initiated in childhood accounts for about 30% of adult obesity (Kang, 2018).

The prevalence of obesity is higher in females than in males and it could be due to sex differences due to hunger and satiety response regulated in the brain. The risk of obesity in females could be due to puberty related factors. A longitudinal study conducted by Laitinen *et al*., (2001) demonstrated the prevalence of obesity at age 31 of females who reached menarche before 11 years to be 15% and those who reached menarche after 15 years of age to be 4%. This could be associated with the accumulation of fat during childhood, increasing the possibility of early menarche or that girls who mature early sexually experience elongated period of positive energy balance (Laitinen *et al*., 2001).

Gestational weight gain has been strongly linked with postpartum weight retention. An estimate of 75% of women are unable to lose postpartum weight after 1 year of giving birth (Endres *et al*., 2015). Gestational weight gain is associated with factors such as high BMI before pregnancy, smoking, lack of exercise, short breastfeeding period and a high calorie intake (Ganapathy, 2019). A study by Zanotti *et al*., (2015) reported a strong correlation between lower weight retention in the postpartum period with higher education levels and being married. In contrast, factors such as normal or below normal BMI before pregnancy, exercise and normal weight gain during pregnancy, lower parity, prolong breastfeeding, low

calorie intake and absence of depression are linked with reduced gestational weight gain (Zanotti *et al*., 2015).

Body adiposity is greatly influenced by age. As age increases, body adiposity also increases. This influence is a result of a decrease in metabolic rate and energy expenditure that requires fewer calories to sustain an optimal body weight. More calories are required by men to sustain their body weight, because their resting metabolic rate is higher compared to that of women. A decrease in metabolic rate and ovarian hormone level alterations, predisposes menopausal women to be obese. For this reason, females especially after the age of 50 years have a higher BMI compared to men (Ali & Crowther, 2009).

2.2.1.5 Psychological factors

Eating habits are often influenced by psychological status and negative emotions cause many people to eat more and this response to stress causes an increase in appetite for caloric-dense food. This is associated with an increase in adrenal glucocorticoids (GC) which can lead to abdominal obesity if not controlled (Ali & Crowther, 2009). It has been previously demonstrated that there is a significant correlation between borderline personality disorder and prevalence of obesity in the normal population (Gerlach *et al*., 2016). Gerlach *et al*., (2016) showed that there is an association between avoidant and antisocial personality disorder and higher BMI in the female population. This study also identified that higher risk obesity was linked with paranoid or schizotypal personality disorder. Higher comorbidity of personality disorders, especially borderline personality disorders and avoidants personality disorders binge-eating disorder are also high in females (Gerlach *et al*., 2016). Success of obesity treatment following weight loss management strategies is poor in both females and males with personality disorders (Gerlach *et al*., 2016).

2.2.1.6 Other factors

Obesity is a multi-factorial disorder and other factors also contributing to obesity are smoking, drugs and the presence of endocrine disruptors in the environment.

Smoking

Smoking cigarettes and obesity has been shown to be a leading cause of preventable morbidity and mortality (Kaufman *et al*., 2011; Watanabe *et al*., 2016). Several studies have shown that there is a strong correlation between smoking behaviour and body weight and obesity (Filozof *et al*., 2004; Dare *et al*., 2015). It has further been shown that former smokers have heavier body weight compared to non-smokers. Although, the link between smoking and

weight is not well understood, biological mechanisms such as energy expenditure, physical activity, metabolic rate and inflammation are thought to be involved (Kaufman *et al*., 2011; Dare *et al*., 2015; Watanable *et al*., 2016). In addition, smoking has been shown to have an impact on glucose tolerance, insulin sensitivity and lipoprotein lipase activity (Kaufman *et al*., 2011).

Pharmaceuticals

Weight gain induced by drugs is a serious adverse effect for many of the commonly used drugs, adversely effecting therapy compliance leading to conditions associated with obesity. Anti-diabetic therapy such as insulin, insulin secretagogues or thiazolidinedione therapy is generally followed by an increase in body weight. Weight gain during anti-diabetic treatment is considered as a detrimental side effect as it affects glucose control, elevates blood pressure and has an adverse effect of a patient's lipid profile. However, strategies such as adherence to diet, physical activity and combination therapy with metformin may lessen or prevent weight gain in diabetic patients (Ness-Abramof & Apovian, 2005). Weight gain is also a common side effect for patients on psychotropic therapy (Shrivastava & Johnston, 2010). Atypical antipsychotic drugs that are known to cause weight gain include clozapine, olanzepine, risperidone and quetiapine. Antidepressants such as mirtazapine and serotonin reuptake inhibitors (SRI) may also increase body weight, although their biological mechanism is not well understood due to patients experiencing depressive symptoms. Mood stabilizer such as lithium, valproic acid and carbamazepine are also known to cause weight gain. Antiepileptic drugs such as valproate, carbamazepine and gabapentin are associated with weight gain (Ness-Abramof & Apovian, 2005).

Endocrine disruptors

Endocrine disruptors, also called "obesegens", are known to induce adipogenesis and cause weight gain. Endocrine disruptors include compounds such as pesticides/herbicides, industrial and household products, plastics, detergents, flame retardants and ingredients in personal care products. The population is exposed daily to these compounds and exposure may contribute to weight gain (Darbre, 2017).

2.2.2 Treatment of obesity

Obesity is associated with cardiovascular complications such as hypertension, dyslipidaemia and insulin resistance. Therefore, any obesity treatment should be assessed for long-term effects, not only on obesity, but also on these complications (Lawlor & Chaturvedi, 2006).

2.2.2.1 Diets

Previous studies have determined the effect of different dietary interventions on weight loss in obese patients. Based on the long-term study by Tobias *et al*., (2015) low carbohydrates diet resulted in a significant weight mean difference of 1.15 kg compared to low fat diet in patients. Low fat diet did not lead to significant difference in weight changes when compared to high fat diet (Tobias *et al*., 2015). Zafar *et al*., (2019) observed a significant improvement in body weight, BMI, LDL and total cholesterol in patients following a low glycaemic index (GI) diet.

2.2.2.2 Over-the-counter dietary supplements

Numerous OTC dietary supplements are available and certain claims about the effectiveness of these supplements have been made (Lawlor & Chaturvedi, 2006). The prevalence of OTC dietary supplement use ranges from 37.3% to 58.9% among Malaysian adults' population (Malik *et al*., 2019). There is conflicting evidence regarding efficacy and safety of supplements such as *ephedra sinica*, conjugated linoleic acid (CLA), ginseng, glucomannan, green tea, hydroxycitric acid (HCA), L-carnitine, psyllium and pyruvate (Saper *et al*., 2004; Lawlor & Chaturvedi, 2006). The use of these products has been linked to adverse effect such as psychiatric, autonomic, cardiovascular and gastrointestinal symptoms (Malik *et al*., 2019). Malik *et al*. (2019) reported that 62 out of 332 participants had used weight loss products during the past year of the study and about 2.4% participants were still taking weight loss products. Among the used weight loss products, dietary supplements were the most common (66.1%). Although many of the participants had pre-existing conditions (75.8%) such as hypertension, diabetes, dyslipidaemia and a history of ischaemic heart disease (8.1%), most participants had not sought medical advice for the use of these supplements (Malik *et al*., 2019).

2.2.2.3 Pharmacological treatment

Various randomized controlled trials have assessed two anti-obesity drugs, orlistat and sibutramine. Orlistat is a pancreatic lipase inhibitor that decreases the amount of dietary fat absorbed, has been shown to have both short and long-term benefits on weight loss, especially when combined with dietary and exercise refinements. The use of orlistat with exercise has been shown to have long-term (18 – 24 months) health benefits on obesity complications such as dyslipidaemia and hypertension. Although, it is not clear if the health benefits are as a result of orlistat or an increase in physical activity (Cavaliere *et al*., 2001; Lawlor & Chaturvedi, 2006). However, the use of orlistat has been associated with toxicity such as nephrotoxicity, hepatotoxicity and gastrointestinal side effects. More studies are still

required to investigate the long-term adverse and/or other beneficial effects of orlistat therapy (Priyadharshini *et al*., 2019).

Sibutramine is a serotonin-norepinephrine reuptake inhibitor that acts on the central nervous system by increasing satiety and promoting energy output. Short-term trials and one long-term trial (over 2 years) showed that sibutramine resulted in sustained effects on weight loss (Arterburn *et al*., 2004). Sibutramine's health benefits on triglyceride levels, high-density lipoprotein cholesterol and glycaemic control has also been supported, but no studies have shown that sibutramine reduces morbidity or mortality associated with obesity (Lawlor & Chaturvedi, 2006).

Sibutramine has been associated with increase in blood pressure due to its norepinephrine effect. For this reason, sibutramine is not recommended for use in obese patients who suffer from hypertension (Lawlor & Chaturvedi, 2006). Sibutramine has been removed from the market due its cardiovascular risks where overdose results in tachycardia, hypertension, headache and dizziness (Pamukcu Gunaydin *et al*., 2015). However, there are still dietary weight loss products on the market that contain sibutramine (Pamukcu Gunaydin *et al*., 2015; Oberholzer *et al*., 2015). Sibutramine has also been shown in rat studies to have several negative effects. A study by Oberholzer *et al*., (2015) conducted on Sprague-Dawley rats, showed that the administration of 1.32 mg/kg sibutramine for 28 days induced lung fibrosis and changes in the morphology of blood platelets. Findings by Van der Schoor, (2015) revealed that sibutramine administration at a low and high dose of 1.32 mg/kg and 13.2 mg/kg respectively in Sprague-Dawley rats had a negative effect on the coagulation system inducing a typical pro-thrombotic state (Van Der Schoor, 2015).

Many new pharmacological treatments such as gut hormones, a cholecystokinin that stimulates satiety and other centrally acting serotonin agents, anticonvulsant medications topiramate and zonisamide, cannabinoids receptor antagonists and drugs that act on other peptide neurotransmitters are under investigation (Lawlor & Chaturvedi, 2006). Micale *et al*., (2019) showed that male C57BL/6N mice given 10 mg/kg rimonabant resulted in weight loss and reduced metabolic syndrome-like symptoms.

2.2.2.4 Surgery

Surgical procedures are considered when a person has a BMI of 35 kg/m^2 with obesity related morbidity or a BMI of 40 kg/m^2 with no related morbidity (Lawlor & Chaturvedi, 2006). Surgical procedures result in weight loss through gastric restriction or malabsorption. Gastric restrictive

procedures include Roux-en-Y gastric bypass (RNYGB), gastric banding and vertical banded gastroplasty. The main function of these methods is to reduce gastric volume, slow gastric movement and stimulate early satiety. Significant weight loss results from restrictive procedures in almost all the patients, but weight regain can still result from this procedure. Weight gain regardless of surgical procedures is mostly encountered with the vertical banded gastroplasty procedure than RNYGB and gastric banding. Therefore, the RNYGB is the most commonly used and is the gold standard method for weight loss. This method has been associated with long-term weight loss, high tolerance from patients and acceptable short- and long-term rate of complications (O'Connell, 2004).

Mortality ranges from 0 – 7.7% among patients receiving RNYGB, in which about 22 patients out of 3,434 died from RYNGB surgical procedure with a 0.64% risk of death. Patients who undergo the RYNGB have been reported to lose about 70% of their excess weight in the first 6 – 12 months (O'Connell, 2004).

2.2.3 Oxidative stress in obesity

Studies have shown that obesity is strongly associated with oxidative stress and there is a significant correlation between oxidative stress and fat accumulation in human and mice. (Furukawa *et al*., 2017). Lipid peroxidation was identified as a marker of oxidative injury in non-diabetic human subjects. ROS production is selectively elevated in adipose tissue of obese mice and accompanied by increased NADPH oxidase (NOX) expression and decreased antioxidant enzymes expression. In cultured adipocytes, increased oxidative stress through NOX activation is result of elevated fatty acids. The resulting oxidative stress caused deregulated adipocytokines production, including adiponectin (Furukawa *et al*., 2017). Using urinary 8-epi-prostagladin-F2α (8-epi-PGF2α) a stable product of arachidonic acid formed by non-enzymatic oxidation as marker of oxidative stress, in 2828 subjects, Keaney *et al*., (2003) observed a strong correlation between indices of obesity [BMI and waist/hip ratio (WHR)] and urinary levels of 8-epi-PGF2α.

With oxidative stress there is an imbalance between ROS production and cellular defence mechanisms, which can scavenge ROS and/or repair the resulting damage. ROS includes superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-). Reactive nitrogen species (RNS) can also form as a result of the reaction between nitric oxide (NO) and O_2^- that forms peroxynitrite (ONOO-) which can also cause nitrosative stress (Le Lay *et al*., 2014).

High-energy intake in obesity has been linked with mitochondrial dysfunction and ROS signalling, subsequently leading to insulin resistance (McMurray *et al*., 2016). ROS is responsible for the regulation of c-Jun N-terminal kinases (JNK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), proteins associated with obesity and obesity-induced insulin resistance. The JNK plays a role in the cellular response to stress stimuli and the regulation of apoptosis. The NF-κB protein complex regulates DNA transcription, production of cytokines and cell survival (Finkel, 2011). Production of ROS results in an activation of JNK through different mechanisms, which may lead to insulin resistance in obese patients. NF-κB contributes to Metabolic Syndrome (MS) through its ability to control neuroendocrine and neural dysfunction in the brain (McMurray *et al*., 2016).

2.2.4 The effect of obesity on coagulation

2.2.4.1 The coagulation cascade

Coagulation is highly regulated and sequential process of proteolytic activation of a series of zymogens necessary to accomplish adequate and timely haemostasis to an injured blood vessel. The coagulation cascade involves both the intrinsic and extrinsic pathways (Figure 2.1) (Adams & Bird, 2009). Tissue factor (TF) is required for the extrinsic pathway and is found in the adventitia of a blood vessel and only following release coagulation is activated. TF apoprotein is an integral glycoprotein that is closely associated with the phospholipids in the membrane. TF binds to factor VII circulates in the blood with high affinity. During vascular injury, TF and factor VII initiate blood clot formation by forming a complex in the presence of calcium ions and this complex catalysis the conversion of factor VII to a serine protease (factor VIIa) by minor proteolysis. The factor VIIa-TF complex facilitates the conversion of factor X to a serine protease (factor Xa). The main function of TF is to act as a co-factor in the activation of factor VII and factor X and catalyses both reactions. The factor Xa binds to Va to form a complex in the presence of calcium ions and phospholipids. The Xa-Va complex, also called the prothrombinase complex, facilitates the conversion of prothrombin to thrombin. Factor Va acts as a co-factor in the activation of prothrombin. Once the thrombin is formed, it then catalyses the conversion of fibrinogen to fibrin by limited proteolysis (Davie *et al*., 1991).

In the intrinsic pathway (Figure 2.1) of the coagulation cascade, the role of factor XI is activated by high molecular weight kininogen. The plasma glycoprotein factor XI circulates in the blood as a precursor of a serine protease and is activated by thrombin generating two subunits of factor XI. The generated factor XI then results in the formation of factor XIa. In a calcium dependent reaction, factor XIa facilitates the conversion factor IX to factor IXa (Davie *et al*., 1991). The activated XI catalyses the activation of factor IX that will then act with its cofactor

(factor VIII) and form a complex on an external surface of phospholipid to active factor X. The newly formed factor X with its cofactor (factor V) combined with tissue phospholipids, platelet phospholipids and calcium form a prothrombinase complex that will catalyse the conversion of prothrombin to thrombin. The formed thrombin activates factor XIII through the cleavage of circulating fibrinogen to insoluble fibrin. The factor XIII will then form crosslinks by covalently binding to fibrin polymers which are incorporated in the platelet plug. Fibrin networks are then formed which will then stabilize the clot and form a definitive secondary haemostatic plug (Palta *et al*., 2014).

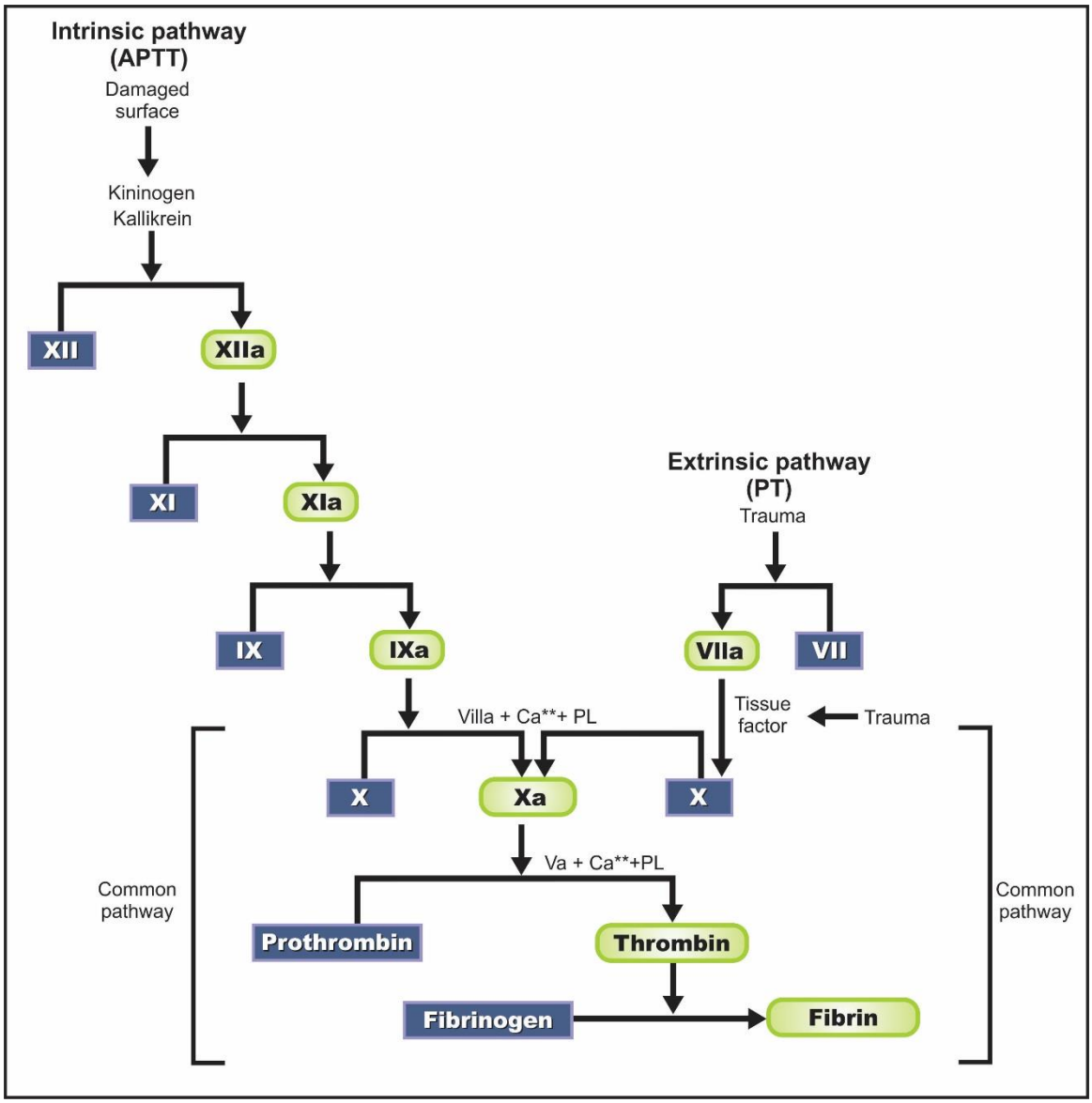


Figure 2.1: The coagulation cascade (Adams & Bird, 2009) PT = prothrombin, PL= phospholipid, APTT= activated partial thromboplastin time.

2.2.4.2 Abnormalities in coagulation and fibrinolysis in obesity

Several studies have demonstrated that there is a strong link between obesity and haemostasis/haemostatic disturbances in the blood coagulation system (De Pergola & Pannaccuilli, 2002). These include enhanced activation of platelets and increased concentrations and activities of plasma coagulation factors (Kaye *et al*., 2012). Disturbances in the coagulation system in obese patients result from both environmental and genetic factors that account for the alteration in the levels of the blood coagulation proteins (Kaye *et al*., 2012).

In a study by De Pergola & Pannaccuilli (2002), patients with obesity are reported to have high plasma concentrations of all pro-thrombotic factors including fibrinogen, von Willebrand factor (vWF antigen and activity) and factor VII than normal weight control group (Figure 2.2). The high plasma concentrations of vWF antigen in obese patients were directly associated with the central fat accumulation measured by waist circumference regardless of the presence of other metabolic and non-metabolic variables (De Pergola *et al*., 1997b).

Von Willebrand factor (vWF) is essential for two important functions in haemostasis. Firstly, it promotes normal platelet aggregation by acting as bridging molecule at the sites of vascular injury under high shear conditions. Secondly, to allow the formation of fibrin by acting as a carrier for factor VIII in the blood thereby, sustaining the normal factor VIII levels (De Meyer *et al*., 2009). Several studies have linked high levels of fibrinogen with development of myocardial infarction and stroke (Mertens & Van Gaal, 2002).

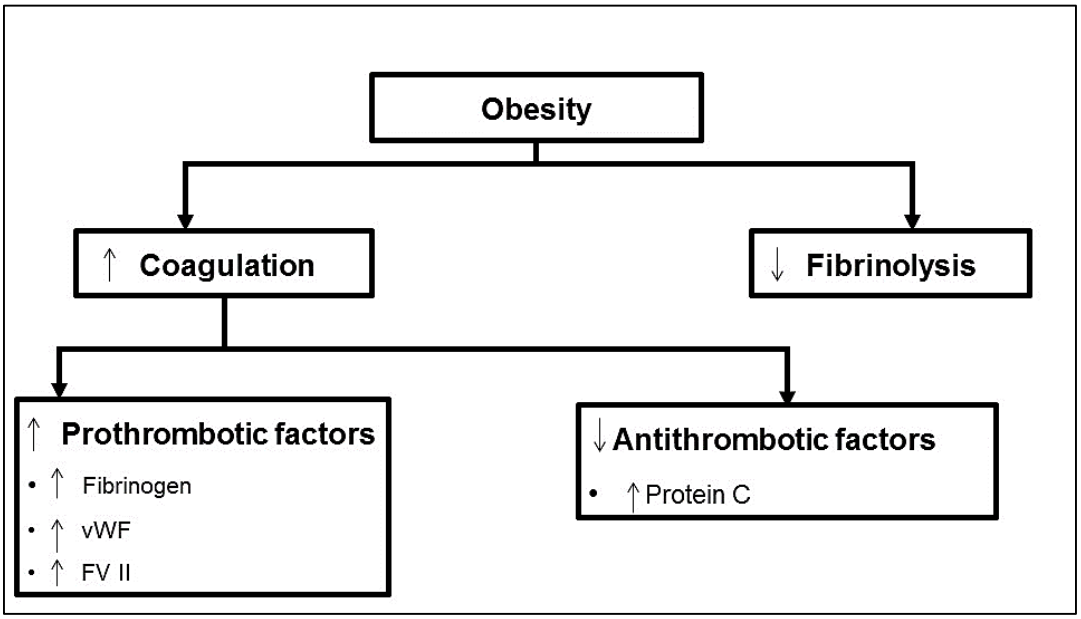


Figure 2.2: Obesity-associated impairment of haemostasis (De Pergola & Pannaccuilli, 2002).

Similarly, De Pergola *et al*., (1997a) observed higher fibrinogen and vWF concentrations with fibrinogen plasma levels associated with central fat as measured by WHR independently of metabolic factors. Men with more visceral fat have significantly higher plasma fibrinogen and factor VIII clotting activity (Cigolini *et al*., 1996). However, the study showed that higher plasma fibrinogen and factor VIII clotting levels were not linked with abdominal visceral fat after regulation of plasma levels of insulin. The conclusion was that the higher activity of fibrinogen and factor VIII in individuals with visceral obesity could result in hyperinsulinemia. Several studies have demonstrated that increased coagulation in obese patients could be related to a cell surface receptor (TF) that results in FVII activation and subsequently acts as a major cellular initiator of the coagulation system (De Pergola & Pannaccuilli, 2002).

Obesity in women was found to be associated with increased plasma concentrations of anti-thrombotic factors such as tissue-type plasminogen activator (t-PA) antigen and protein C than non-obese women (De Pergola *et al*., 1997a). However, there was no association between these anti-thrombotic factors and with waist circumference or the WHR. Men with more visceral fat also have higher plasma t-PA antigen levels (Cigolini *et al*., 1996, De Pergola *et al*., 1997a). Although the plasma t-PA antigen levels are higher in obese men, activity was still low (Cigolini *et al*., 1996). Therefore, these abnormalities in anti-thrombotic factors have an unfavourable overall influence on coagulation balance (De Pergola & Pannacuilli, 2002).

Abdominal obesity and thickness of epicardial fat increases the risk of the development of venous thromboembolism by approximately two-fold (Samad & Ruf, 2003, Nieuwdorp *et al*., 2005). Dysfunction of adipocytes (i.e. accumulation of visceral fat), inflammatory activation and changes at various levels of the coagulation cascade is associated with this pro-thrombotic state resulting in increased generation of thrombin, decreased fibrinolysis and hypercoagulability of platelets (Nieuwdorp *et al*., 2005).

Not only is obesity associated with changes in the intrinsic and extrinsic pathways of coagulation, but OTC and medication can increase thrombotic risk. In male Sprague-Dawley rats consuming a high fat diet, sibutramine was found to alter the ultrastructure of platelets and fibrin networks resulting in a pro-thrombotic state (Van Der Schoor *et al.,* 2014). This is of concern as obese patients may inadvertently be exposed to sibutramine as a hidden ingredient in OTC products or may use OTC products that may aggravate a pre-existing pro-thrombotic state.

OTC weight loss products may have beneficial weight loss effects. However, in obese patients the health risk may be increased due to the presence of ROS and an increased risk for

thrombosis. As the focus of this study is on the oxidant/antioxidant, cytotoxicity, lipid accumulation in adipocytes and blood haemostasis, the known effects of four common ingredients in weight loss products will be discussed in greater detail.

2.3 Conjugated linoleic acid

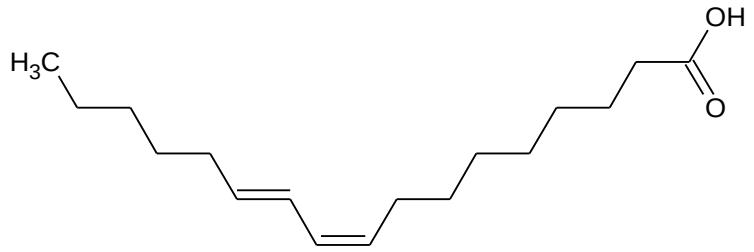
2.3.1 Background

Pariza and colleagues discovered conjugated linoleic acid (CLA) in 1987, and it was shown to have anti-cancer properties (Ha *et al*., 1987). Subsequent studies were conducted, and these studies showed that CLA also exhibited anti-atherosclerotic and anti-obesity properties. Interest in CLA as a weight loss supplement has been increasing as the result of considerable increase in the prevalence of obesity in the past 30 years. CLA has two major isomers with the *trans*-10, *cis*-12 isomer being responsible for the anti-obesity properties. CLA supplementation has been used as a mixture (i.e. equal parts of the *trans*-10, *cis*-12 and *cis*-9, *trans*-11 isomers) (Kennedy *et al*., 2010). The use of the *trans*-10, *cis*-12 isomer alone has been linked with decreased adiposity, browning in white adipose tissue (WAT) and low-grade inflammation in 129Sv mice (Shen *et al*., 2013).

2.3.2 Structure and source of CLA

CLA is a group of conjugated octadecadienoic acid isomers originating from linoleic acid, which is a fatty acid that consists of 18 carbons and two double bonds in the *cis* configuration at the 9^{th} and 12^{th} carbon (i.e. *cis*-9, *cis*-12 octadecadienoic acid). Linoleic acid is converted in the gastrointestinal tract of ruminant animals by microbes to form CLA isoforms through the process of biohydrogenation. During biohydrogenation process, the position and configuration of double bonds change into single bonds that forms between one or both of the double bonds [i.e. *cis*-9, *trans*-11 (9, 11) or *trans*-10, *cis*-12 (10,12) octadecadienoic acid] (Figure 2.3 A & B) (Kennedy *et al*., 2010).

A



B

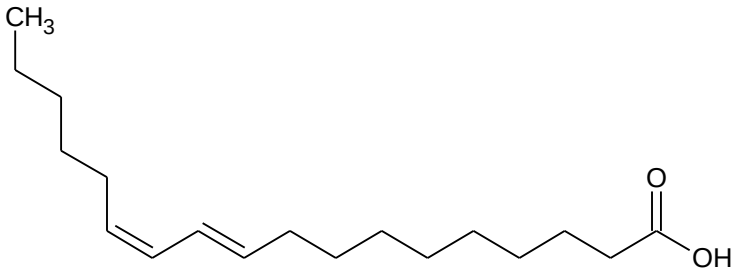


Figure 2.3: CLA isomers. **A**: CLA-9-*cis* and 11-*trans*. **B**: CLA 10-*trans* and 12-*cis* (Lehnen *et al*., 2015).

CLA can be commercially prepared from linoleic acid found in sunflower oils under alkaline conditions. The CLA mixture obtained from this type of process consists of approximately 40% of the *cis*-9, *trans*-11 isomer and 44% of the *trans*-10, *cis*-12 isomer. The commercially prepared CLA may also contain trace amounts of other isomers (Kennedy *et al*., 2010). The 9, 11 isomer is also called the rumeric acid and is the most abundant form of CLA found in naturally occurring foods such as meats (beef and lamb) and dairy products (milk and cheese). Approximately 90% of CLA of the 9, 11 isomers are found in ruminant meat and dairy products and the other 10% is from the 10, 12 isomers. There are other CLA isoforms that are known including *trans*-9, *trans*-11; *cis*-9, *cis*-11; *trans*-10, *trans*-11; *cis*-10, *cis*-12, however the 9, 11 and 10, 12 isomers are known to be the most biologically active (Kennedy *et al*., 2010).

The proportion of CLA in the dairy product's total fats ranges from 0.34% to 1.07% and from 0.12% to 0.68% of the raw processed beef product's total fats. However, several factors affect the CLA content of foods and this includes the season and the breed of animal, nutritional status and age. Non-vegetarian individual consumes an average of 152 mg to 212 mg CLA daily and human levels of serum ranges from 10 µmol/L to 70 µmol/L (Kennedy *et al*., 2010).

2.3.4 Anti-obesity mechanisms of CLA

CLA decreases energy intake

Energy balance is a function of energy consumption relative to energy output. Body weight and body fat mass (BFM) increases when energy input is higher than energy expenditure. CLA exerts its anti-obesity effect by decreasing energy intake or increasing energy expenditure (Kennedy *et al*., 2010). Zhang *et al*., (2008) observed a significant decrease in body weight of birds fed with 2.5% *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA for 56 days compared with control group. Weight loss was linked to decrease in energy intake (Zhang *et al*., 2008). So *et al*., (2009) reported a reduction of food intake by 23.6% in mice that consumed a low-fat diet supplemented with *trans*-10, *cis*-12 CLA.

Proopiomelanocortin is a precursor protein that produces many biologically active peptides such as melanocyte-stimulating hormones (MSHs), corticotrophin (ACHT) and β-endorphin (Millington, 2007). Mice that consumed *trans*-10, *cis*-12 CLA showed a significant decrease in the gene expression of proopiomelanocortin to neuropeptide Y (NYP) ratio, which is a potent orexigenic peptide expressed in the hypothalamus that plays a role in regulating eating behaviour. These results suggested that CLA exerts its anti-obesity effect on the genes that regulate appetite in the hypothalamus (So *et al*., 2009).

CLA increases energy expenditure

Basal metabolic rate (BMR), adaptive thermogenesis and exercise are functions of energy expenditure. Several studies have demonstrated that CLA exerts its anti-obesity effect by increasing energy expenditure through increased BMR, thermogenesis or by increasing oxidation of lipids in animals (Kennedy *et al*., 2010). Terpstra *et al*., (2003), reported that male BALB/c mice that consumed a CLA mixture for 6 weeks showed a significant decrease in body fat by 50% compared to the controls and this reduction in body fat was associated with increased BMR. Cui *et al*., (2017) noted a significant decrease in body weight in obese rats within weeks following consumption of 6g/kg/d CLA after exercise, 5 times/week. Reduced body weight was correlated with increase in energy metabolism of the resting state (Cui *et al*., 2017). Den Hartigh *et al*., (2017) reported that *trans*-10; *cis*-12 CLA isomer caused weight loss along with increased energy expenditure, respiratory quotients reduction, increased fat oxidation and browning of subcutaneous WAT in obese mice that were given high fat high sucrose diet (36% fat, 36.2% sucrose and 0.15% cholesterol) with 0.5 or 1% of *cis*-9, *trans*-11 or *trans*-10, *cis*-11 isomer for 12 weeks.

The upregulation of uncoupling proteins (UCPs) is thought to be responsible for increased thermogenesis. This protein complex is in the inner mitochondrial membrane and is responsible for the transportation of protons across the membrane, thereby redirecting energy

from the synthesis of adenosine triphosphate (ATP) to the production of heat (Kennedy *et al*., 2010). Baraldi *et al*., (2016) demonstrated that male C57B1/6 mice fed with 3 g/kg of *cis*-9, *trans*-11; *trans*-10, *cis*-12 CLA for 60 days developed insulin resistance, increased body weight and lipid content through induced liver UCP expression and activity. Rossignoli *et al*., (2018) also observed an increase in the expression and activity of UCP in male C57B1/6 mice fed with 3 g/kg for 60 days.

CLA inhibits adipogenesis

Transcription factors such as PPARγ and CAAT/enhancer binding proteins (C/EBPs) are essential for the conversion of preadipocytes to adipocytes. During differentiation, the activity of C/EBPβ and C/EBPδ increases, thereby inducing transcription of C/EBPα and PPARγ, the master regulators of differentiation of adipocytes (Kennedy *et al*., 2010). Yeganeh *et al*., (2016) showed that treatment with 60 µM *trans*-10, *cis*-12 CLA isomer in 3T3-L1 adipocytes reduce lipid accumulation by increasing β-catenin and down-regulating PPARγ. Meadus *et al.,* (2017) showed that 100 µM *trans*-10, *cis*-12 CLA isomer inhibit 3T3-L1 adipogenesis and caused significant increase in lipid hydroperoxide activity. In a study by Miller *et al*., (2008), treatment of human adipocytes or mature 3T3-L1 adipocytes with *trans*-10, *cis*-12 CLA resulted in a significant decrease in PPARγ expression and activity and a decrease in the expression of PPARγ target genes and lipid content. However, 100 µM CLA supplementation did not only inhibit the expression of PPARγ but it also reversed the adipogenic process by suppression of activity of PPARγ. The reduction in the target gene expression of PPARγ may be associated with decrease in the expression of PPARγ or to post-translational inhibition activity of PPARγ per se (Miller *et al*., 2008).

CLA suppresses lipogenesis

The major function of adipocytes is to store fatty acids as triglycerides. Lipogenesis involves numerous proteins such as lipoprotein lipase (LPL), acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and stearoly-CoA desaturase (SCD). Supplementation of the CLA mixture (*cis*-9, *trans*-11 and *trans*-10, *cis*-12) or *trans*-10, *cis*-12 CLA isomer alone has been shown to decrease these proteins. Maslak *et al*., (2015) showed that 1% of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers prevented excessive accumulation of glycogen in the liver of male Wistar rats after 4 weeks of feeding. The effects of these isomers, specifically *trans*-10, *cis*-12 was as the result of reduced serum triacylglycerol, LDL, very low density lipoprotein (VLDL) cholesterol, and elevated HDL and reduced the lipid content in the liver and composition of fatty acids through downregulation of liver SCD-1 and FAS expression (Maslak *et al*., 2015). PPARγ is essential for the activation of numerous lipogenic genes such as glycerol-3-

phosphate dehydrogenase (GPDH), LPL, and lipin. PPARγ is also responsible for encoding proteins associated with formation of lipid droplets such as perilipin, adipocyte differentiation-related protein (ADRP) and cell death-inducing DFFA-like effector c (CIDEC). CLA may exert its potent anti-lipogenic activity through inhibition of PPARγ activity (Kennedy *et al*., 2010).

CLA stimulates lipolysis

Lipolysis involves the breakdown of stored triglycerides into free fatty acids and glycerol through catalytic action of hormone-sensitive lipase (HSL). With energy depletion, lipolysis is upregulated through c-AMP-mediated signalling. CLA has been shown to promote breakdown of triglycerides in white adipose tissue (WAT) through the activation of pro-inflammatory pathways, thereby releasing free fatty acids for uptake in metabolically active tissues such as the liver and muscle (Kennedy *et al*., 2010). CLA at a concentration of 100 µM significantly reduced fat accumulation in *Caenorhabditis elegans* by 29% through ATP synthase B homolog (Shen *et al*., 2018).

CLA induces apoptosis of (pre)adipocyte

CLA reduces BFM by inducing apoptosis (Kennedy *et al*., 2010). Recent studies have shown that the CLA mixture of *trans*-10, *cis*-12 isomers induce apoptosis in adipocytes. Meadus *et al*., (2017) showed that 100 µM of *trans*-10, *cis*-12 CLA isomer added to 3T3-L1 cells induced apoptosis through expression of apoptotic genes and proteins such as caspase 3, Bcl-2 and BAXs. Wang *et al*., (2017) reported a stronger induction of apoptosis in the adipocytes by *cis*-9, *trans*-11 CLA isomer compared with *trans*-10, *cis*-12 CLA isomers in mice given 15 g/kg, 3 times for 45 days through suppression of miR-23a which plays a critical role in post-transcriptional expression of target genes. A summary of the anti-obesity mechanisms of CLA is shown in Figure 2.4 below.

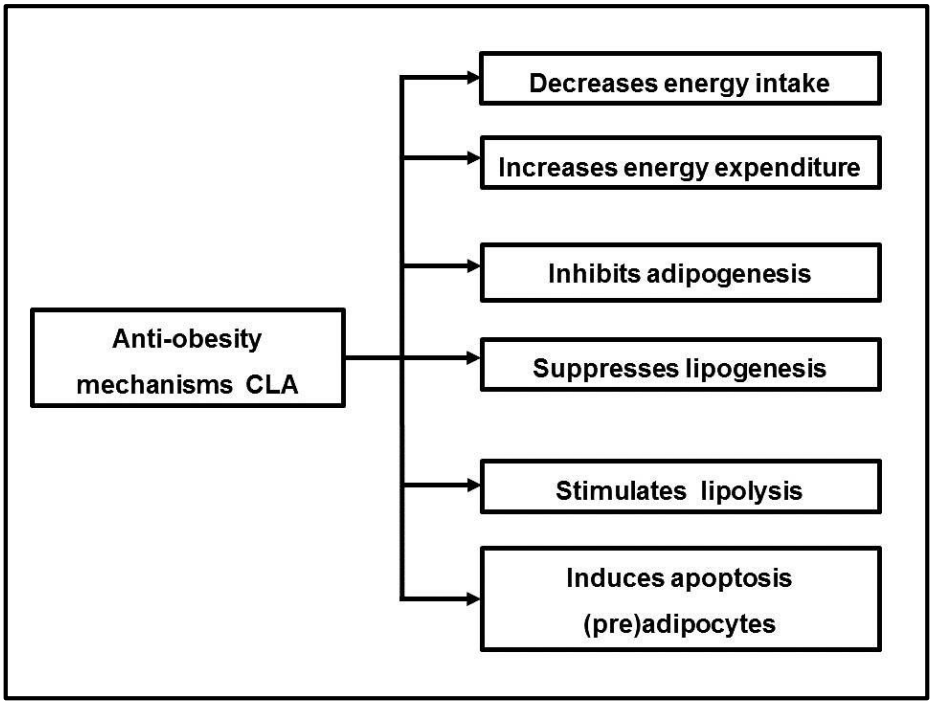


Figure 2.4: Summary of anti-obesity mechanisms of CLA.

2.3.5 The effects of CLA in humans

The interest on CLA due to its effects on body composition, especially in body fat mass reduction and increase in lean body mass has been increasing. Despite the multitude of animals studies showing that CLA is effective in reducing fat mass, human clinical trials are controversial. Recently, the sale of CLA dietary supplements or CLA-containing products as a weight loss agent has been increasing on numerous internet websites and health food stores (Rainer & Heiss, 2004).

Ribeiro *et al*., (2016) showed that consumption of 3.2 g/d of CLA while performing an 8 week protocol of aerobic exercise in obese women has no effect on body fat reduction and lipid profile improvements over placebo in young adult obese women. Similarly, Shahmirzadi *et al*., (2019) found that administration of 3000 mg of CLA supplement, 3 times daily for 3 months in obese adults did not reduce body fat and serum leptin. In a meta-analysis study conducted by Terpstra, (2004) reported that the effect of CLA on body weight and body fat in humans is significantly lower than those seen in mice although the administered CLA doses used in these studies is comparable. The differences between mice and humans is considered to be as the result of differences in metabolic rates as mice have considerably higher metabolic rate. Furthermore, it was noted that the results obtained from human studies did not show any correlation between the CLA doses and the effect on body weight and fat. In addition, the effect of CLA on body composition was shown to be rather small due to possible variations in energy consumption and expenditure interfering with the effects of CLA (Terpstra, 2004). In

contrast, Watras *et al*., (2007) revealed that consumption of 4 g/day of 78% active CLA isomer (39.2% *cis*-9, *trans*-11 and 38.5% *trans*-10, *cis*-12) among overweight adults for 6 months significantly reduced body fat and prevented weight gain during the holiday season.

2.4 L-carnitine

2.4.1 Background

L-carnitine was first discovered by two Russian scientists in human muscle extracts and was named carnis, which is the Latin word for meat or flesh. The structure of L-carnitine was established in 1927, and the first article about this substance was published in 1935 (Karlic & Lohninger, 2004). In 1959, Fritz demonstrated that L-carnitine promotes the oxidation of long-chain fatty acids in the liver and heart (Fritz, 1959). L-carnitine was also called vitamin B T (T = Tenebrio) as the larva of black beetle *Tenebrio molitor* requires L-carnitine as a growth stimulator (Karlic & Lohninger, 2004).

2.4.2 Structure and function of L-carnitine

Carnitine (β-hydroxy-γ-N-trimethyl-ammonium butyric acid) is a quaternary amine with a molecular weight of 161.2 g/mol (Figure 2.5). Synthetic carnitine is found in both D and L isomers; however, the only physiologically active form is the L-carnitine. The primary function of carnitine is to facilitate the oxidation of lipid through transportation of long-chain fatty acids into the mitochondrial matrix where β-oxidation takes place. In order to produce energy from fatty acids (from food intake or adipose tissue) fatty acids must be converted into acetyl CoA derivatives prior to β-oxidation. However, acetyl CoA derivatives cannot cross cell membranes and therefore carnitine is required for the transportation through the mitochondrial matrix. In a deficiency of carnitine dietary lipids cannot be utilised as an energy source and subsequent accumulation of fatty acids causes obesity. In humans, the small intestinal mucosa is responsible for the absorption of carnitine through sodium-dependent active transport and by passive transport (Cha, 2008).

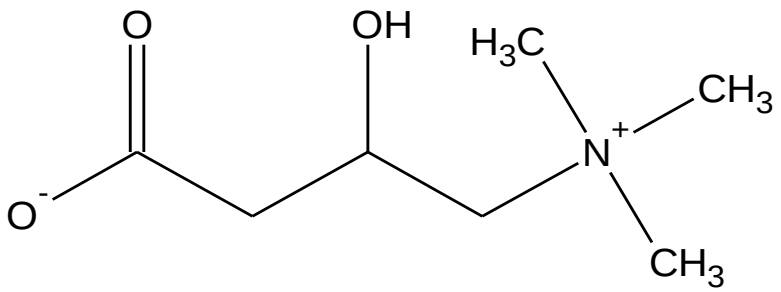


Figure 2.5: Structure of L-carnitine (Cha, 2008).

2.4.3 Carnitine as weight loss agent

The mechanism of action of carnitine as a weight loss agent is that increased intake of L-carnitine promotes fat oxidation, thereby reducing the amount of reserved fat (Karlic & Lohninger, 2004). Studies have demonstrated that high doses of L-carnitine regulate the function of glucocorticoid receptor and mimicking some of the glucocorticoid's biological activities, thereby promoting the breakdown of triglycerides in adipose tissue. Supplementation with L-carnitine has been shown to stimulate carnitine acyltransferases thereby resulting in hyperlipidaemia (Karlic & Lohninger, 2004). A meta-analysis randomized controlled trial by Pooyandjoo *et al*., (2016) revealed that participants that received carnitine significantly lost body weight and showed a decrease in BMI compared with the control group. A propensity score-matched study by Zhang *et al*., (2019) showed that ingestion of L-carnitine by patients after finishing fasting therapy caused significant decrease in weight, BMI and triglyceride levels compared with the control.

2.4.4 Safety aspects of L-carnitine

L-carnitine belongs to the OTC nutritional supplements and is also a prescribed drug. The lethal dosage (LD) for carnitine in rats was determined at 8.9 to 9.1 g/kg, which is equivalent to 630 g/d in humans. However, clinical (pharmacological) dosage for dialysis patients is 48 mg or 50 to 350 mg of carnitine per kilogram of body weight. Extrapolation of these dosages to 70 kg individual gives a dose of 3 to 3.5 g/d as the minimum at which a pharmacological effect can be expected. High dosage L-carnitine supplementation of greater than 4 g/d may induce slight gastrointestinal distress and a low dose of 2 g/d is sufficient for reducing gastrointestinal distress (Karlic & Lohninger, 2004).

2.5 Hydroxycitric acid

2.5.1 Background

(-)-Hydroxycitric acid [(-)-HCA] is found in the fruit rinds of certain garcinia species such as, *G. cambogia*, *G. indica* and *G. atroviridis*. Garcinia (family: *Guttiferae*) belongs to the large genus of polygamous trees or shrubs found in tropical Asia, Africa and Polynesia. In the past, several garcinia species have been used to isolate small and complex molecules such as xanthones and xanthone derivatives. The isolation of (-)-HCA from few garcinia species and its pharmacological activity have led to research on its weight loss properties (Jena *et al*., 2002).

(-)-HCA has been studied for its physiological and biochemical effects on synthesis of fatty acids, lipogenesis, appetite and weight loss. (-)-HCA derivatives have been used in various pharmaceutical preparations in conjunction with other ingredients claimed to have beneficial effects such as promotion of weight loss, cardioprotection, correcting abnormal lipid profiles and endurance in physical activity (Jena *et al*., 2002).

2.5.2 Structure of HCA

The dried fruit of *G. cambogia* consists of approximately 10 to 30% citric acid, most of which is HCA, whose structure is similar to citric acid. HCA consist of 4 isomers: (-)-HCA, (+)-HCA, (+)-allo-HCA and (-)-allo-HCA. The main acid found in the fruit of *G. cambogia* is the (-)-HCA (Figure 2.6) (Marquez *et al*., 2012).

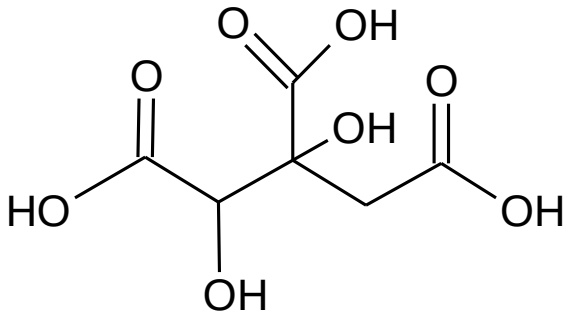


Figure 2.6: Structure of (-)-hydroxycitric acid (Marquez *et al*., 2012).

2.5.3 Mechanism of action

In the late 1960s, Watson and Lowenstein identified (-)-HCA as a potent competitive inhibitor of the extramitochondrial enzyme adenosine triphosphate-citrate (pro-3S)-lyase, which is critical for synthesis of fatty acids outside the mitochondria (Watson & Lowenstein, 1970). (-)-HCA promotes a reduction of Acetyl-CoA, thereby limiting fatty acids and cholesterol biosynthesis in different tissues both *in vitro* and *in vivo* (Marquez *et al*., 2012).

(-)-HCA has been shown to have an inhibitory effect on glycolysis and increases the rate of hepatic glycogen synthesis. Glycogen influences the liver glucoreceptors, promoting satiation via the vagus nerve, thereby contributing to decreased energy intake and reducing body weight gain (Marquez *et al*., 2012). In a study by Marquez *et al*., (2012), (-)-HCA increased the release and availability of serotonin which is a neurotransmitter required for appetite regulation. Ohia *et al*., (2001) investigated the effects of administration of 1 mM (-)-HCA *in vitro* on the cerebral cortex of rats and found that there was a significant increase in the release and availability of serotonin (Ohia *et al*., 2001).

(-)-HCA has also been shown to regulate body weight by its ability and efficiency in the regulation of genes that are essential for metabolism of lipids and carbohydrates as we as in cell communication (Marquez *et al*., 2012). Treatment with (-)-HCA on human adipocytes significantly downregulated 348 and upregulated 366 fat and obesity associated genes such as HSL, PPARγ, leptin and hypoxia-inducible factor-1 gene. These results indicated that (-)-HCA has antilipolytic and antiadipogenic effects (Roy *et al*., 2007).

2.5.4 Safety aspects of HCA

Several animal studies have shown that the toxicity of (-)-HCA, is low and is similar to that of citric acid, which is found in many foods. (-)-HCA is a natural component of products used in Indian cuisine and for medicinal purposes. Administration of 5000 mg/kg of body weight of (-)-HCA showed no toxicity symptoms or death in laboratory animals. This dosage corresponds to 350 g or 233 times the dosage of 1.5 g/day of (-)-HCA that might be consumed by an adult of average weight (Jena *et al*., 2002).

In the past, *G. cambogia* has been used as a flavouring agent, preservative and herbal tonic and very little toxicity data is available. Excessive ingestion of (-)-HCA may have negative effects and intolerance can be easily reversed by decreasing the dosage. However, bowel intolerance has not been described in any *in vivo* studies at the dosages that were necessary to decrease food intake in humans (Clouatre & Preuss, 2013). Nevertheless, despite the lack of evidence of its safety, (-)-HCA, like all other diet products, is not recommended for certain individuals. (-)-HCA has a negative effect on fatty acid and cholesterol production, thereby directly influencing sterol production and reducing steroid hormone production. For this reason, products containing (-)-HCA are not recommended during pregnancy and breast-feeding (Jena *et al*., 2002).

(-)-HCA reduced the number and total area of lipid droplets in chicken adipocytes by reducing supply of acetyl CoA through ATP-citrate lyase inhibition and increasing energy metabolism (Li *et al*., 2017). Administration of (-)-HCA at 0 – 3000 mg/kg for 28 days in broiler chickens significantly altered the metabolic pathways associated with amino acid metabolism, protein synthesis and the citric acid cycle as well as uric acid and fatty acids synthesis. (-)-HCA administration resulted in an inhibition of fatty acid synthesis by enhancing the citric acid cycle, causing reduction in cytosolic acetyl CoA content (Peng *et al*., 2018). Kim *et al*., (2013) showed that C57BL/6J mice fed with 1% of (-)-HCA in conjunction with a high fat diet (45 kcal% fat) for 16 weeks induced hepatic fibrosis, inflammation and oxidative stress.

2.6 Ephedrine

2.6.1 Background

Ephedra sinica (called *Ma haung* in Chinese) is a medicinal plant native to China and Mongolia that consists of ephedra alkaloids which is a sympathomimetic compound (Saper *et al*., 2004; Diepvens *et al*., 2007). Ephedra has been used since the third millennium BC to present day as an antitussive, antipyretic, sudorific and anti-inflammatory agent (Miller, 2004).

2.6.2 Structure

Ephedrine-type alkaloids consist of 6 isomers, (-)-ephedrine, (+)-pseudoephedrine, (-)-norephedrine, (+)-norpseudoephedrine, (-)-N-methylephedrine and (+)-N-methyl-pseudoephedrine (Figure 2.7) that are sold OTC as natural remedies and dietary supplements. OTC ephedrine-type alkaloids are sold either as a concentrated extract or as raw botanicals or powdered plant material and packaged as capsules, tablets, powders and liquids (Miller, 2004).

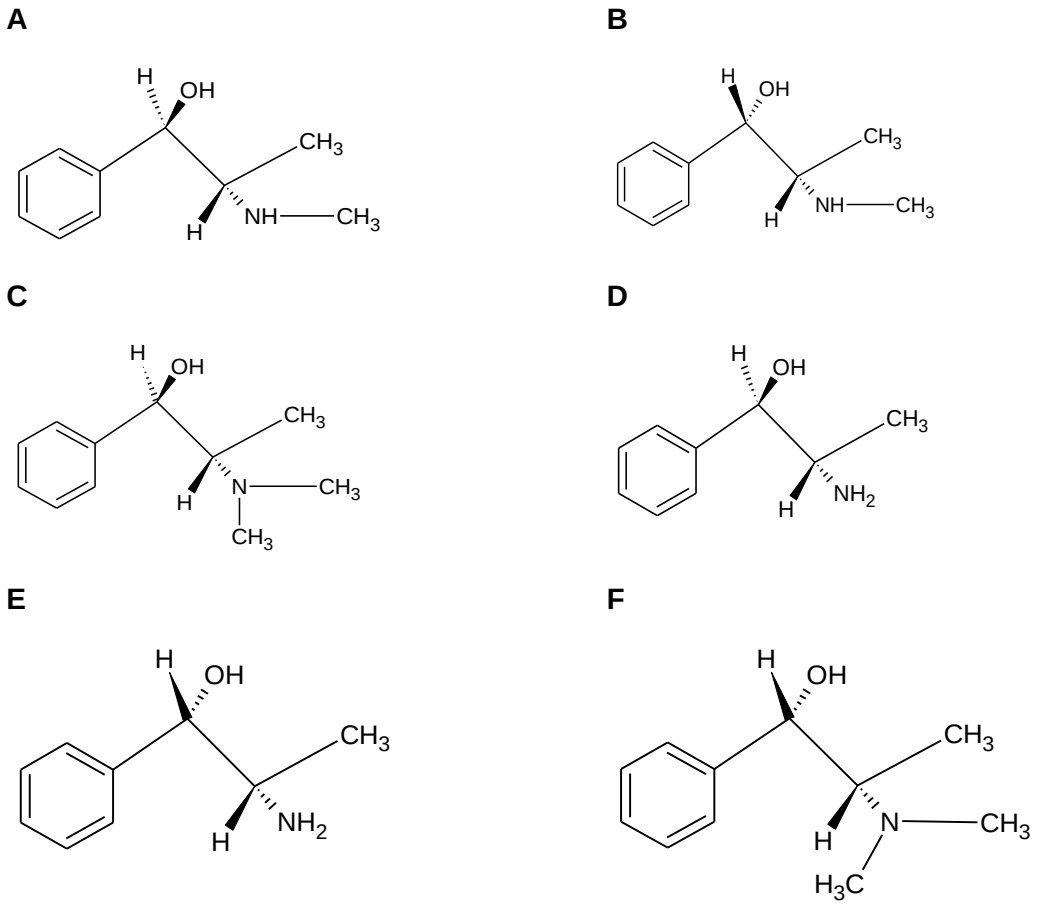


Figure 2.7: Ephedrine-type alkaloids isomers. **A**:(-)-ephedrine. **B**: (+)-pseudoephedrine. **C**: (-)-methylephedrine. **D**: (-)-norephedrine. **E**: (+)-norpseudoephedrine**. F**: (+)-methylpseudoephedrine (Miller, 2004).

2.6.3 Ephedra as a weight loss agent

The active components of ephedra are ephedrine-type alkaloids, which are contained in many OTC weight loss products (Boozer *et al*., 2001). Increase in brown adipose tissue activity is regarded as possible strategy for developing therapeutics targeting obesity. Carey *et al*., (2013) reported that oral administration of 2.5 mg/kg ephedrine to lean and obese young men significantly increased brown adipose activity in the lean group, but activity was unchanged in the obese participants.

Boozer *et al*., (2001) designed a study to investigate short-term safety and efficacy for Ma Huang and guarana (i.e. ephedrine alkaloids and caffeine), and other ingredients in overweight individuals. Treatment with 72 mg/day ephedra/guarana resulted in significant weight loss (-4.0 ± 3.4 kg) and fat (-2.1 ± 3.0% fat) over the 8-week treatment period compared to the control placebo group (-0.8 ± 2.4 kg and 0.2 ± 2.3% fat). A significant reduction in hip circumference and levels of serum triglyceride was also observed (Boozer *et al*., 2001).

2.6.4 Safety aspects of ephedrine

In April 2004, the FDA banned the sale of ephedra and ephedrine-containing supplements because of safety concerns, although it had been shown to be effective for modest weight loss (Pittler & Ernst, 2004, Saper *et al*., 2004). Shekelle *et al*., (2003) found that supplementation of ephedra and ephedrine-containing supplements has been linked with 2.2 to 3.6-fold increase in the odds for psychiatric, autonomic, gastrointestinal symptoms and heart palpitations. The use of these supplements containing ephedra and its associated alkaloids have been associated with severe cardiovascular and central nervous system events (Haller & Benowitz, 2000). Haller *et al*., (2005) showed that supplementation of an ephedra/caffeine mixture (325/90 mg) at two doses (5 h apart) resulted in a persistent increase in heart rate and blood pressure as well as an increase in glucose and a decrease in potassium levels.

2.7 Aim and objectives

The aim of this study was to provide evidence regarding the effect and safety of ephedrine (weight loss control) and three common weight loss OTC dietary supplements, CLA, L-carnitine and HCA with specific focus on the antioxidant properties, cell viability, adipogenicity and haemostatic effects.

The objectives of this study, for each weight loss supplement, was to:

1. Determine and compare the antioxidant activity of low (0.75 – 4 µg/mL) and high (25 – 250 µg/mL) concentrations of ephedrine, CLA, L-carnitine and HCA using the oxygen radical absorption capacity (ORAC) assay.
2. Determine and compare the cellular antioxidant activity in 3T3-L1 preadipocytes of low (7.5 – 42.5 µg/mL) and high (250 – 2500 µg/mL) concentrations of ephedrine, CLA, L-carnitine and HCA using 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay.
3. Determine and compare the effect of 24 and 48 h exposure to low (1.5 – 8.5 µg/mL) and high (50 – 500 µg/mL) concentrations of ephedrine, CLA, L-carnitine and HCA on cell viability in the L929 and preadipocyte 3T3-L1 cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.
4. In mature, differentiated 3T3 L1 adipocytes determine and compare the effects 10 and 100 µg/mL ephedrine, CLA, L-carnitine and HCA following 48 h exposure on lipid levels and cell viability determined with Oil Red O (ORO) staining and the MTT assay, respectively.
5. Determine the effects of exposure to 10 and 100 µg/mL ephedrine, CLA, L-carnitine and HCA during differentiation (day 4, 7 and 10) on lipid levels determined with ORO staining.
6. Determine and compare the effect of 30 min, 24 and 48 h exposure to low (1.5 – 8.5 µg/mL) and high (50 – 500 µg/mL) concentrations of ephedrine, CLA, L-carnitine and HCA on erythrocytes evaluated with the haemolysis assay.
7. Using whole blood, evaluate and compare the effect of 10 and 100 µg/mL ephedrine, CLA, L-carnitine and HCA on erythrocyte morphology evaluated with scanning electron microscopy (SEM).
8. Using whole blood, evaluate and compare the effect of 10 and 100 µg/mL ephedrine, CLA, L-carnitine and HCA on fibrin network formation evaluated with SEM.

Chapter 3: Chemical and cellular antioxidant activity and cytotoxic effects of ephedrine, CLA, L-carnitine and HCA

3.1 Introduction

Obesity is characterised by increased oxidative stress and related complications due to the antioxidant defence mechanisms of obese patients being inadequate and this is directly proportional to central adiposity (Savini *et al*., 2013). Obesity-related complications include hyperglycaemia, hypertension, low HDL levels and hypertriglyceridemia which are collectively known as MS. Increased oxidative stress in obesity has been shown to impair the secretion of insulin in pancreatic β-cells and transportation of glucose to muscles and adipose tissue (Rudich *et al*., 1998; Maddux *et al*., 2001). Various biochemical pathways such as generation of superoxide from NOX, oxidative phosphorylation, glyceraldehyde auto-oxidation, activation of protein kinase C, polyol and hexosamine pathways are involved in the induction of systemic oxidative stress linked to obesity (Savini *et al*., 2013; Serra *et al*., 2013). Other factors including hyperleptinemia, impaired antioxidant defence mechanism, chronic inflammation and generation of postprandial ROS are also known to contribute to obesity related oxidative stress. It has been recently demonstrated that the pathophysiological mechanisms of obesity and its associated co-morbidities are highly regulated by adipose tissue. Therefore, it is critical to understand the factors that contribute to the development of obesity and complications in order to adequately establish the platform that will lead to prevention of obesity and its related health issues (Manna & Jain, 2015).

A low antioxidant defence system in obese patients could be as a result of a low consumption of antioxidant and phytochemical-rich foods including fruits, vegetables and legumes (Savini *et al*., 2013). Phytochemicals such as catechins, anthocyanins, resveratrol, and curcumin have been shown to have beneficial effects in obese patients including the reduction of oxidative stress (Meydani & Hasan, 2010). CLA isomers (mainly *cis*-9, *trans*-11) have been shown to have protective effects against oxidative stress and lipid peroxidation in laboratory animals such as Wistar rats (Andreoli *et al*., 2010; Chinnadurai *et al*., 2013). Likewise, L-carnitine has been shown to reduce oxidative stress in several animal models such as the Wistar rats and aged rats (Kumaran *et al*., 2003; Ueno *et al*., 2013; Boyacioglu *et al*., 2014). In rat models, HCA has been shown to increase the levels of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) (Amin *et al*., 2011; Kim *et al*., 2013). Ephedrine has been reported to trigger mitochondrial oxidative stress depolarization in cultured cells (Lee *et al*., 2017).

Therefore, the aim of the research presented in this chapter was to investigate and compare the chemical and cellular antioxidant activity as well as the cytotoxic effects of weight loss molecules ephedrine, CLA, L-carnitine and HCA found in weight loss dietary supplements.

The specific objectives of this chapter are to:

1. Determine and compare the antioxidant activity of low and high concentrations of ephedrine, CLA, L-carnitine and HCA using the ORAC assay.
2. Determine and compare the cellular antioxidant activity in 3T3-L1 preadipocytes of low and high concentrations of ephedrine, CLA, L-carnitine and HCA using the DCFH-DA assay.
3. Determine and compare the effect of 24 and 48 h exposure to low and high concentrations of ephedrine, CLA, L-carnitine and HCA on cell viability in the L929 and preadipocyte 3T3-L1 cell lines using the MTT assay.

3.2 Materials

3.2.1 Weight loss products

Ephedrine (285749), conjugated (9Z, 11E) - linoleic acid, conjugated (10E, 12Z) - linoleic acid, L-carnitine hydrochloride and Garcinia acid (44929), were obtained from Sigma-Aldrich, Atlasville, South Africa (SA).

3.2.2 Reagents, equipment and disposable plastic ware

Dulbecco's modified Eagle's medium (DMEM), foetal calf serum (FCS), antibiotic solution (streptomycin, penicillin and fungizone), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,2'-azobis(2-amidinopropane) dichloride (AAPH), sodium phosphate dibasic dehydrate ($Na_2HPO_4 \cdot 2H_2O$), sodium phosphate (NaH_2PO_4), sodium hydroxide (NaOH), Potassium chloride (KCl), sodium carbonate ($NaHCO_3$) and sodium chloride (NaCl), sodium dodecyl sulfate (SDS) were obtained from Sigma-Aldrich, Atlasville, SA. Dimethyl sulphoxide (DMSO) was obtained from Merck, SA. Trypsin was obtained from Life Technologies Laboratories and was supplied by Gibco BRL products, Johannesburg, SA. Ethanol (EtOH) was obtained from Merck, SA. Water was double distilled and de-ionised (ddH_2O) with continental water system and medium and buffers were sterilized by filtration through a Millex 0.2 µm filter. Glassware was sterilized at 121˚C for 30 min in a Prestige Medical Autoclave series 100 from Labotec, Midrand, SA.

Equipment include: Lambda LS5OB spectrophotometer from Perkin Elmer, Boston, MA, USA supplied by Separations Scientific, Honeydew, SA, a BioTek plate reader purchased from

Analytical and Diagnostic Products (ADP) Johannesburg, SA. A Zeiss Axio cam Erc5s, Olympus IX71 light microscope (Oberkochen, Germany) was used. A plate shaker and Eppendorf pipettes from Eppendorf AG Hamburg, Germany were supplied by the LASEC, Cape Town, SA. A FLUOstar OPTIMA plate reader from BMG lab technologies, Offenburg, Germany. A Hermle Z300 centrifuge and Eppendorf pipettes from Eppendorf AG Hamburg, Germany were supplied by the Scientific Laboratory Equipment Company (LASEC), Cape Town, SA.

Disposable plastic ware used included: 96-well plates, 25 cm^2 tissue culture flasks, polypropylene tubes (0.6, 1.5, 15 and 50 mL) and pipette tips (10, 25, 100, 200, and 1000 µL) were obtained from Greiner Bio-one also supplied by LASEC, Cape Town, SA.

3.2.3 Cell lines

The cell lines used in this study were L929 (mouse fibroblast) and preadipocytes 3T3-L1 (murine fibroblast) and were purchased from CELLONEX Separations, Johannesburg, SA.

3.2.4 Laboratory facilities

All research was conducted in the research facilities of the Section of Cell Biology, Histology and Embryology in the Department of Anatomy of the Faculty of Health Sciences, University of Pretoria.

3.3 Methods

3.3.1 Sample preparation

Stock solutions of the weight loss products, ephedrine, CLA (10Z, 12E), CLA (9Z, 11E), L-carnitine hydrochloride and HCA were made to starting concentrations of 5000 µg/mL in PBS. CLA was mixed to a 1:1 ratio (10Z, 12E/ 9Z, 11E) as recommended by Lehnen *et al.*, 2015. Starting concentrations were 30.26 mM, 17.83 mM, 31.02 mM and 24.02 mM, ephedrine CLA, L-carnitine and HCA, respectively and the solutions were stored at -20°C.

The concentrations of CLA, L-carnitine and HCA used in this study were based on the recommended dosages provided by SOLAL™ products (https://www.wellnesswarehouse.com/brands/solal/), as shown in Table 3.1. A theoretical exposure was based on the average mass of an individual of 70 kg. Two dosages were calculated, and these were blood levels if bioavailability is 100% after taking i) a single tablet and ii) the accumulated daily dosage as shown in Table 3.1. In this study a concentration of

15, 21, 42 and 84 µg/mL represent single low dose and 500, 1000 and 5000 µg/mL represented a higher, daily accumulated dose. In molar concentrations these range from as low as 55 µM to as high as 31 mM. A similar dosage range was used for ephedrine used as weight loss control.

Table 3.1: The calculated low and high dosages based on dosage recommendation of SOLAL™ weight loss products.

Product	CLA	L-carnitine	HCA
Capsule content (mg)	1000	746	660
Dosage (µg/mL)	200	149,2	132
Daily dosage	1 capsule, 3 x day	2 capsules, 2 x day	1 capsule, 3 x day
Total dosage (mg)	3000	2984	1980
Dosage (µg/mL)	600	596,8	396

3.3.2 The L929 and 3T3-L1 cell lines maintenance and growth

The 3T3-L1 preadipocytes and L929 cells were maintained in DMEM supplemented with 10% FCS and 1% antibiotic solution (DMEM/FCS). Vials were thawed rapidly in warm water at 37˚C and then the cells were suspended in 5 mL DMEM/FCS and were collected by centrifugation at 7000 ×g. The supernatant was removed, and the cells were re-suspended in fresh DMEM/FCS before being plated in a 25 cm^2 culture flask. The flasks were then incubated at 37˚C at 5% CO_2. Once the cells were confluent, the medium was removed and the cells were passaged with a 1 mL of a 5% trypsin solution prepared in PBS. Following detachment, 5 mL fresh DMEM/FCS was added to the flask and then the cells were collected via centrifugation at 800 ×g for 2 min, before the pellet was re-suspended in 5 mL DMEM/FCS. The cell concentration was determined by counting a 10 µL aliquot of cells with a haemocytometer.

For determining cellular antioxidant activity and cell viability as described in the following sections, cells were plated at a cell concentration of 4×10^3 cells per 100 µL and 90 µL, respectively in 96-well flat bottom plates and were incubated for 24 h at 37˚C and 5% CO_2 to allow the cells to attach to the tissue culture surface before conducting each experiment. The cells were used between the passage values of P44 to P54 for L929 cells and P41 to P49 for the 3T3-L1 cells.

3.3.3 Determination of antioxidant activity

3.3.3.1 Chemical antioxidant activity: ORAC assay

The ORAC assay is based on free radical generation by AAPH and a decrease in fluorescence is measured in the presence of free radical scavengers (Alam *et al*., 2013). AAPH degrades either by hydrolysis or thermally forming nitrogen gas and two carbon-centred radicals (Werber *et al.*, 2011) which can react and form stable products or generate peroxyl radicals through interaction with molecular oxygen (Buys *et al*., 2013). The ability of the antioxidants to protect fluorescein against AAPH generated peroxyl radicals is the basis of the ORAC assay.

The method used is based on a modified version of the method of Ou *et al*., (2002). AAPH was used as a peroxyl radical generator and Trolox as a standard control (0 – 0.05 mM) to determine the Trolox Equivalence (TE) and fluorescein as a fluorescent probe and PBS was used as the blank. A 165 µL volume of 0.139 nM solution of fluorescein was added to 10 µL PBS, or 10 µL Trolox (serial dilution) or 10 µL of each concentration of ephedrine, CLA, L-carnitine and HCA. AAPH was added (25 µL of a 0.24 µM solution) to the experimental groups and the positive control (fluorescein and AAPH only). Samples were mixed well, and the microplate was placed into the plate reader and incubated at 37°C. The fluorescence was measured using a FLUOstar OPTIMA plate reader at an excitation wavelength (Ex) of 485 nm and an emission wavelength (Em) of 520 nm, every min for 2 h. The final ORAC values of the samples were calculated by using the net area under the decay curve (AUC) and the data was expressed as mM TE.

3.3.3.2 Cellular antioxidant activity: DCFH-DA assay

The DCFH-DA assay is a fluorometric assay for the detection of oxidative damage using either hydrogen peroxide or AAPH as a radical generator. When DCFH-DA crosses the membrane due to its hydrophobicity, it is hydrolysed by intracellular esterases to a non-fluorescent DCFH. During oxidative stress, which produces excessive ROS, DCFH will undergo oxidation to produce fluorescent dichlorofluorescein (DCF). The production of DCF fluorescence can be quantified as a measure of oxidative stress (Wang & Joseph, 1999). In this study, two strategies were used, (i) no AAPH was added to determine if the weight loss supplements can induce oxidative stress and (ii) with the addition of AAPH, the weight loss supplements have antioxidant activity i.e. reduce oxidative stress.

For determining the oxidative and protective effects to different concentrations of ephedrine, CLA, L-carnitine and HCA, a 50 µL volume of 75 µM of the DCFH-DA prepared in PBS (final concentration 25 µM) was added to each well containing $4x10^3$ undifferentiated 3T3-L1 cells per 100 µL and then the cell culture plates were incubated for 45 min at 37˚C. The medium

containing DCFH-DA was then carefully removed and then the cell culture plates were carefully rinsed with PBS, before being blotted dry.

For determining possible oxidative effects, a volume of 20 µL PBS was added to each well followed by 20 µL of low (0.75 – 4 µg/mL) and high (25 – 250 µg/mL) concentrations of ephedrine, CLA, L-carnitine and HCA. To determine if the weight loss supplements have protective effects, a volume of 20 µL of each concentration of ephedrine, CLA, L-carnitine and HCA were added to each well followed by a 20 µL volume of a 0.1 mg/mL AAPH solution.

For the positive control, 20 µL PBS was added to each well followed by 20 µL of a 0.1 mg/mL AAPH solution. For the negative control, 40 µL of PBS was added to each well. The change in fluorescence was measured immediately over 0 – 60 min, every 2 min with the FLUOstar Omega micro-plate reader at an Ex of 485 nm and Em of 520 nm was used. The gradient of the change in fluorescence was calculated, and the data was expressed as % damage where AAPH alone causes 100% damage.

3.3.4. Determination of cytotoxicity

3.3.4.1 MTT assay

The MTT assay is based on the principle that mitochondrial activity of viable cells is constant and that there is a direct relationship between mitochondrial activity and the number of viable cells (Van Meerloo *et al*., 2011). However, studies have indicated that MTT is not reduced in the mitochondria but in the cell cytoplasm, as the mitochondria have high oxidative capacity (Bernas & Dobrucki, 2002b). Reduction of MTT results in the formation of insoluble formazan crystals which are solubilized to ensure homogenous measurements and the absorbance is measured at 570 nm (van Meerloo *et al*., 2011).

For cell viability, both the L929 and 3T3-L1 preadipocytes cell lines were exposed to 10 µL of the increasing concentrations of ephedrine, CLA, L-carnitine and HCA for 24 and 48 h (with and without AAPH) at 37°C and 5% CO_2. To measure viability with the MTT assay, a 1 mg/mL MTT solution in PBS was prepared. A 10 µL volume of the MTT solution was added to each well and the cell culture plates were maintained for 3 h at 37˚C. The medium was then carefully removed, and plates were then blotted dry. The MTT formazan crystals were dissolved with 100 µL of 25% DMSO in ethanol by shaking the plates for 10 min. The absorbance was read at 570 nm on a Biotek plate reader. The cell viability was calculated as the percentage of the control (no weight loss supplement added).

3.3.5 Data management and statistical analysis

All data obtained is the average of three experiments and each measurement was done in triplicate, thereby resulting in nine data points. The results are expressed as mean ± standard error of mean (SEM). The GraphPad Prism 6 statistical program was used. Analysis of variance (ANOVA) was employed for data analysis for this study design followed by post hoc testing for determining statistical differences between treatments with weight loss dietary supplements vs. control. Testing was done at a 95% confidence level ($p<0.05$).

3.4 Results

3.4.1 Chemical antioxidant activity: ORAC assay

Increased ROS production in cells can result in oxidative stress and antioxidants are important molecules due to their potential to scavenge ROS. Radical scavenging activity of weight loss dietary supplements, ephedrine, CLA, L-carnitine, and HCA was assessed with the ORAC assay (Figure 3.1). A dose-response characteristic of antioxidant capacity was observed for CLA (0.065 – 0.88 mM TE), at high concentrations of 25 – 250 µg/mL but not at low concentrations. Ephedrine, L-carnitine and HCA exhibited no radical scavenging ability.

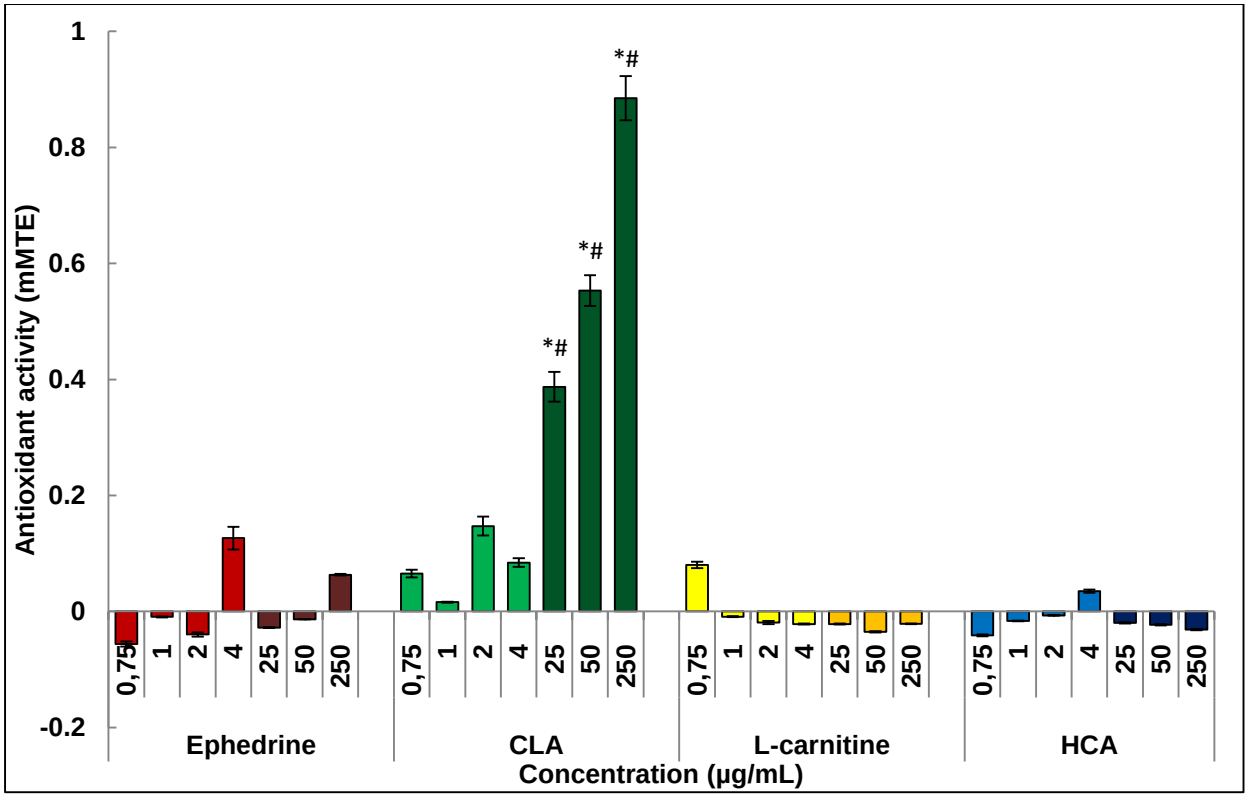


Figure 3.1: Oxygen radical absorbance capacity of low (0.75 – 4 µg/mL) and high (25 – 250 µg/mL) concentrations of ephedrine, conjugated linoleic acid, L-carnitine and hydroxycitric acid. All data is an average of at least three experiments and represented as mean ± SEM. * indicates differences compared to the control and # compared to the same concentrations of ephedrine ($p<0.05$).

3.4.2 Cellular antioxidant activity: DCFH-DA assay

The potential of weight loss dietary supplements to induce ROS in undifferentiated 3T3-L1 cells was assessed with DCFH-DA assay (Figure 3.2). Cells treated with CLA showed a significant increase in ROS production compared to the control (no CLA added) from a concentration as low as 10.5 µg/mL (≥100%) with large variability of results obtained. Ephedrine, L-carnitine and HCA at all concentrations evaluated did not induce ROS. CLA compared to all weight loss supplements caused a significant increase in ROS at all concentrations evaluated.

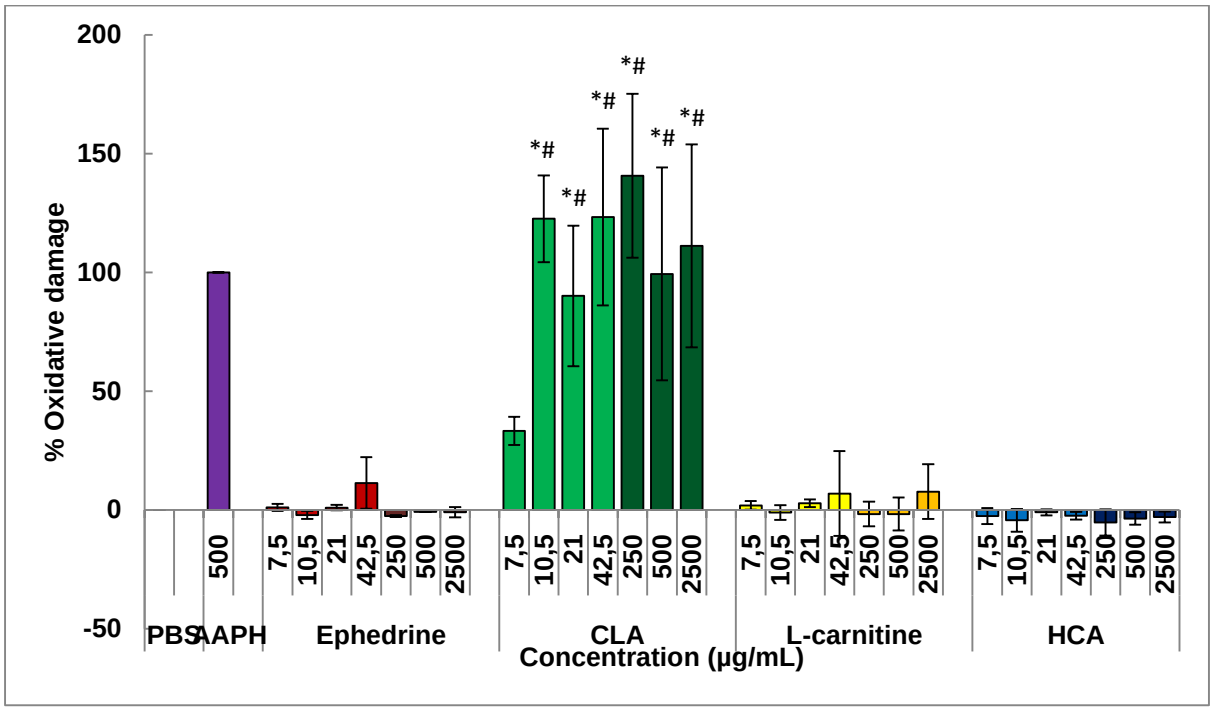


Figure 3.2: The oxidative effects of low (7.5 – 42.5 µg/mL) and high (250 – 2500 µg/mL) concentrations ephedrine, conjugated linoleic acid, L-carnitine and hydroxycitric acid in undifferentiated 3T3-L1 cells. Data presented are means ± SEM of three independent experiments performed in triplicates. * indicates significant differences compared to the control, # compared to the same concentrations of all supplements ($p<0.05$).

To assess the potential protective effect of the weight loss dietary supplements against AAPH induced oxidative damage the DCFH-DA assay was employed (Figure 3.3). Ephedrine did not protect 3T3-L1 cells against oxidative damage. For CLA at low concentrations and 250 µg/mL, no protection but rather an increase in oxidative damage is observed. At high concentrations the percentage oxidative damage is reduced, although not statistically significant. High concentrations of L-carnitine induced an increase in the percentage oxidative damage. Only HCA at 500 and 2500 µg/mL protected 3T3-L1 cells against oxidative damage. Likewise, HCA showed higher oxidative damage compared to ephedrine at concentration of 7.5 and 42.5 µg/mL.

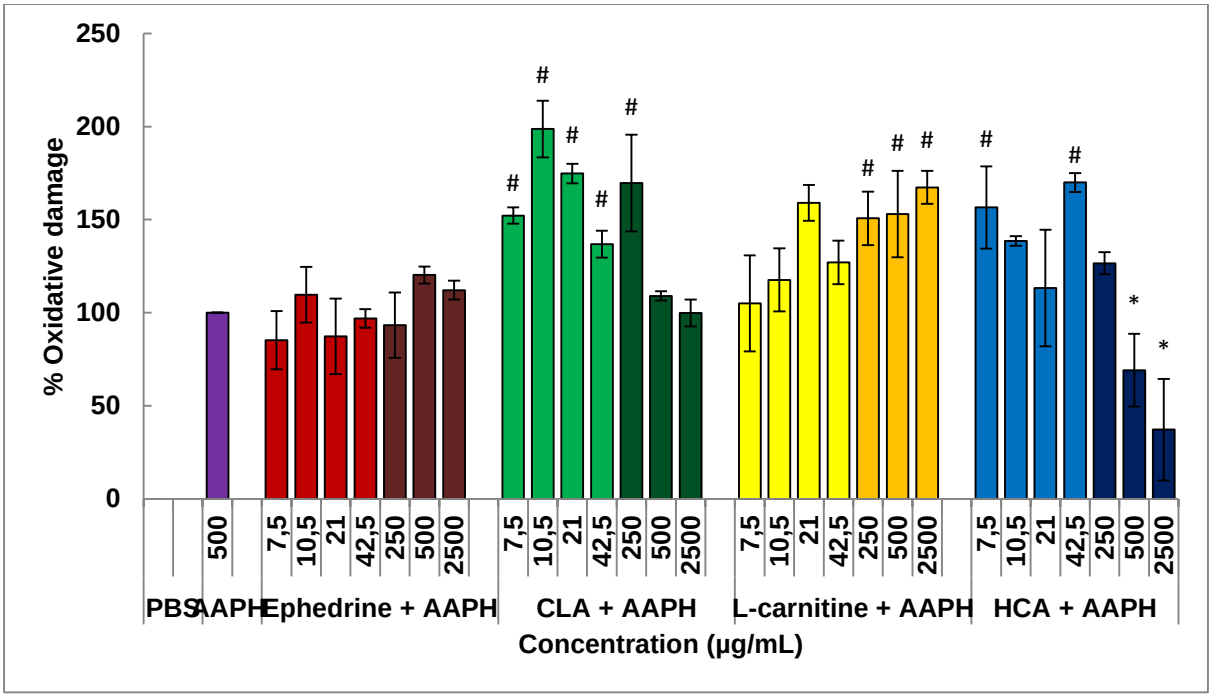


Figure 3.3: The protective effect of low (7,5 – 42,5 µg/mL) and high (250 – 2500 µg/mL) concentrations ephedrine, conjugated linoleic acid, L-carnitine and hydroxycitric acid against AAPH induced oxidative damage in undifferentiated 3T3-L1 cells. Data presented are means ± SEM of three independent experiments performed in triplicates. * Represents a statistically significant difference relative to AAPH # represents a statistically significant difference compared to the same concentrations of ephedrine ($p < 0.05$).

3.4.3 *In vitro* cytotoxicity: MTT assay

The cytotoxicity screening of the weight loss dietary supplements was assessed in the L929 cell line using the MTT assay after 24 and 48 h exposure (Figure 3.4). The treatment of L929 cells with ephedrine showed significant cytotoxicity compared to control (100% viability) at the highest concentration of 500 µg/mL at 24 and 48 h with measured cell viability of 75.30% and 60.24%, respectively. CLA similarly showed cytotoxicity at a concentration of 500 µg/mL of 76.94% and 47.53% after 24 and 48 h, respectively. No changes in cell viability was observed for L-carnitine and HCA at all concentrations, evaluated after 24 and 48 h. Over a concentration range of 1.5 – 100 µg/mL no differences were observed between L929 cells treated with L-carnitine and HCA compared with ephedrine exposed L929 cells. However, the highest concentration of 500 µg/mL ephedrine showed significant cytotoxicity compared to L-carnitine and HCA at the same concentrations.

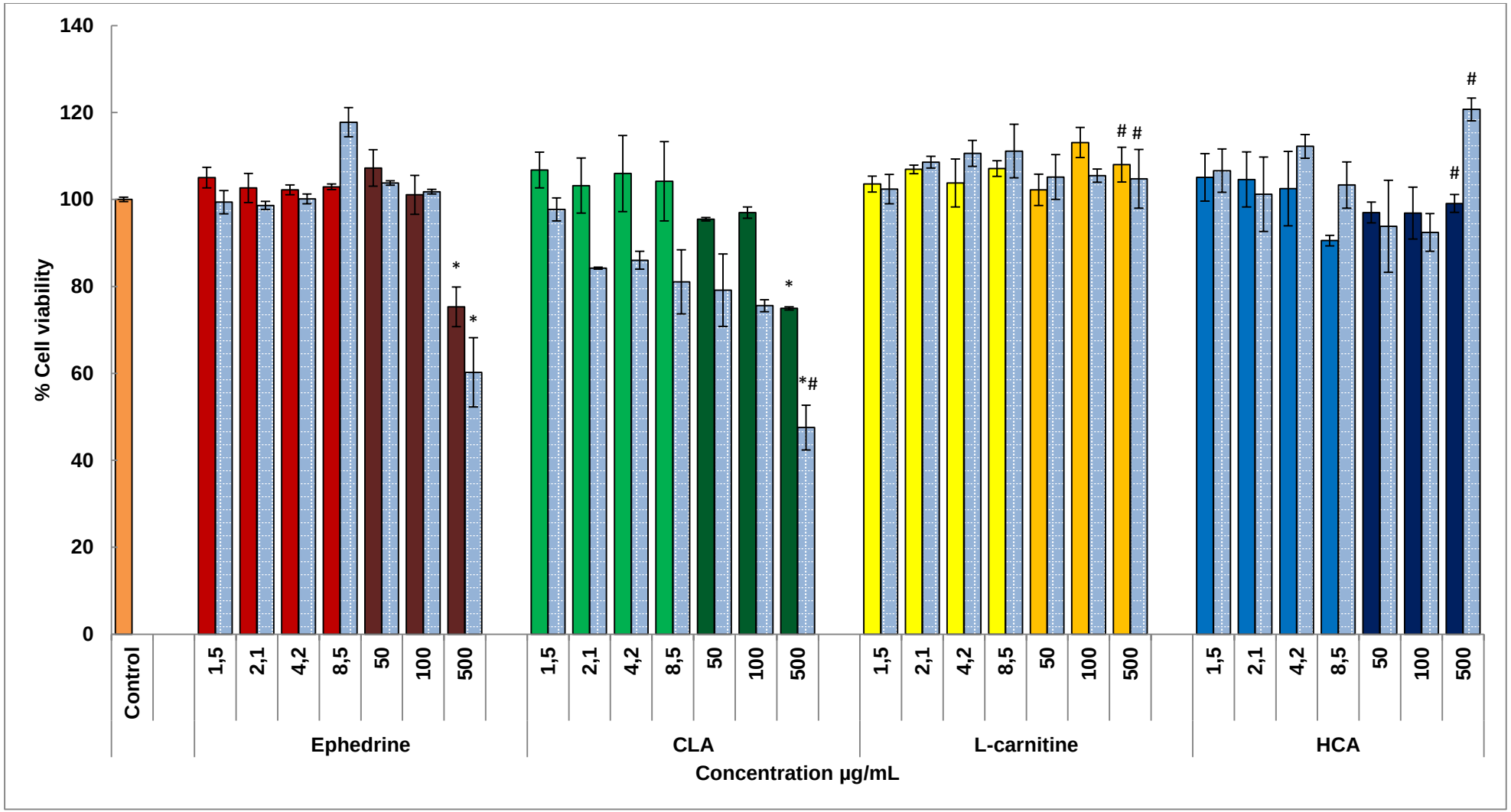


Figure 3.4: *In vitro* cytotoxic activity of low (1.5 – 8.5 µg/mL) and high (50 – 500 µg/mL) concentrations of ephedrine, conjugated linoleic acid, L-carnitine and hydroxycitric acid against L929 cells after 24 h (bold colours) and 48 h (striped). Data presented are means ± SEM of the three independent experiments performed in triplicates. * Represents statistical difference compared to the 100% cell viability control and, # represent differences observed between 24 and 48 h only at 5000 µg/mL between ephedrine and CLA, and L-carnitine and HCA ($p<0.05$).

Protective effect of ephedrine, CLA, L-carnitine and HCA against AAPH-induced cytotoxicity in L929 cell line was assessed using MTT assay (Figure 3.5). The results revealed that viability of cells treated with 50 µg/mL AAPH for 48 h was reduced to 78.68%, however, this decrease was not statistically significant compared to the control. Exposure to 1.5 – 100 µg/mL ephedrine and CLA increased the cell viability compared to AAPH exposed cells. However, exposure to 500 µg/mL ephedrine and CLA in the presence of AAPH caused a significant decrease in cell viability to 62.08% and 62.59%, respectively after 48 h exposure. Compared with ephedrine, L-carnitine and HCA at 500 µg/mL was not cytotoxic. At several concentrations the weight loss supplements caused an increase in cell viability compared to L929 cells exposed to 50 µg/mL AAPH although these effects were not statistically significant.

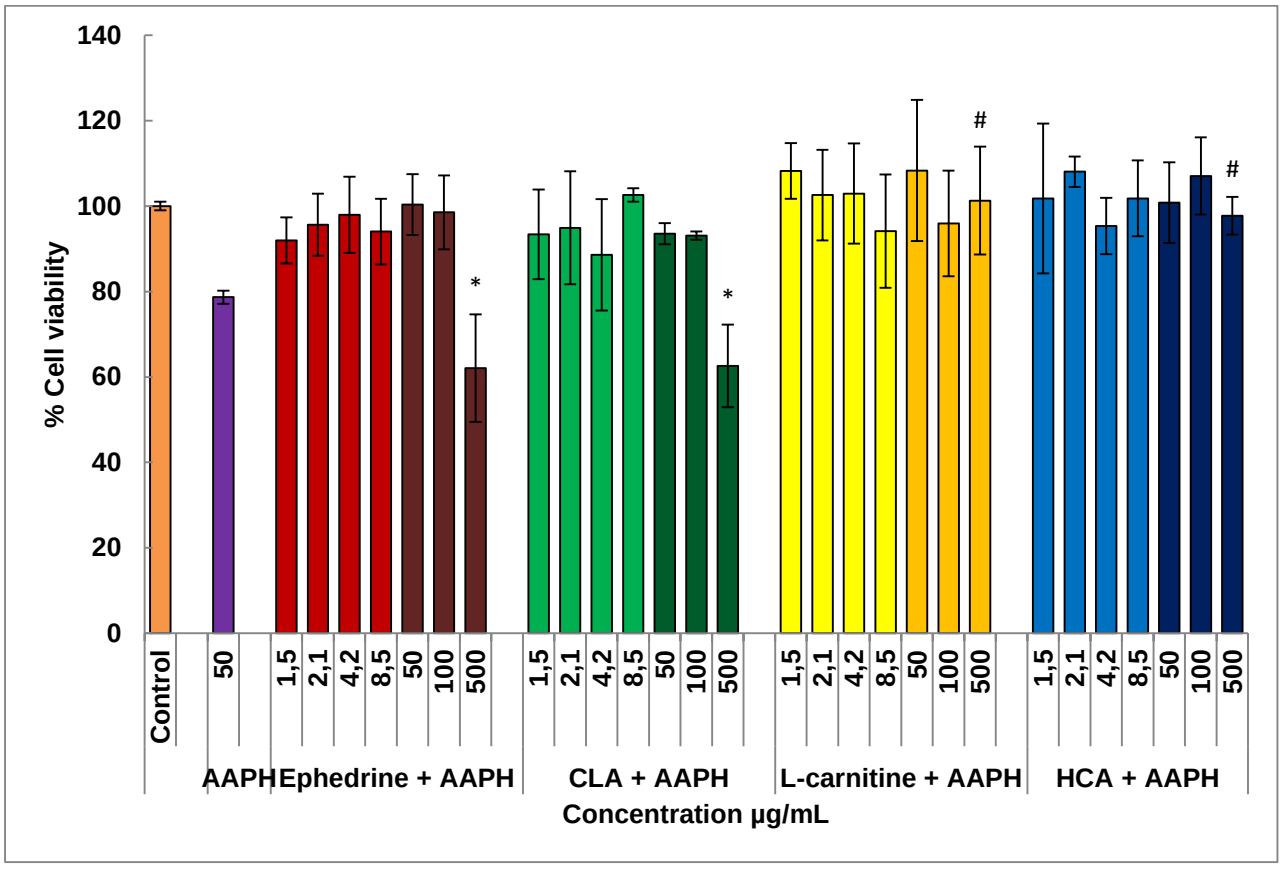


Figure 3.5: *In vitro* cytotoxic activity of low (1.5 – 8.5 µg/mL) and high (50 – 500 µg/mL) concentrations ephedrine, conjugated linoleic acid, L-carnitine and hydroxycitric acid against AAPH-induced cytotoxicity in L929 cells after 48 h. Data presented are means ± SEM of the three independent experiments performed in triplicates. * Represents statistical difference compared to the 100% cell viability control and # represents a statistically significant difference compared to the same concentrations of ephedrine ($p<0.05$).

The effect of the weight loss dietary supplements on cell viability on preadipocytes 3T3-L1 cells after 24 and 48 h exposure was also determined (Figure 3.6). For ephedrine, CLA, L-carnitine and HCA at concentrations of 1.5 – 100 µg/mL no change is cell viability was

observed. A concentration of 500 µg/mL ephedrine and CLA after 48 h exposure caused a significant loss in cell viability to 54.64% and 56.21%, respectively. L-carnitine and HCA at all concentrations did not adversely affect cell viability. Differences between ephedrine and CLA was not significant but were significant between ephedrine and L-carnitine and HCA at a concentration of 500 µg/mL after 48 h exposure. The L929 cell line was more sensitive to the effects of ephedrine and CLA than the 3T3-L1 cell line. A decrease in L929 cell viability was observed after 24 and 48 h exposure to 500 µg/mL ephedrine and CLA while a decrease in 3T3-L1 viability was only observed after 48 h exposure to 500 µg/mL ephedrine and CLA.

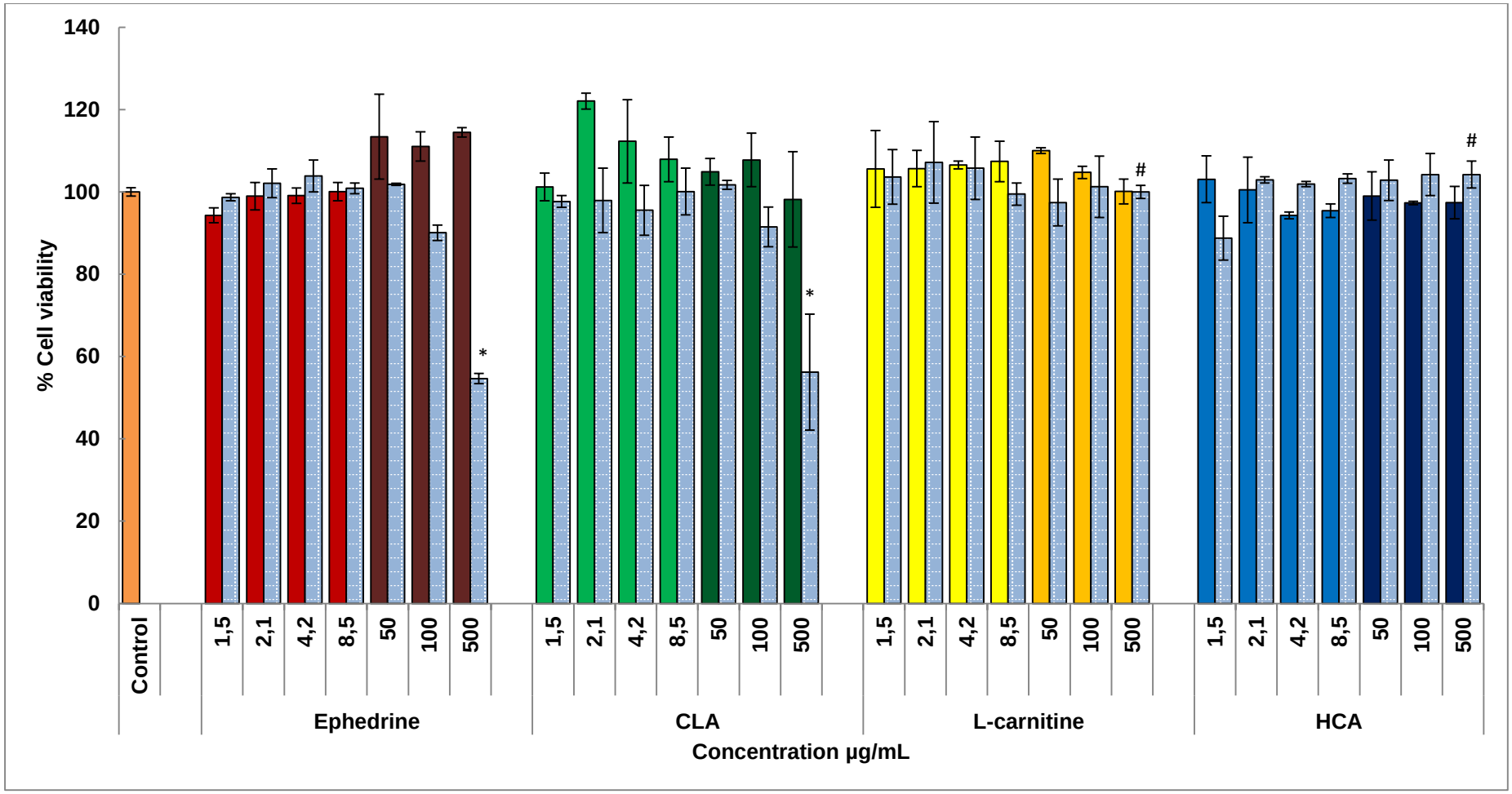


Figure 3.6: *In vitro* cytotoxic activity of low (1.5 – 8.5 µg/mL) and high (50 – 500 µg/mL) concentrations ephedrine, conjugated linoleic acid, L-carnitine and hydroxycitric acid against 3T3-L1 preadipocytes after 24 h (solid colours) and 48 h (striped). Data presented are means ± SEM of the three independent experiments performed in triplicates. * Represents statistical difference compared to the 100% cell viability control and, # represent differences observed between 24 and 48 h only at 5000 µg/mL between ephedrine and CLA, and L-carnitine and HCA ($p<0.05$).

The protective effect on cell viability of ephedrine, CLA, L-carnitine and HCA against AAPH-induced cellular damage was also determined in 3T3-L1 cells (Figure 3.7). Exposure to 50 µg/mL AAPH caused a reduction in cell viability to 83.61% cell viability although differences between the control and 3T3-L1 cells exposed to AAPH was not significant. No changes in viability was observed for ephedrine, L-carnitine and HCA in combination with AAPH. Although, not statistically significant, cell viability was increased. At 500 µg/mL, exposure to ephedrine for 48 h caused some loss of cell viability although not significant compared with the control. At the same concentration CLA caused significant loss in cell viability compared with 3T3-L1 cells exposed to AAPH. Treatment with different concentrations of ephedrine, L-carnitine and HCA improved cell viability although the increase in cell viability was not statistically significant. Compared to ephedrine at all concentrations cell viability was unchanged for ephedrine, CLA, L-carnitine and HCA in combination with AAPH.

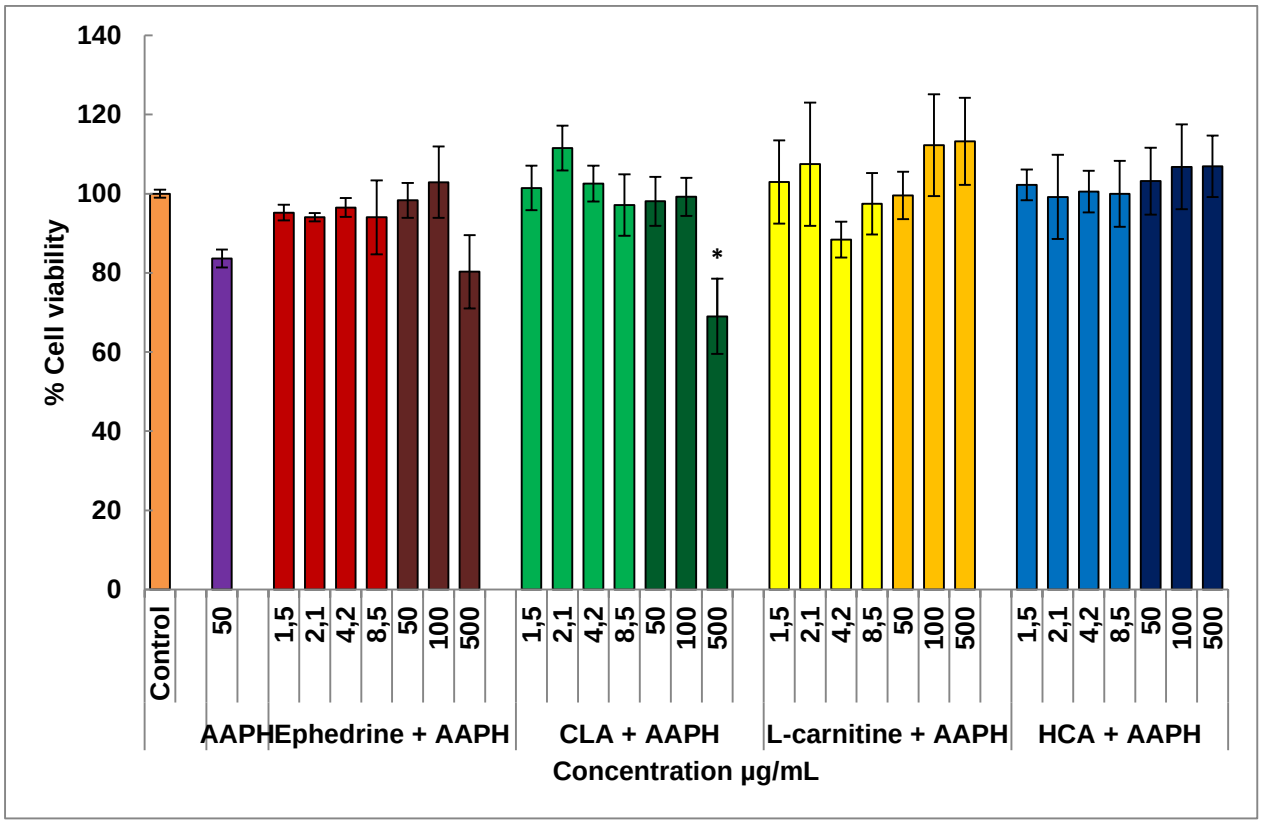


Figure 3.7: *In vitro* cytotoxic activity of low (1.5 – 8.5 µg/mL) and high (50 – 500 µg/mL) concentrations of ephedrine, conjugated linoleic acid, L-carnitine and hydroxycitric acid against AAPH-induced cytotoxicity in 3T3-L1 preadipocytes after 48 h. Data presented are means ± SEM of the three independent experiments performed in triplicates. * Represents statistical difference compared to the 100% cell viability control ($p<0.05$).

3.5 Discussion

3.5.1 Chemical antioxidant activity: ORAC assay

Oxidative stress is associated with several diseases such as CVD, neurodegenerative diseases, cancer, and MS (Wlodarczyk & Nowicka, 2019). Antioxidants are classified as either endogenous or exogenous antioxidants. Endogenous antioxidants are enzymes such as CAT, GPx and SOD while exogenous antioxidants, are present in the diet and include vitamin C, vitamin E, zinc, selenium and carotenoids. Protection can occur as a result of direct and indirect neutralization of free radicals, inhibition of chain reactions and binding of iron or copper, that are catalysts of the Fenton reaction, subsequently preventing the formation of free radicals. Secondly, antioxidants through cellular metabolism and repairing damage protect cells against the effects of free radicals. Finally, antioxidants can protect macromolecules by preventing free radicals from attacking lipids, amino acids, and polyunsaturated fatty acid double bonds and DNA bases thereby preventing cell death (Marcadenti & Coelho, 2015).

Extensive studies have been conducted on determining antioxidant activity of pure compounds, foods and dietary supplements as well as newly identified or synthesized antioxidants (Lui & Finley, 2005). Several chemical-based methods can be used to assess the antioxidant properties of natural products (Lopez-Alarcon & Denicola, 2013). The ORAC assay is a widely used chemical-based method for determining chemical antioxidant activity in foods, food ingredients, plant extracts, dietary supplements and biological samples as it uses the biological relevant free radicals, peroxyl radicals. This assay is the gold standard for the determination of antioxidant activity as it is a hydrogen atom transfer (HAT) based assay, and the mechanism involved is physiologically relevant (Pisoschi *et al*., 2016).

The protective effect of CLA, L-carnitine and HCA against oxidative stress is not fully understood (Pignatelli *et al*., 2003; Ali *et al*., 2012; Basirico *et al*., 2017). In this study, a dose-response characteristic of antioxidant capacity was observed for CLA. Ali *et al*., (2012) investigated the antioxidant activity of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 as single or mixed CLA isomers at two ratios 1:6 and 1:13 (*trans*-10, *cis*-12/ *cis*-9, *trans*-11) at concentrations of 2.5, 5, 10, 20, 40, and 80 mg/mL in ethanol using the DPPH radical scavenging assay and calculating the inhibitory concentration (IC_{50}). All tested CLA isomers showed a dose and time dependent scavenging ability. Based on the IC_{50} values, CLAs are effective antioxidants with the *cis*-9, *trans*-11 (11.1 ± 3.8 mg/mL) being the most effective followed by *trans*-10, *cis*-12 (12.6 ± 3.4 mg/mL) and then the isomer mixture with IC_{50} value of 16.3 ± 1.1 mg/mL. Similarly, Fagali & Catalia, (2008) observed that the single isomers of CLA, *trans*-10, *cis*-12 and *cis*-9, *trans*-11 have free radical scavenging properties against the stable radical ($DPPH^{\cdot}$) from 5 to 25 mM (1402 to 7010 µg/mL). Yu *et al*., (2002) identified that CLA provided immediate protection against DPPH free radicals in a dose and time-dependent manner using electron

spin resonance (ESR) spectrometry and spectrophotometric methods at 5000 – 80000 µg/mL. Kadidrareddy *et al*., (2016) evaluated the scavenging activity of *Lactobacillus plantarum*-CLA (LP-CLA) at concentrations of 25 – 200 µg/mL using the DPPH assay. *Lactobacillus plantarum* was used as a microbial model for the bioconversion of linoleic acid into conjugated linoleic acid. A dose-dependent quenching of DPPH was observed for 25 and 200 µg/mL LP-CLA with 18% and 98% inhibition. The IC_{50} for DPPH scavenging ability was 74.94 µg/mL of LP-CLA. Therefore, the researchers suggested that LP-CLA has a proton donating ability and acts as free radical scavenger (Kadidrareddy *et al*., 2016).

In a study by Leung & Liu, (2000), the antioxidant activity of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers using highly purified CLA isomers (>98%) by the total ORAC assay was investigated. The concentrations used were 2 – 200 µM (0.56 – 56.09 µg/mL). The *trans*-10, *cis*-12 CLA showed antioxidant activity at all tested concentrations, while *cis*-10, *trans*-11 CLA showed weak antioxidant activity and strong pro-oxidant activity at 200 µM. The researchers suggested that the potential antioxidant activity of CLA may be due to the balance between the antioxidant capacity of *trans*-10, *cis*-12 CLA and pro-oxidant activity of *cis*-9, *trans*-11 CLA (Leung & Lui, 2000). Data from previous studies suggest that the chemical antioxidant activity exerted by CLA is through direct neutralization of free radicals. In the present study, at concentrations of 25 – 250 µg/mL CLA has antioxidant activity confirming the antioxidant activity of CLA.

L-carnitine has been shown to decrease oxidative stress through direct free radical scavenging properties. In support to direct free radical scavenging properties of L-carnitine, Gulcin, (2006), evaluated antioxidant properties of L-carnitine using total antioxidant activity assay which was measured according to the ferric thiocynate method where alpha-tocopherol and Trolox (water-soluble analogue of vitamin E) were used as reference compounds. This assay measures the ability of antioxidants to prevent lipid hydroperoxides (LOOH) mediated oxidation of ferrous to ferric ion. At concentrations of 15, 30 and 45 µg/mL L-carnitine had 94.6%, 95.4% and 97.1% antioxidant activity against lipid peroxidation respectively compared to controls showing 88.8% for alpha-tocopherol and 86.2% for Trolox. Solarska *et al.*, (2010), reported that L-carnitine quenched the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{\cdot+}$) radicals at concentrations of 5 – 20 µM (0.8 – 3.2 µg/mL). In addition, L-carnitine protected fluorescein against AAPH induced oxidative damage by AAPH-derived peroxyl radicals at concentrations of 5 – 20 µM (0.8 – 3.2 µg/mL). L-carnitine also was shown to protect thiol groups against oxidation induced by H_2O_2 at 1 – 4 µM (0.16 – 0.64 µg/mL) (Solarska *et al*., 2010). The lack of antioxidant activity observed in the present study is contradictory as similar and higher concentrations were used (0.75 – 250 µg/mL).

HCA is found in the fruit rinds of certain species of *garcinia*, which include *G. cambogia*, *G. indica*, *G. atroviridis*, *G. pedunculata* and *G. cowa* (Jena *et al*., 2002; Sharma *et al*., 2014). HCA has been identified as an active compound in extracts of the rinds of *garcinia* fruits and is used as weight controlling agent (Jena *et al*., 2002, Hayamizu *et al*., 2003). In the present study, HCA showed no chemical antioxidant activity against free radical generator, AAPH using the ORAC assay. Minakshi *et al*., (2015) evaluated the antioxidant activity of an extract from *G. indica* fruit. A significant dose dependent inhibition of the DPPH radical at concentrations of 6.25 – 200 µg/mL was observed. The IC_{50} value of *G. indica* fruit extract was 50.34 g/mL compared with standard ascorbic acid which was found to be 13.74 at 200 µg/mL. In addition, *G. indica* fruits extract showed a significant superoxide radical scavenging activity at a concentration of 200 µg/mL. The researchers identified that the free radical scavenging activity of *G. indica* fruit was due to its high polyphenol content (Yamaguchi *et al*., 2000). Hassan *et al*., (2013), evaluate the antioxidant activity of *G. parvifolia* extract and found that this extract exhibited significant scavenging activity against the DPPH free radical. These researchers also identified that phenolic compounds, flavonoids, anthocyanins and carotenoids contribute to the antioxidant activity of the *G. parvifolia* fruit extract. The researchers reported that there is a strong correlation between DPPH free radical scavenging activity and total phenolic content (r=0.887 and r=0.962 for that of total flavonoid content) (Hassan *et al*., 2013). The present study confirms that HCA does not contribute significantly to the antioxidant activity of these extracts.

The use of ephedrine-containing dietary products, due to adverse side effects, were banned for sale in 2004 by the U.S. FDA. In 2005, the U.S. Federal District Court concluded that the need for analysis of dose specific adverse effects of ephedrine containing products is still required to better understand the mechanism of toxicity (Lee *et al*., 2017). In the present study ephedrine was used as a weight loss control and it did not have any antioxidant activity measured with the ORAC assay. In contrast, several studies have shown that ephedrine has potent chemical antioxidant activity as it has been shown that ephedrine is present together with a mixture of phenolic compounds, such as flavonoids and some alkaloids (Al-Rimawi *et al*., 2017; Gul *et al*., 2017; Kallassy *et al*., 2017, Khan *et al*., 2017).

3.5.2 Cellular antioxidant activity: DCFH-DA assay

Antioxidant capacity is not limited to inhibition of oxidation of a targeted molecule but also involves antioxidant upregulation and enzyme detoxification, redox cell signalling modulation and gene expression. Therefore, it is important to move to cellular-based methods for evaluation of antioxidant capacity of a compound or extract (Lopez-Alarcon & Denicola, 2013).

In the current study, each weight loss compound was evaluated for its ability to induce ROS, a pro-oxidant effect or inhibit ROS, an antioxidant effect.

CLA induced oxidative stress with low repeatability of the results obtained. Likewise, Basirico *et al*., (2017) reported that exposure of BME-UV1 cells (primary bovine mammary epithelial cells) to 50 µM (14.02 µg/mL) CLA isomers (*cis*-9, *trans*-11 and *trans*-10, *cis*-12) for 48 h caused an increase in ROS production when compared with the control. Kadidrareddy *et al*., (2016) reported that following exposure of human breast cancer cell line (MDA-MB-231 cells) to increasing concentrations of LP-CLA (20, 40 and 60 µg/mL) for 24 h, CAT activity was significantly increased by 1.63-fold. Implying that CAT inhibited the accumulation of H_2O_2 during oxygen transport (Scibor & Czeczot, 2006). CLA did not provide any protective effect against oxidative damage caused by AAPH. Induction of antioxidant enzymes is not an immediate response to oxidative damage and is time dependent therefore, the lack of an antioxidant effect may be due to a lack of enzyme induction. Large variability of the results obtained with DCFH-DA assay in this study, could be related to the DCFH-DA probes which are sensitive to photo-oxidation and chemical environment (Pavelescu, 2015).

L-carnitine also did not induce significant oxidative damage in 3T3-L1 cells or protected these cells against oxidative damage. A beneficial effect identified in several studies was that L-carnitine is a powerful antioxidant (Moosavi *et al*., 2016; Canbolat *et al*., 2017; Wang *et al*., 2017). The protective effect of L-carnitine supplementation against oxidative stress was the result of the stabilisation of free radicals by the carbonyl group of L-carnitine via conjugation, therefore protecting cell components against the toxic actions of ROS and RNS (Gülcin, 2006, Kolodziejczyk *et al.*, 2011).

The protective effect of L-carnitine is also related to its ability to protect the structure and function of the mitochondria against oxidative stress. Mitochondrial dysfunction results in an imbalance between the production and the removal of ROS (Surai, 2015). Shen *et al*., (2008) observed that pre-treatment with 10 – 100 µM (1.61 – 16 µg/mL) L-carnitine reduced ROS formation and increased mitochondrial membrane potential in insulinoma and islet cells isolated from rats chronically exposed to oleic acid. Similarly, Ye *et al*., (2010) found that L-carnitine at concentrations of 10 – 100 µM (1.61 – 16 µg/mL) provided protective effects against H_2O_2- induced injury in human proximal tubule epithelial cells by preventing oxidative damage, mitochondrial dysfunction and inhibition of apoptosis. Zhang *et al*., (2010) observed that pre-treatment with L-carnitine for 12 h in human neuroblastoma cells prevented H_2O_2 - induced cytotoxicity, intracellular ROS generation and lipid peroxidation in a dose-dependent manner. Furthermore, L-carnitine was reported to prevent mitochondrial dysfunction and

associated apoptosis, loss of membrane potential, Bcl-2 downregulation and Bax upregulation as well as cytochrome release. Lee *et al*., (2014), found that L-carnitine reduced markers of oxidative stress and increased antioxidant enzymes activities in patients with coronary artery disease. L-carnitine supplementation at a dose of 1000 mg/d significantly reduced MDA levels and increased the levels of antioxidant enzymes, CAT and SOD. In the present study, L-carnitine did not protect cells against oxidative damage and at high concentrations a pro-oxidant effect was observed (Figure 3.2). This may be related to the concentrations used, where an antioxidant effect is observed at low concentrations and an oxidative effect at high concentrations. In the above studies, an antioxidant effect was observed for 1.61 – 16 µg/mL L-carnitine, concentrations that are several folds less than that used in the present study and may explain the lack of cellular antioxidant activity.

HCA is considered a safe dietary supplement used for weight loss and reduction of blood cholesterol levels (Goudarzvand *et al*., 2016). The current study confirms that HCA does not induce oxidative damage in 3T3-L1 cells. In the present study, HCA showed a dose-dependent response (250 – 2500 µg/mL) against AAPH-induced oxidative damage. Minakshi *et al*., (2015) also found that pre-treatment of *G. indica* fruit extract in human monocytic (THP-1) cells resulted in a significant dose-dependent reduction against H_2O_2 - induced intracellular ROS production using flow cytometry with DCFH-DA. Ghosh & Mukherjee, (2017) observed a significant increase in ROS production in human lymphocytes following treatment with *G. cambogia* containing 50.9% HCA at concentrations of 40 and 100 µg/mL after 3 and 24 h using DCFH-DA quantified by flow cytometry. This ROS production caused significant damage to cellular DNA evaluated with the comet assay.

Goudarzvand *et al*., (2016) reported that *G. cambogia* extract containing 50% HCA (2 g/kg once daily for 3 weeks in C57BL/6 male mice) used to treat autoimmune encephalomyelitis, induced oxidative stress. Supplementation with this extract was shown to significantly reduce NO production by decreasing the levels of inflammatory cytokines such as TNF-α and inducible nitric oxide synthase (iNOS) (Nikolaos *et al*., 2000). Treatment with this extract increased SOD activity and decreased serum MDA levels. Amin *et al*., (2011), showed that treatment with 50 mg/d of *G. cambogia* in male Wister rats for 12 weeks significantly decreased MDA levels and CAT activity in rats consuming a high fat and sucrose diet. These researchers indicated that the observed beneficial effects may also be related to decreased food intake by the rats (Amin *et al*., 2011). Although these extracts also contain polyphenols, the presence of HCA has a beneficial antioxidant effect as observed in the present study.

In the present study, ephedrine did not induce cellular oxidative damage or protected 3T3-L1 production in the 3T3-L1 cell line. Lee *et al*., (2017) showed that treatment of LX-2 hepatic stellate cells with 50 – 1600 µg/mL ephedrine induced ROS reaching the highest level after 6 h. Differences between this study and the findings of the present study may be related to cell type (hepatocyte vs adipocyte) and/or duration of exposure (1 h vs. 6 h). No antioxidant effects have been reported.

3.5.3 *In vitro* cytotoxicity: MTT assay

In vitro cytotoxicity tests are based on the ability of toxic compounds to induce cell lysis, reduce mitochondrial function, alter cellular morphology and cell replication. Different methods can be used to assess cell viability and includes quantifying the release of ^{51}Cr-labeled protein following cell lysis, measuring radioactive nucleotides incorporation and measuring colorimetric changes based on enzymatic activity in metabolically active cells. Colorimetric methods employing tetrazolium salts are frequently used as these assays do not utilize any hazardous radioactive materials and can be used for testing large numbers of samples (Fukushima, 2004).

In this study, the MTT assay was used to assess the *in vitro* cytotoxicity of weight loss dietary supplements (ephedrine, CLA, L-carnitine and HCA) in the L929 and 3T3-L1 cell lines. Only CLA was cytotoxic and a significant decrease in L929 viability was observed following exposure to 500 µg/mL CLA for 24 and 48 h. In contrast, in 3T3-L1 preadipocytes cellular activity at the same concentration was only reduced after 48 h exposure. In the presence of AAPH, toxicity was observed for both cell lines exposed to 500 µg/mL of CLA for 48 h. Cellular death may be the consequence of oxidative stress induced by CLA.

Melake *et al*., (2012) reported that CLA isomers (*cis*-9, *trans*-11, *trans*-10, *cis*-12 and mixed isomers) reduced cell viability evaluated with the MTT assay in a dose-dependent manner in human hepatoma cancer cells (HepG2) at concentrations of 5 – 180 µg/mL after 48 h exposure Using the terminal deoxynucleotidyl transferase-mediated d-UTP nick-end labelling (TUNEL) and Acridine orange/propidium iodide (AO/PI) staining, at all concentrations, the CLA isomers increased the number of apoptotic cells compared to the control. The authors further observed that the CLA isomers induced cell cycle arrest in the $G_{0/1}$ phase possibly activating apoptosis. The authors concluded that the reduced cell viability of HepG2 cells by CLA isomers could be related to cell cycle arrest and induction of apoptosis (Melaku *et al*., 2012).

Achenef & Arifah, (2012), showed that treatment of human breast cancer cells (MCF7) with 10 – 80 µg/mL CLA isomers (*cis*-9, *trans*-11, *trans*-10, *cis*-12 and mixed isomers) reduced cell viability with the MTT assay in a dose-dependent manner after 72 h. Induction of apoptosis was also investigated using the TUNEL assay and a significantly higher number of apoptotic cells compared to the control was observed. Furthermore, CLA isomers elicited cell cycle arrest in $G_{0/1}$ (Achenef & Arifah, 2012). Incubation of rat hepatic stellate cells (HSC-T6) with 10 – 80 µM (2.8 – 22.4 µg/mL) of 98% *trans*-10, *cis*-12, 96% *cis*-9, *trans*-11 and mixed isomers (41% *cis*-9, *trans*-11: 44% *trans*-10, *cis*-12) for 24 h reduced cell viability in a dose-dependent manner (Yu *et al*., 2008). The *cis*-9, *trans*-11 CLA isomer was the most cytotoxic on HSC-T6 cells, where 80 µM (22.4 µg/mL) of this isomer reduced cell survival to 60% while the survival rate was only slightly affected by the mixed CLA isomers. CLA induced cell death was further investigated by measuring DNA fragmentation using 4',6-diamidino-2-phenylindole staining. Findings were that the *cis*-9, *trans*-11 isomer at 80 µM induced severe DNA fragmentation in the HSC-T6 cells. The authors further evaluated the protective effect of CLA on 10% carbon tetrachloride induced hepatic fibrosis *in vivo*. With CLA supplementation there was a significant reduction of α-smooth muscle actin-positive cells around the portal veins of the rat livers. In addition, collagen fibres were not detected in the CLA treated group (Yun *et al*., 2008). In contrast to the current study, Cusack *et al*., (2005) found that incubation with increasing concentrations 0 – 5 µM (0 – 1.4 µg/mL) of CLA either as pure *cis*-9, *trans*-11, *trans*-10, *cis*-12 and mixed isomers did not induce any significant cytotoxicity in human osteoblast-like cell lines (SaOS2 and MG63). In addition, in both cell lines, the CLA isomers did not cause DNA fragmentation. The lack of cytotoxic effects of CLA on human osteoblast-like cells identified that CLA could have a beneficial effect in bone (Cusack *et al*., 2005). The lower cytotoxic effects seen in the 3T3-L1 preadipocytes compared with L929 cells as well as the findings of other studies indicates that CLA maybe cell type specific.

There is still little evidence concerning the effect of L-carnitine *in vitro* and *in vivo*. This study was conducted to provide evidence on the possible cytotoxic effects of L-carnitine. L-carnitine was found not to induce cytotoxicity in the L929 and 3T3-L1 cells after 24 and 48 h of exposure. Several studies have reported L-carnitine as a protective agent against induced cytotoxicity. Yu *et al*., (2011) revealed that pre-treatment with L-carnitine in neuroblastoma cells (SH-SY5Y) after 3 h inhibited H_2O_2 induced cell viability loss, morphological changes, production of intracellular ROS, and lipid peroxidase in a dose-dependent manner. L-carnitine further promoted endogenous antioxidant defence components including total antioxidative capacity, increased GSH levels as well as the induction of CAT and SOD. This protective effect of L-carnitine against H_2O_2 induced cell viability loss was related to its antioxidant action against H_2O_2 induced oxidative stress and regulation of the Bcl_2 family thereby preventing apoptosis

of neuronal cells (Yu *et al*., 2011). As a result, L-carnitine was identified to have beneficial effects against oxidative stress associated with neurodegenerative disease. Abdoli *et al*., (2015), observed that administration of L-carnitine reduced cell death in rat hepatocytes exposed to statin, an anti-hyperlipidemic drug known to cause hepatotoxicity. The protective effect of L-carnitine may be related to its capacity to facilitate the entry of fatty acids into the mitochondria and to limit the ROS inducing effects of statins (Abdoli *et al*., 2015). Li *et al*., (2012) showed that supplementation with L-carnitine at low concentrations of 0.01 – 3 mM (1.61 – 484 µg/mL) did not provide cytoprotection against on H_2O_2 induced toxicity in human normal hepatocyte cells (HL7702), while at a higher concentration of 5 mM (806 µg/mL) a significant inhibitory effect of L-carnitine was observed after 24 h using the MTT assay. In addition, pre-treatment with 0.1 – 1 mM (16.1 – 161 µg/mL) L-carnitine reduced H_2O_2 induced LDH release in a dose-dependent manner in HL7702 cells. The protective effect of L-carnitine may be related to its antioxidant capacity (Li *et al*., 2012). In the present study, no protection against AAPH induced damage was observed and this may be due to the experimental design, where in many of the above studies, cells are pre-treated with L-carnitine before exposure to an oxidant such as H_2O_2.

Regarding the toxicity and safety of *G. cambogia* extracts most studies have not provided enough evidence regarding toxicity (Marquez *et al*., 2012). According to the results obtained from this study, exposure to HCA did not induce any significant cytotoxicity against L929 and 3T3-L1 cell lines after 24 and 48 h exposure. Ghosh and Mukherjee, (2017) assessed the cytotoxic effect of *G. cambogia* (containing 50.9% HCA) at concentrations of 0 – 100 µg/mL for 3 h and 24 h in human lymphocytes using the trypan blue dye exclusion test and the MTT assay. With the trypan blue dye exclusion assay, only at 100 µg/mL there was a decrease in cell viability to 89.88% and 80.83% after 3 and 24 h exposure respectively. Furthermore, treatment with this extract at 100 µg/mL failed to provide protection against H_2O_2 induced cytotoxicity. This cytotoxic effect was linked to ROS generation due to the high flavonoid content and an associated pro-oxidant effects (Ghosh & Mukherjee, 2017).

Varalakshmi *et al*., (2011) evaluated the cytotoxic effects of *G. indica* rind extract at concentrations of 80, 160 and 240 µg/mL on Balb/c 3T3 mouse fibroblasts using the MTT assay. *G. indica* rind extracts reduced cell viability nearly by 50% and 80% after 60 days of exposure to 80 µg/mL and 240 µg/mL extract (Varalakshmi *et al*., 2011). Li *et al*., (2017) observed no significant difference in viability of chicken hepatocytes at concentrations of 1 – 50 µM (0.208 – 10.40 µg/mL) of HCA after 24 h using MTT assay. Furthermore, the rate of cellular death was not affected by HCA exposure (Li *et al*., 2017). Likewise, in the present study, HCA was not cytotoxic.

Ephedrine-type alkaloids are the active ingredients of ephedra and it is known to contain other phytoconstituents, which may contribute to its pharmacological and toxicological effects (Food & Drug, 1997). More than 800 studies have reported the adverse effects associated with consumption of ephedrine and have been received by the U.S. FDA. However, further studies are required to elucidate the mechanism of toxicity (Lee *et al*., 2000). Similar to CLA, ephedrine caused a significant decrease in L929 cell viability following exposure to 500 µg/mL ephedrine for 24 and 48 h. At the same concentration in 3T3-L1 preadipocytes cellular activity was only reduced after 48 h exposure. In the presence of AAPH, toxicity was observed for both cell lines exposed to 500 µg/mL for 48 h. Lee *et al*., (2017) showed that ephedrine reduced cell viability in a dose-dependent manner at concentrations of 50 – 1600 µg/mL following 48 and 72 h exposure. Ephedrine was found to also decrease ATP production after 24, 48 and 72 h. Morphological studies showed damage to mitochondria morphology in a time-dependent manner and a reduction in mitochondrial DNA (Lee *et al*., 2017). Kallassy *et al.,* (2017) observed a significant reduction in cell viability of Jurkat cells (human leukaemia T cell line) treated with 5 – 200 µg/mL methanol and ethanol *ephedra campylopoda* plant extract following of 24, 48 and 72 h exposure. However, the aqueous extracted ephedrine did not exert any significant cytotoxicity. Milena *et al*., (2010) did not observe a significant reduction in cell viability of isolated human lymphocytes exposed to 0.0005 – 500 µM (0.000083 – 82.6 µg/mL ephedrine for 1 h. Compared to ephedrine, which was found to be cytotoxic, only CLA had a similar toxicity profile.

3.6 Conclusion

Both L-carnitine and HCA did not have antioxidant activity although at high concentrations, HCA protected 3T3-L1 preadipocytes against oxidative damage. Neither, L-carnitine nor HCA caused oxidative damage. Even at high concentrations no cytotoxic effects without and with AAPH was observed in L929 and 3T3-L1 cell lines. In contrast, CLA was found to have antioxidant activity at 50 – 500 µg/mL, however induced oxidative damage at these concentrations in 3T3-L1 preadipocytes. No antioxidant or oxidative effects were observed for ephedrine and CLA, however at 500 µg/mL, both weight loss compounds were cytotoxic with the L929 cell line being more sensitive. Of the weight loss compounds evaluated, although CLA showed antioxidant activity (ORAC assay), this did not translate into cellular protection. At high concentrations, CLA is as cytotoxic as ephedrine while L-carnitine and HCA, have no effects.

Chapter 4: Effects of ephedrine, CLA, L-carnitine and HCA on accumulated lipid droplets and adipocyte differentiation in differentiated 3T3-L1 cells

4.1 Introduction

Adipocytes are derived from mesenchymal stem cells (MSCs), which undergo differentiation into lipoblasts, then preadipocytes and finally into mature adipocytes. This differentiation process involves complex multi-steps which includes a cascade of transcription factors required for the activation of gene expression for specific proteins (Ruiz-Ojeda *et al*., 2016b). The two master transcription factors involved in adipocyte differentiation are PPRAγ and C/EBP family, which regulates gene expression for the formation and maintenance of adipocytes phenotype and insulin-responsive glucose transporter 4 (GLUT 4), stearoyl-CoA desaturase (SCD) 1 and 2 (Wang *et al*., 2007). PPRAγ is also involved in the storage and breakdown of fatty acids (Lehnen *et al*., 2015). The presence of GLUT 1 protein stimulates insulin for transportation of glucose to adipose tissue (Hauner *et al*., 1998). SCD 1 is a lipogenic enzyme that controls membrane lipid homeostasis and plays a role in the conversion of saturated fatty acids into monosaturated fatty acids. SCD 2 regulates the maintenance of gene expression specific to adipocytes in highly differentiated 3T3-L1 adipocytes (Christianson *et al*., 2008).

Research has revealed that a prolonged state of obesity may stimulate the body to recruit new preadipocytes to differentiate into mature adipocytes, thereby increasing the population of adipocytes (Tung *et al*., 2013; Wang *et al*., 2013). In these adipocytes, the continuous deposition of triglycerides, results in the expansion of the size of the adipocytes. Regulation of excessive expansion of adipose tissue could have beneficial effects in managing obesity and related diseases. Anti-obesity treatment involves two mechanisms, appetite suppression and fat intake reduction. Associated with a reduction of fat intake, the size of adipocytes is reduced, but the adipocytes population remain unchanged. Therefore, discontinuation of anti-obesity treatment can result in adipocytes regaining their original size. A more effective strategic approach in controlling the expansion of the adipocyte population or reducing the adipocyte population by cutting off their supply or lower their inventory (Tung *et al*., 2017).

Natural products including crude extracts and isolated pure natural compounds are reported to stimulate a reduction in body weight and prevent diet induced obesity by suppressing the growth of adipose tissue, preventing preadipocyte differentiation, stimulation of lipolysis and inducing apoptosis of existing adipocytes (Mohamed *et al*., 2014). Anti-adipogenic properties

of CLA isomers have been previously described in cultured adipocytes (Den Hartigh *et al*., 2013; Kumari Ramiah *et al*., 2017). Positive effects of L-carnitine on body weight have been reported in animal models (rats and mice). To our knowledge, *in vitro* evidence regarding anti-adipogenic properties of L-carnitine in differentiated adipocytes is still limited to a study by Siegner *et al*., (2010). *Garcinia* extract has been widely used as a potential weight loss control agent. However, the efficacy and mechanism of action of one of its active ingredients, HCA in weight loss is still questionable. Previous studies have supported the anti-adipogenic effect of HCA in cultured adipocytes (Roy *et al*., 2007; Li *et al*., 2017).

Gaps in knowledge exists on the effects of CLA, L-carnitine and HCA on pre-adipocytes differentiation, lipolysis and adipocytes viability. Therefore, the aim of this chapter was to determine if ephedrine, CLA, L-carnitine and HCA reduces lipid levels and cellular viability of differentiated 3T3-L1 adipocytes and 3T3-L1 adipocytes undergoing differentiation.

The specific objectives of this chapter are:

1. In mature, differentiated 3T3-L1 adipocytes to determine the effects of ephedrine, CLA, L-carnitine and HCA following 48 h exposure on lipid levels and cell viability determined with ORO staining and the MTT assay, respectively.
2. To determine the effects of exposure to ephedrine, CLA, L-carnitine and HCA during differentiation (day 4, 7 and 10) on lipid levels determined with ORO staining.

4.2 Materials

4.2.1 Reagents, equipment and disposable plasticware

The same weight loss samples and reagents were used as indicated in chapter 3 (Section 3.2.1 and 3.2.2). Additional reagents including insulin, isobutylmethylxanthine (IBMX), dexamethasone (DEX), rosiglitazone, Oil Red O (ORO) powder and isopropanol were purchased from Sigma-Aldrich Company, Atlasville, SA.

Additional equipment included: Zeiss Axio cam Erc5s, Olympus IX71 light microscope (Oberkochen, Germany).

4.2.2 Laboratory facilities

The experiments were conducted in the Cell Biology laboratory in the Department of Anatomy as well as at the unit for Microscopy and Microanalysis, University of Pretoria.

4.3 Methods

4.3.1 Differentiation of 3T3-L1 pre-adipocytes

The 3T3-L1 cell line is the most commonly used pre-adipose cell line for studying adipogenesis and the pathophysiology of obesity (Scott *et al*., 2011; Ruiz-Ojeda *et al*., 2016a). The 3T3-L1 line is derived from 17 to 19 day old murine Swiss 3T3 mouse embryos and has a characteristic fibroblast-like morphology with the ability to change its phenotype to adipocyte-like cells. Differentiation of 3T3-L1 cells to mature adipocytes requires the necessary adipogenic agents or supplements such as insulin, DEX and IBMX (Ruiz-Ojeda *et al*., 2016b). Zebisch *et al*., (2012), reported that the differentiation of 3T3-L1 preadipocytes can be achieved within 10 to 20 days when using rosiglitazone as an additional adipogenic supplement. Rosiglitazone is an antidiabetic agent that sensitizes adipose tissue to insulin by activating adipocyte PPARγ thereby increasing lipid droplet accumulation (Wang *et al*., 2007). Insulin at high concentrations has been known to act as insulin-like growth factor-1 resulting in a stimulation of mitogen-activated protein kinase pathway. DEX is an anti-inflammatory steroid agent that is essential for both osteogenic and adipogenic differentiation depending on cell-type, time and concentration. A combination of DEX and IBMX promotes adipogenesis through the regulation of PPARγ. In addition, both play a major role in inducing transcription factors for growth and differentiation such as C/EBPδ and C/EBPβ. IBMX is a competitive, non-selective phosphodiesterase inhibitor that increases cAMP and the protein kinase (PKA) signalling pathway necessary for activation of transcription factor PPARγ required for the expression of genes required for adipogenesis (Scott *et al*., 2011).

The composition of the media required for differentiation are presented in Table 4.1. All media was filtered using a Millex 0.2 µm filter and stored at 4°C. Differentiation of 1×10^3 3T3-L1 preadipocytes was performed according to a modified protocol of Zebisch *et al*., (2012) and the differentiation process is provided in Table 4.2.

Table 4.1: Differentiation medium

Medium	Composition
Basal medium I (BMI)	DMEM + 10% foetal calf serum (FCS) + 1% antibiotic
Differentiation medium I (DMI)	DMEM +10% FBS + 1% antibiotics + insulin (10 µg/mL) + IBMX (0.5 mM) + DEX (1 µM) + rosiglitazone (2 µM)
Differentiation medium II (DMII)	DMEM +10 FBS + 1% antibiotics + insulin (10 µg/mL)

Table 4.2: Differentiation of 3T3-L1 adipocytes

Day	**Exposure after differentiation**	**Exposure during differentiation**
1	Cell cultured in BMI	Cell cultured in BMI
2	Cells allowed to reach confluence	Cells allowed to reach confluence
3	Plated at 1×10^3 per 100 µL in 96-well plate (BMI)	Plated at 1×10^3 per 100 µL in 96-well plate (BMI)
4	Change medium to DMI	Change medium to DMI and add 10 µL of each supplement at 10 and 100 µg/mL
5		
6		
7	Change medium to DMI (Intracellular droplets should have appeared)	Change medium to DMI and add 10 µL of each supplement at 10 and 100 µg/mL
8		
9		
10	Change medium to DMII	Change medium to DMII and add 10 µL of each supplement at 10 and 100 µg/mL
11		
12		
13	Change medium to BMI	Change medium to BMI
14	Change medium to BMI and add 10 µL of supplements at concentration of 10 and 100 µg/mL for 48 h	Change medium to BMI

Two different experimental strategies were used i) to expose differentiated 3T3-L1 cells to the weight loss compounds or ii) to add the weight loss compounds during differentiation. The first strategy is to determine the effect on mature adipocytes and the second to evaluate the effect on adipocytes while they undergo differentiation. In the latter experiment, medium without and with 10 µg/mL and 100 µg/mL (that represent low and high dosages, without causing cytotoxicity on undifferentiated cells) of the weight loss products were added on day 4, 7 and 10. After day 14, cell viability and the effect on lipid accumulation was determined with the MTT and ORO assays, respectively.

4.3.3 Lipid content: Oil Red O staining

ORO is hydrophobic dye that binds to lipids within cells or tissue sections resulting in red staining of the lipid in the lipid droplets of adipocytes (Mehlem *et al*., 2013). Extraction of the ORO in an organic solvent allows the quantification of the levels of cellular lipids.

Stock solution of ORO was prepared by dissolving 0.5 g of ORO powder in 100 mL of 60% (w/v) isopropanol. The stock solution was stored at room temperature. The working solution was made by diluting the stock solution 1.7 times with distilled water, yielding a final concentration of 0.3% ORO in 60% isopropanol. The working solution was prepared freshly

each time and filtered immediately before use. To stain the 3T3-L1 cells after exposure the cells were fixed with 11 µL of 20% (v/v) of formaldehyde, (final concentration 2%) and incubated for 45 min at 37ºC, 5% CO_2. The medium was then removed and 100 µL of ORO working solution was added and then the cell culture plates were incubated for 1 h at room temperature. The cells were then washed with tap water to remove excess dye. For imaging differentiated 3T3-L1 cells were photographed at 20× and 40× machine magnification using Zeiss Axio cam Erc5s, Olympus IX71 light microscope. For lipid content quantification, the dye was dissolved with 100 µL of 60% (v/v) isopropanol for 10 min at room temperature on an orbital shaker. Absorbance was measured at 405 nm using a FLUOstar OPTIMA plate reader. The data was expressed as percentage of ORO staining relative to untreated control 3T3-L1 cells at the same level of differentiation.

4.3.4 Cellular toxicity: MTT assay

The MTT assay was performed following the same methods as described in chapter 3. In brief, 11 µL of 1 mg/mL MTT solution was added to each well and plates were incubated for 3 h at 37°C, 5% CO_2. The medium was then removed and the MTT formazan crystals were dissolved with 100 µL of 25% DMSO in ethanol. The absorbance was determined at 570 nm using a Biotek plate reader. The cell viability was calculated with the untreated control cells as 100% cell viability.

4.3.5 Data management and statistical analysis

All data management and statistical methods used were similar as described in chapter 3. Briefly, ANOVA was employed for data analysis for this study design followed by post hoc testing for determining statistical differences between treatment with weight loss dietary supplements vs. control (no weight loss supplement added) and ephedrine vs. the tested weight loss dietary supplements using the GraphPad Prism statistical software. Testing was done at the 95% level of confidence ($p<0.05$).

4.4 Results

4.4.1 Differentiation of 3T3-L1 preadipocytes

The 3T3-L1 preadipocytes cells were cultured and differentiated according to standard protocol (Zebisch *et al*., 2012). Undifferentiated 3T3-L1 preadipocytes have a fibroblast-like structure. After 14 days of differentiation, the 3T3-L1 preadipocytes were differentiated into mature adipocytes as shown in figure 4.1. Two types of differentiated adipocyte phenotypes could be identified in the confluent cultures. These were rounded adipocytes with intense red

staining (Figure 4.1a and d) and flat rounded adipocytes with cytoplasmic staining (Figure 4.1a, b, c, and d). The highly differentiated adipocytes were found in groups (Figure 4.1a). Some cells are spindled shaped (Figure 4.1c) and this together with differences in staining intensity indicates that some cells may not be fully differentiated.

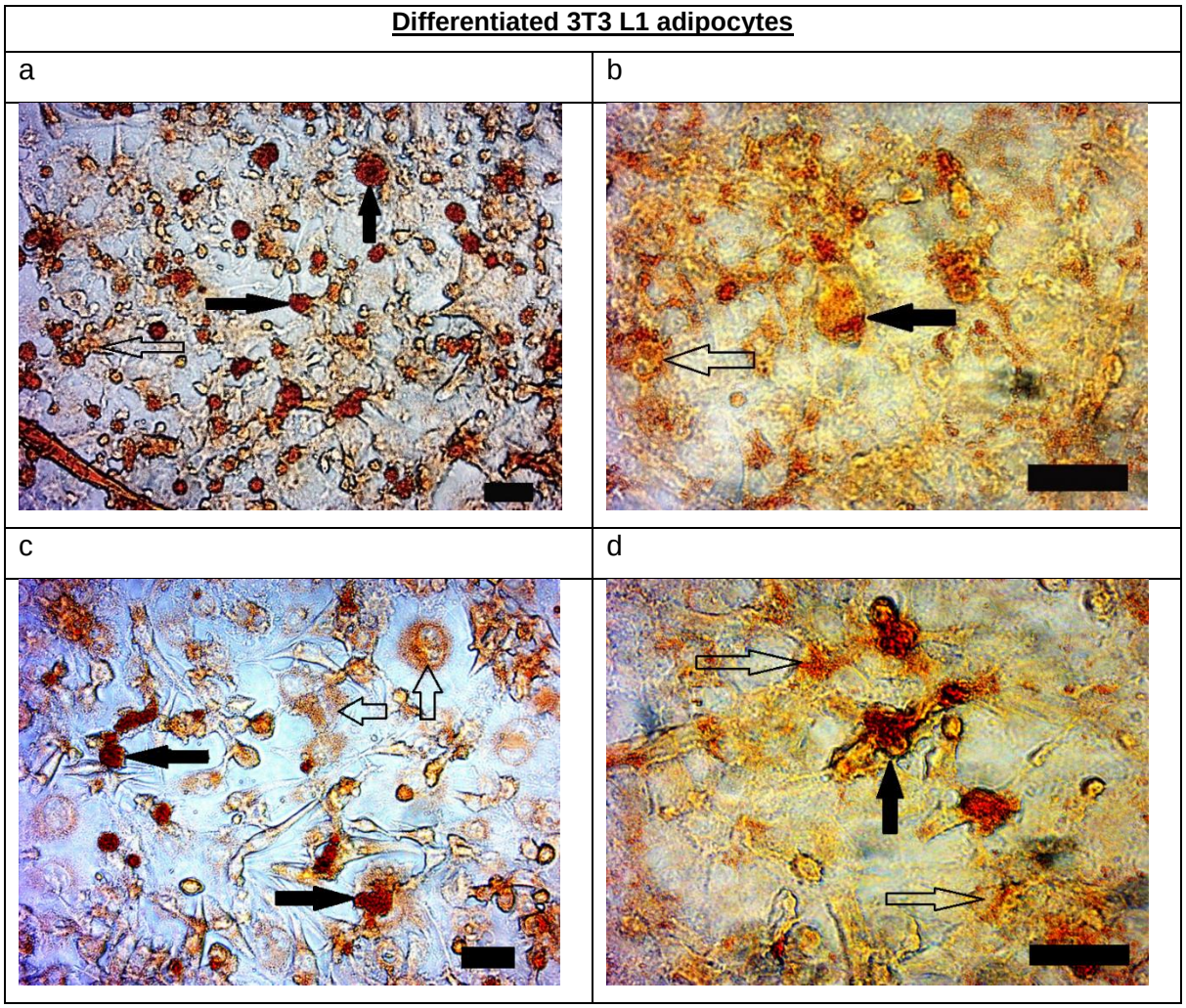


Figure 4.1: Phase contrast microscopic images of Oil Red O stained 3T3-L1 adipocytes after a differentiation period of 14 days. Differentiated adipocytes have a rounded morphology (black arrows) with lipid droplets accumulating in the cytoplasm (open arrows) although staining intensity was variable. **Scale bars:** 50 µm.

4.4.2 Effect of ephedrine, CLA, L-carnitine and HCA on differentiated 3T3 L1 adipocytes lipid content and cell viability

The ability of ephedrine, CLA, L-carnitine and HCA after 48 h exposure to reduce the amount of preformed lipid was determined following the extraction of ORO stain (Figure 4.2). There was no significant reduction in lipid content in mature adipocytes exposed at concentrations of 10 and 100 µg/mL compared with the control. No significant differences were observed in lipid content between ephedrine treated adipocytes and the investigated weight loss dietary

supplements. Furthermore, no significant differences were observed in lipid content between adipocytes exposed to 10 and 100 µg/mL of the ephedrine, CLA, L-carnitine and HCA.

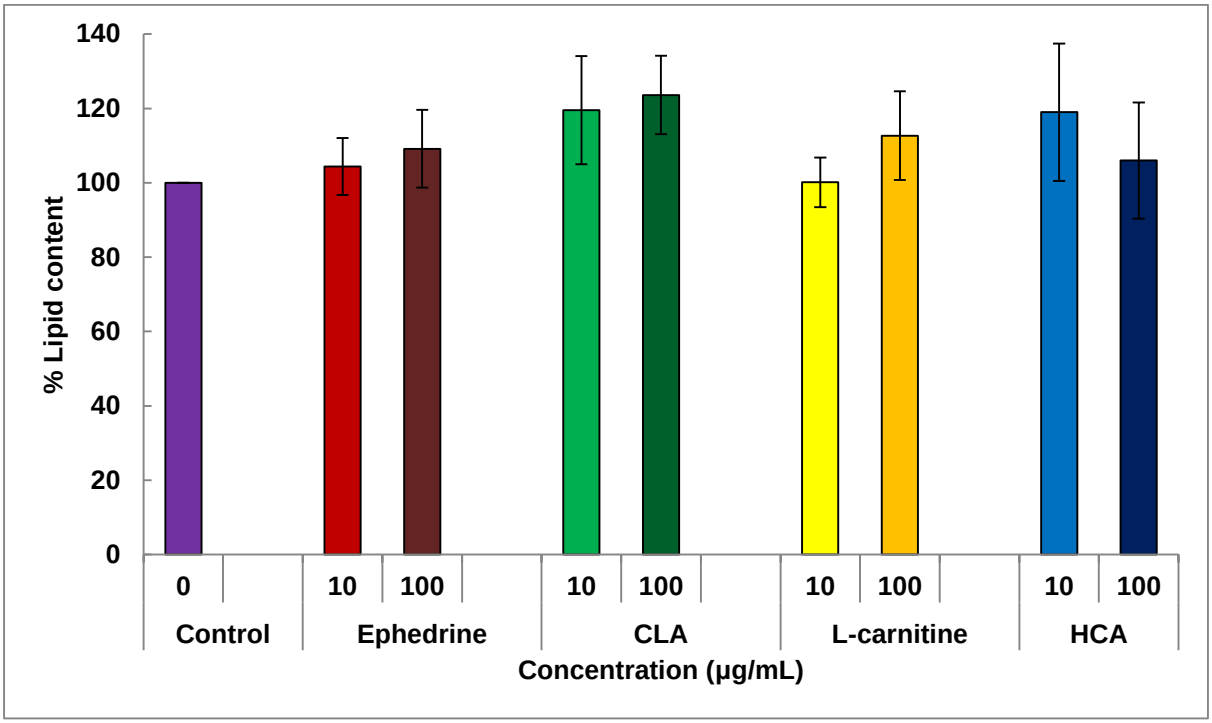


Figure 4.2: Effect of ephedrine, conjugated linoleic acid, L-carnitine and hydroxycitric acid on the lipid content of differentiated 3T3-L1 adipocytes at 10 and 100 µg/mL (representative of a low and high concentration) after 48 h exposure. Data presented are means ± SEM of the three independent experiments performed in triplicate. Differences between the control and weight loss compounds and between exposure to 10 and 100 µg/mL was not significant ($p<0.05$).

One of the strategic approaches in controlling the expansion of the adipocyte population is to reduce the total number of adipocytes (Tung *et al*., 2017). Therefore, the MTT assay was used to measure if cell viability was reduced (Figure 4.3). The MTT assay revealed that treatment with CLA, L-carnitine and HCA did not induce a significant reduction in cell viability of adipocytes after 48 h exposure at both tested concentrations of 10 and 100 µg/mL compared to untreated cells. No significant difference was observed between ephedrine and each compound as well as between low and high concentrations.

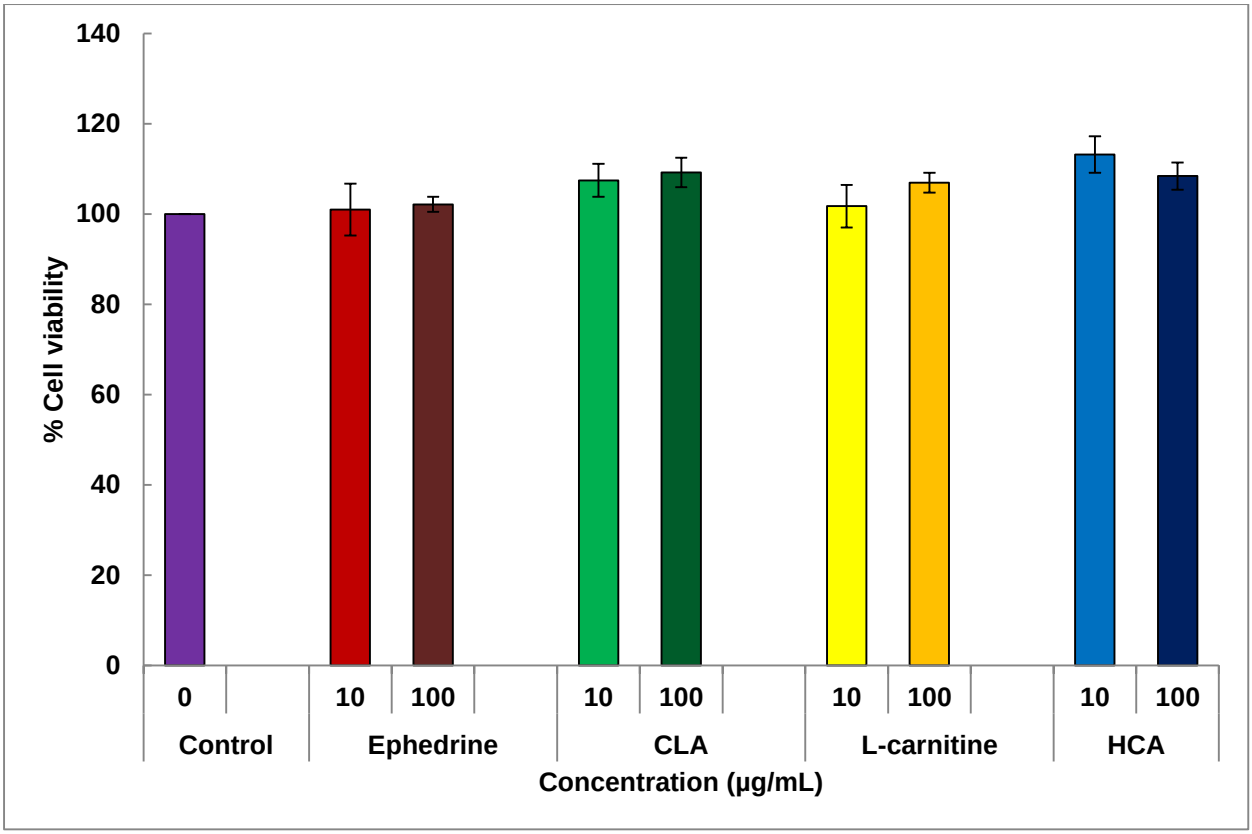


Figure 4.3: Effect of ephedrine, conjugated linoleic acid, L-carnitine and hydroxycitric acid on the viability of differentiated 3T3-L1 adipocytes at 10 and 100 µg/mL (representative of a low and high concentration) after 48 h exposure. Data presented are means ± SEM of the three independent experiments performed in triplicate. Differences between the control and weight loss compounds and between exposure to 10 and 100 µg/mL was not significant ($p<0.05$).

4.4.3 Effect of exposure to ephedrine, CLA, L-carnitine and HCA on lipid content and cell viability during 3T3 L1 adipocyte differentiation

The ability of the investigated weight loss dietary supplements (ephedrine, CLA, L-carnitine and HCA) to inhibit adipogenesis in 3T3-L1 cells was also assessed (Figure 4.4). CLA, L-carnitine and HCA were added during the process of adipocyte differentiation. No significant suppression of adipocytes differentiation was observed. No significant differences in lipid content was found between the control and exposed adipocytes, between ephedrine and CLA, L-carnitine and HCA as well as between low and high concentrations.

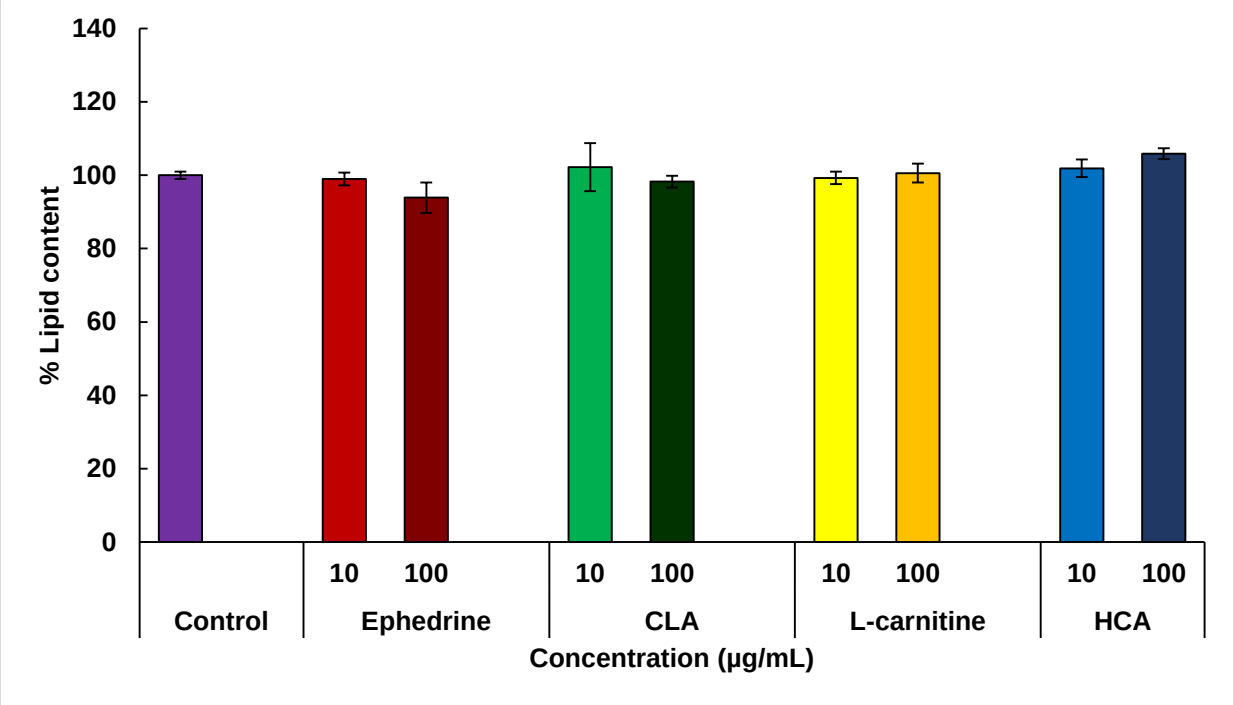


Figure 4.4: Effect of exposure to 10 and 100 µg/mL (representative of a low and high concentration) ephedrine, conjugated linoleic acid, L-carnitine and hydroxycitric acid during 3T3-L1 adipocytes differentiation. Data presented are means ± SEM of the three independent experiments performed in triplicate. Differences between the control and weight loss compounds and between exposure to 10 and 100 µg/mL was not significant ($p<0.05$).

4.5 Discussion

4.5.1 Imaging of lipids

Accumulation of pathological lipids is a characteristic for development of metabolic diseases including T2DM, non-alcoholic fatty liver disease and MS. There is an increasing need for more research to understand the mechanisms behind the metabolic dysfunction associated with lipid accumulation. The ORO staining method has been shown to be the most reliable method for detection and quantification of lipid droplets (Mehlem *et al*., 2013). ORO binds specifically to triglycerides, the degree of differentiation is directly proportional to the degree of ORO staining and can be used for quantification purposes (Dragunow *et al*., 2017).

The degree of 3T3-L1 adipocytes following ORO staining was evaluated. The 3T3-L1 adipocytes were filled with lipid droplets localised in the cytoplasm 14 days after the induction of differentiation. Differentiated adipocytes were rounded with variable staining indicating different degrees of lipid accumulation. Some spindle shaped undifferentiated cells were also present.

Zhang *et al*., (2014) observed highly differentiated 3T3-L1 cells after 7 days of induction of differentiation using differentiation media with 1 µg/mL insulin and without rosiglitazone. More than 90% of the cells exhibited a phenotype associated with mature adipocytes consisting of a large number of lipid droplets accumulated in the cytoplasm. With differentiation the expression of two key transcription factors, PPARγ and C/EBPα were elevated. Boschi *et al*., (2014) achieved complete differentiation of 3T3-L1 adipocytes after 8 days using 0.2 mM IBMX, 10 µM rosiglitazone, 1 µM DEX and 10 µg/mL insulin. Kong *et al*., (2017) observed highly differentiated 3T3-L1 adipocytes after 15 days using a differentiation cocktail consisting of 10 µg/mL insulin, 1 µM DEX and 0.5 mM IBMX. With real-time PCR, the expression of differentiation transcription factors, PPARγ and C/EBPα was found to be increased (Kong *et al.*, 2017). In the present study, the method of Zebisch *et al*., (2012) was used, and the media contained 10 µg/mL insulin, 0.5 mM IBMX, 1 µM DEX and 2 µM rosiglitazone. Differences in formulation clearly indicates that successful differentiation is a function of laboratory conditions, cell line and other factors such as the source of media and supplements as well as passage number. Successful differentiation of the 3T3-L1 adipocytes was achieved and these cultures were then used to determine the effects of ephedrine, CLA, L-carnitine and HCA on formed lipid in differentiated adipocytes and on adipocytes undergoing differentiation.

4.5.2 The effect of compounds to adipocytes – compounds added after differentiation

4.5.2.1 Determination of lipid content

Various dietary supplements are claimed to increase fat oxidation, increase metabolic rate or inhibit hepatic lipogenesis. These dietary supplements are marketed as weight loss agents, but there are still insufficient scientific studies supporting that CLA, L-carnitine and HCA promote weight loss via an effect on lipid accumulation or on adipocytes. Therefore, the lipid content quantification of the mature 3T3-L1 adipocytes following treatment with weight loss dietary supplements (ephedrine, CLA, L-carnitine and HCA) was assessed using ORO staining.

The CLA isomer *trans*-10, *cis*-12 has been reported to exhibit health benefits such as a reduction in adiposity however, adverse effects include systematic inflammation, insulin resistance and dyslipidemia (Den Hartigh *et al*., 2013). In the present study, the combination of CLA isomers (1 *cis*-9, *trans*-11: 1 *trans*-10, *cis*-12) caused no reduction in the levels of lipid that had accumulated in matured 3T3-L1 adipocytes. In contrast, Den Hartigh *et al*., (2013) evaluated the effect of 25 mM (7010 µg/mL) *trans*-10, *cis*-12 CLA isomer (in glucose containing medium) and *cis*-9, *trans*-11 CLA (used as a negative control) for 7 days on triglyceride levels in differentiated 3T3-L1 adipocytes. Total intracellular triglycerides levels

were significantly reduced. Treatment with *trans*-10, *cis*-12 CLA induced lipolysis of the stored lipids, decreased plasma membrane expression of the major adipocytes' glucose transporters, increased fatty acid oxidation and reduced the expression of adipogenic gene PPARy. Compared to this study, this effect is possibly due to the higher concentrations used.

Thiyam, (2011) investigated the role of PPARγ and activation of fatty acid storage in the regulation of hepatic lipid droplet formation induced by CLA isomers (*cis*-9, *trans*-11 and *trans*-10, *cis*-12) in an *in vitro* model. Rat hepatocytes (H4IIE) exposed to CLA isomers (0.4% *cis*-9, *trans*-11 or 0.4% *trans*-10, *cis*-12) for 24 h revealed a dose-dependent increase in lipid formation. The role of PPARα hepatic steatosis was also investigated by feeding obese fa/fa Zucker rats CLA isomers (0.4% *cis*-9, *trans*-11 or 0.4% *trans*-10, *cis*-12) for 8 weeks. The results showed that only the *trans*-10, *cis*-12 CLA isomer significantly reduced the expression of PPARα and no significant changes were observed with the *cis*-9, *trans*-11 CLA isomer. It was concluded that the CLA isomer directly promotes lipid droplet formation in H4IIE cells by activating lipid storage pathways (Thiyam, 2011). Sakuma *et al*., (2010) showed that treatment with 100 µM (28 µg/mL) *cis*-9, *trans*-11 CLA isomer in the presence of differentiation cocktail (containing insulin, DEX and IBMX) significantly stimulated triacylglycerol accumulation in differentiated mouse 3T3-L1 mature adipocytes. Microscopy analysis of ORO stained adipocytes showed that the isomer significantly increased the total number and size of small mature adipocytes that secrete adiponectin when compared to the controls. Furthermore, treatment with the *cis*-9, *trans*-11 CLA isomer increased adipocyte levels of PPARγ. It was concluded that the therapeutic effects of *cis*-9, *trans*-11 CLA isomer on lifestyle related diseases might be due to the induction of small adipocytes from preadipocytes via the stimulation of PPARγ (Sakuma *et al*., 2010).

Simon *et al*., (2005) showed that hamsters fed with 0.5% *trans*-10, *cis*-12 CLA for 6 weeks had a significant reduction in adipose tissue mass compared to the control. No significant differences were observed for basal lipolysis in CLA fed hamsters. The DNA content per gram tissue as an indirect reverse index of adipocyte size was significantly elevated in hamsters supplemented with CLA. Microscopy analysis did not reveal differences in the diameter of the medium mature adipocytes and the cell size distribution compared to the control. The authors concluded that the reduced size of adipose tissue might not be related to changes in lipolysis but delayed differentiation of preadipocytes to mature adipocytes (Simon *et al*., 2005). Martin *et al*., (2017) showed that 3T3-L1 adipocytes treated with 50 µM (14 µg/mL) of *trans*-10, *cis*-12 CLA isomer during 11 days of differentiation caused the greatest reduction in lipid accumulation compared to the control. Furthermore, the *trans*-10, *cis*-12 CLA isomer

decreased the levels of genes during lipogenesis but did not up-regulate HSL and PPARγ associated with lipolysis and fatty acid β-oxidation, respectively (Martins *et al*., 2017).

The *cis*-9, *trans*-11 CLA isomer has been shown to have anti-adipogenic properties. Ramiah *et al*., (2017) reported that treatment with 1.51% of *cis*-9, *trans*-11 significantly decreased the size of chicken adipocytes and reduced the number of adipocytes. In contrast, *trans*-10, *cis*-12 treated group, did not alter chicken adipocytes size and diameter compared to the control group. Furthermore, treatment with 2.56% *cis*-9, *trans*-11 CLA isomer significantly downregulated the expression of LPL. No significant changes in gene expression were observed with *trans*-10, *cis*-12 treatment (Ramaih *et al*., 2017).

The lack of an effect of CLA on preformed lipids in the present study may be related to the concentrations used where the beneficial effect observed by Den Hartigh *et al*., (2013) was at CLA levels fourteen times greater than used in this study. A further explanation for the lack of effect is that the CLA used in the present study was a mixture of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA. As it has been proven that the lack of inconsistency and contradictory results on the efficacy of CLA could be as the results of consumption of CLA mixture. The *trans*-10, *cis*-12 CLA isomer is known to exerts its specific effect on adipocytes and liver, while the *cis*-9, *trans*-11 CLA isomer active in preventing carcinogenesis (Benjamin *et al*., 2015). In addition, observed effects were related to gene expression (Lehnen *et al*., 2015) and adipocyte size (Ramiah *et al*., 2014) rather than lipid accumulation.

The supplementation of carnitine as weight loss control is based on the principle that daily oral ingestion will increase intracellular levels hence, activating fatty acid oxidation and reducing fat storage in adipose tissue (Pooyandjoo *et al*., 2016). *In vitro* studies supporting the efficacy of L-carnitine are still limited. In this study, supplementation with L-carnitine did not reduce lipid content of differentiated adipocytes. Similarly, Brandsch & Eder, (2002), observed no significant reduction in triglyceride levels, total cholesterol and free fatty acids in rats supplemented with 5 g/kg of L-carnitine for 23 days. The researchers concluded that the synthesis of endogenous L-carnitine in the animals was sufficient for β-oxidation of fatty acids during the catabolic phase (Brandsch & Eder, 2002).

Conversely, some studies have reported that L-carnitine has a positive effect on body weight. Jain and Singh, (2015) showed that supplementation with 100 mg/kg of L-carnitine for 5 days under a calorie restricted diet resulted in a decline in the body weight of male Sprague-Dawley rats. However, the blood glucose levels of these rats compared to the untreated group was raised and liver glycogen levels were reduced (Jain & Singh, 2015). Jang *et al*., (2014)

reported that administration of L-carnitine at 300 mg/kg in high fat induced obesity in male mice (C57BL/6J) with 5 days exercise for 9 weeks significantly decreased both the amount of fat and body weight which was associated with a decline in serum glucose and the levels of leptin (Jang *et al*., 2014). Leptin is a hormone that is critical for regulation of energy balance for long-term by acting as appetite suppressant thereby promoting weight loss (Klok *et al.*, 2007). Siegner *et al*., (2010) showed that treatment with 0.01% of L-carnitine alone and in combination with 0.5% lotus leaf extract solution reduced triglyceride accumulation in differentiated human adipocytes. It was concluded that reduced triglyceride accumulation might be as the result of a synergistic effect between the lotus leaf extract and L-carnitine leading to the modulation of lipolysis activity and L-carnitine increasing β-oxidation (Siegner *et al.*, 2010). The contradicting effects found between studies and the lack of an effect in the present study highlights the complexity of obesity, where the interplay between several factors such as a calorie restricted diet and exercise plays an important role.

Garcinia extract which contains HCA has been widely used as potential weight loss control agent; however, its efficacy and mechanism of action is still questionable. This study revealed that exposure of adipocytes to HCA prior to and during differentiation did not reduce lipid levels. In contrast, a study conducted by Roy *et al*., (2007) showed that treatment with the potassium salt of HCA (HCA-SX) in human differentiated adipocytes caused lipid droplet dispersion in the adipocytes, facilitating lipase action. Furthermore, the treatment with HCA-SX also induced leptin expression and this anti-lipolytic and anti-adipogenic effect of HCA-SX was concluded to be the result of increased leptin expression and activation of lipase in the adipocytes (Roy *et al*., 2007). Li *et al*., (2017) demonstrated that treatment with 10 and 50 µM (2.08 µg/mL and 10.40 µg/mL, respectively) of (-)-HCA significantly reduced the number of lipid droplets in a time period between 3 to 24 h in cultured primary chicken hepatocytes compared to untreated cells. Likewise, 10 and 50 µM of (-)-HCA treatment significantly reduced the total area of lipid droplet when compared to the untreated group during 1 to 24 h of the experimental period. Furthermore, the triglyceride content was significantly reduced compared to the untreated cultured cells from 1 to 24 h. The mechanism of reduced fat accumulation in the chicken hepatocytes was further investigated and was due to the probable inhibition of fatty acid synthesis, increased glucose metabolism and energy metabolism (Li *et al.*, 2017). Mahmoud & Amer, (2013) observed a significant reduction of plasma triglyceride levels in male albino rats treated with 200 mg/kg of *G. cambogia* (containing 50% w/w HCA) for three weeks. Furthermore, rats treated with 400 mg/kg for 2 weeks showed a significant elevation of triglycerides and total cholesterol however, the total body weight was significantly reduced. It was concluded that the effect of HCA on body weight and lipid profile is dose and duration dependent (Mahmoud & Amer, 2013). Likewise, in the present study, the lack of

evidence showing that HCA reduces lipid levels in adipocytes may be a function of dosage and duration of exposure, as well as the method used to quantify effects where HCA had the greatest effect on lipid droplet size.

Ephedrine has been previously called the “energizer” as it stimulates the central nervous system, elevates the heart rate and often increases blood pressure. It’s efficacy on weight loss in animal and humans has been well described (Malchow-Møller *et al*., 1981, Toubro *et al*., 1993, Boozer *et al.,* 2001). In a more recent studies, Slocum *et al*., (2013) showed that mice given 18 mg/kg/day of ephedrine orally, treadmill exercise (10 m/min, 1 h/day) and dietary restriction for 7 days significantly reduced brown adipose tissue and lipid accumulation in the liver and increased oxygen consumption. A human study conducted by Liu *et al*., (2013) showed that participants that were treated with 200 mg of caffeine and 20 mg of ephedrine (orally) 3 times a day had a significant reduction in body weight after 24 weeks. However, no significant changes were observed in blood glucose, insulin, triglycerides and LDL cholesterol. Interestingly, 57% of the participants dropped out from the study due to intolerance to the forced dose titration of the caffeine/ephedrine schedule. Adverse effects experienced by subjects treated with caffeine/ephedrine included insomnia and anxiety (Liu *et al*., 2013). The present study confirms that the weight loss effects of ephedrine is not as a result of the effects on adipocytes. Likewise, it was found that, weight loss compounds CLA, L-carnitine and HCA did not reduce the levels of preformed lipid or inhibited lipid accumulation. Lack of evidence may be related to exposure time, concentration and the quantification method used.

4.5.2.2 Determination of cytotoxicity

Recent research has revealed that adipocyte mitochondria might be essential for development of obesity due to defects in mitochondrial lipogenesis and lipolysis, regulation of adipocyte differentiation, apoptosis, ROS production, oxidative phosphorylation efficiency and the control of the conversion of white adipocytes into brown-like adipocytes (De Pauw *et al*., 2009, Yin *et al*., 2014). Therefore, therapeutic strategies that could intervene into any of these mitochondrial processes without affecting cell viability could be useful for reducing adiposity (Cedikova *et al*., 2016). In addition, another beneficial effect is to inhibit the expansion of the adipocyte population or reducing the total number of adipocytes (Tung *et al*., 2017).

Previous research has demonstrated that CLA can induce apoptosis in fat cells in animals and 3T3-L1 adipocytes (Meadus *et al*., 2017; Wang *et al*., 2017). In contrast, in the current study, even at 100 µg/mL CLA containing CLA (1:1 ratio, 10Z, 12E/ 9Z, 11) did not cause any changes in cell viability. Adipose cells of pigs fed 2% of CLA isomers (1 *cis*-9, *trans*-11: 1

trans-10, *cis*-12) exhibited typical apoptotic characteristics such as cell shrinkage, chromatin condensation, and DNA degradation (Qi *et al*., 2014). The mRNA and protein levels of Bcl-2, a key mitochondrial inhibitor of apoptosis was reduced while the expression of Bax, a pro-apoptotic molecule and p53 was increased. The release of cytochrome c into the cytoplasm and the activity of caspase-9 and caspase-3 were also significantly increased. The researchers concluded that CLA induced apoptosis of pig adipocytes might be related to its ability to intervene in mitochondrial signalling pathway (Qi *et al*., 2014). Zhai *et al*., (2010) showed that treatment with 75.4 µM (21.15 µg/mL) in the differentiation medium of individual CLA isomers (*cis*-9, *trans*-11 and *trans*-10, *cis*-12) induced apoptosis of 3T3-L1 adipocytes. The CLA induced apoptosis in 3T3-L1 adipocytes was associated with increased levels of TNFα, a cytokine that is essential for activation of lipolysis and apoptosis in adipocytes. Although no toxicity was observed in this part of the study, CLA did reduce the L929 and preadipocytes viability following 48 h exposure to 500 µg/mL. In the present study, lack of toxicity at the concentrations evaluated may be related to the higher concentration used.

L-carnitine is required for oxidation of fatty acids in the mitochondria and defects in this process could result in the release of fat from the adipose tissue and the accumulation of this fat in the liver, skeletal muscles and heart during the fasting state (Longo *et al*., 2016). In this study, the effect of L-carnitine on cell viability of adipocytes was evaluated. Treatment with L-carnitine for 48 h did not have any effect on cell viability of differentiated 3T3-L1 adipocytes. In agreement to this study, Farahzadi *et al*., (2016) observed that 0.1 – 0.4 mM (1.61 – 6.44 µg/mL) of L-carnitine had no significant effect on the proliferation of adipose tissue derived from human mesenchymal stem cells compared to untreated cells after 24 and 72 h using the MTT assay. However, 48 h treatment of adipose tissue derived from human mesenchymal stem cells with 0.1 – 0.4 mM (16.1 – 64.4 µg/mL) L-carnitine caused a significant increase in cell proliferation which was associated with elevated expression of the human telomerase reverse transcriptase gene and increased telomere length (Farahzadi *et al*., 2016). Mobarak *et al*., (2017) showed that 48 h treatment with 0.2 mM (32.2 µg/mL) of L-carnitine caused a significant increase in the proliferation of rat adipose tissue derived mesenchymal stem cells and this effect was related to its antioxidant activity (Mobarak *et al*., 2017). The lack of evidence in the present study could be related to different cell lines and concentrations used.

In the current study the effect of HCA on cell viability of differentiated 3T3-L1 adipocytes was assessed using MTT assay. HCA did not cause a reduction in the adipocyte cell viability after 48 h treatment. There are still insufficient studies conducted on understanding the effect of HCA on adipocyte viability. Nisha *et al*., (2014) showed that treatment with HCA at concentrations of 5 – 100 µM (1.04 – 20.80 µg/mL) for 24 h did not cause significant cell death

in 3T3-L1 adipocytes using the MTT assay. Furthermore, the protective effect by HCA against ER stress induced by tunicamycin were associated with improved antioxidant activity, mitochondrial function and inflammatory response (Nisha *et al*., 2014).

There are no studies that provide evidence on the effect of ephedrine on the cell viability of adipocytes. In this study, ephedrine was used as a weight loss control for comparing the effect of CLA, L-carnitine and HCA on cell viability of differentiated 3T3-L1 adipocytes. Ephedrine, at the concentrations evaluated, did not have an effect on the cell viability of differentiated adipocytes. However, ephedrine did reduce the viability of L929 and preadipocytes following 48 h exposure to 500 µg/mL.

4.5.3 The effect of compounds on adipocytes – compound added during differentiation

4.5.3.1 Determination of lipid content

The anti-obesity treatment by caloric restriction is based on the reduction of lipids in the adipocytes by increasing lipolysis and breakdown of the hepatic fatty acids to ketones. However, adipocytes retain their capacity to re-synthesise lipids as these cells retain their lipogenic enzymes, which probably explains the high rate of relapse following the treatment (Zhou *et al*., 1999). Inhibition of differentiation of adipocytes represents a potential to inhibit obesity. Activation of key transcription factors such as PPARγ and CAAT/enhancer binding proteins play a critical role in adipocytes differentiation. Increased activity of C/EBPβ and C/EBPδ during adipogenesis promotes the transcription of C/EBPα and PPARγ which are master regulators of adipocyte differentiation (Kennedy *et al*., 2010). Therefore, in this study the potential of CLA, L-carnitine and HCA to inhibit differentiation of 3T3-L1 cells was assessed. In the present study, none of these weight loss dietary supplements inhibited adipocyte differentiation.

Several previous studies have reported the effect of CLA on the differentiation of adipocytes, yet, the available evidence on CLA is still not clear. Similar results to the current study were observed by McNeel *et al*., (2003), who showed that human preadipocytes cultured with 50 µM (14.02 µg/mL) *cis*-9, *trans*-11 CLA/*trans*-10, *cis*-12 CLA had three to four times increased cell number during 17 days of differentiation compared to the control. Furthermore, human preadipocytes treated with *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA had increased differentiation triacylglycerol increased activity of GPDH, a marker of adipocytes differentiation activity, as well as PPARγ and LPL compared to control. These results indicate that during initiation of differentiation when *de novo* fatty acid synthesis is restricted and competition for fatty acids by membrane and pathways for triacylglycerol synthesis are elevated, human pre-

adipocytes do not differentiate unless there is an addition of a PPARγ ligand (McNeel *et al*., 2003).

Iga *et al.*, (2009) demonstrated that ovine preadipocytes and 3T3-L1 preadipocytes exposed to 12.5, 25 and 50 µM (3.5, 7 and 14 µg/mL) *trans*-10, *cis*-12 CLA for 10 days in addition to standard differentiation medium showed more mature adipocytes with large lipid droplets compared to the untreated preadipocytes. Ovine and 3T3-L1 preadipocytes treated with *cis*-9, *trans*-11 CLA had no visible effect on adipocyte differentiation. GPDH activity was significantly increased in both ovine and 3T3-L1 adipocytes treated with *trans*-10, *cis*-12 CLA during differentiation process compared to the control. Treatment further upregulated the expression of $PPAR\gamma_2$ and adipophilin (belongs to PAT protein family and is located on the surface of intracellular lipid droplets) mRNA in a dose-dependent manner. However, a decrease in $PPAR\gamma_2$ and adipophilin mRNA expression was observed in 3T3-L1 preadipocytes treated with *trans*-10, *cis*-12 during differentiation. The researchers concluded that CLA can regulate GPDH activity and adipogenic genes such as $PPAR\gamma_2$ and adipophilin (Iga *et al*., 2009). In agreement to this study, Ding *et al*., (2000) showed that porcine stromal vascular cells treated with 0 – 300 µM (0 – 84 µg/mL) of *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA isomers in addition to differentiation medium for 24 h, significantly increased differentiation. Treatment with CLA isomers did not have any effect on PPARγ mRNA concentration. However, a significant increase in LPL activity and adipocyte fatty acid binding protein (aP2) mRNA levels was observed (Ding *et al*., 2000).

However, previous studies have supported the efficacy of CLA to inhibit adipocyte differentiation in both animal and human studies. In contrast to the current study, Miller *et al*., (2008) showed that 3T3-L1 cells differentiated in the presence of 100 µM (28 µg/mL) *cis*-9, *trans*-11, *trans*-10, *cis*-12 CLA mixed isomers, 10 µM troglitazone (PPARγ agonist) or 1 – 10 µM GW9662 (PPARγ antagonist) and adiponectin (an insulin-sensitizing protein) caused a profound reduction in cellular triglyceride mass. The anti-obesity effect of CLA isomers was thought to be due to its ability to change metabolism of adipocyte adiponectin via PPARγ-dependent and PPARγ-independent mechanisms (Miller *et al*., 2008). The results of this study are in direct contrast to that from Satory & Smith, (1999) who showed that 3T3-L1 preadipocytes treated with 5.0 and 10.0 µg/mL of 41% *cis*-9, *trans*-11/*trans*-9, *cis*-11, 44% *trans*-10, *cis*-12, and 10% *cis*-10, *cis*-12 CLA isomers during differentiation promoted lipid accumulation in 3T3-L1 preadipocytes. This finding was supported by three independent mechanisms and these were an increase in *de novo* lipogenesis from glucose, elevated absolute fatty acid concentrations and enlargement of lipid droplets. Furthermore, treatment with 0.5, 1.0, 5.0 and 10.0 µg/mL CLA significantly inhibited proliferation by 8, 12, 31 and 26%,

respectively. The data showed that CLA prevents the proliferation and promotes *de novo* lipogenesis and lipid accumulation in 3T3-L1 preadipocytes indicating that CLA inhibits the total fat mass in animals by obstructing stromal vascular preadipocytes hyperplasia (Satory & Smith, 1999). The differences between this study and that of Satory & Smith, (1999) could be as the result of additional adipogenic agent such as L-glutamine in differentiation medium which is essential for increasing adiposity in cultured cells.

The anti-obesity effect due fatty acid oxidation of L-carnitine has been previously investigated in differentiated adipocytes. Little information is available on the effect of L–carnitine during adipocyte differentiation. However, one study conducted by Siegner *et al*., (2010) showed that treatment with 0.01% L-carnitine in combination of 0.5% lotus leaf extract solution in human preadipocytes resulted in pronounced reduction in the accumulation of triglyceride during differentiation compared to incubation of 0.01% L-carnitine and 0.5% lotus leaf extract solution alone. The anti-obesity effect of the combination of L-carnitine and lotus leaf extract solution could be related to simultaneous stimulation of different pathways linked to fat storage providing potential for the prevention and treatment of obesity (Siegner *et al*., 2010). The lack of evidence in this study might be due to the use of lotus leaf extract solution in combination with L-carnitine. Furthermore, the differences could be as the results of different cell lines used.

Over the past decade the anti-adipogenic effect of herbal extracts such as *G. cambogia* has been extensively investigated. The findings of the present study is in contrast to that of Sharma *et al*., (2018), who demonstrated that 3T3-L1 preadipocytes with differentiating medium in the presence of 30 µg/mL pear pomace extract, 60 µg/mL of *G. cambogia* extract and the mixture of the herbal extract for 8 days during differentiation significantly inhibited adipocytes differentiation. Lipid accumulation was inhibited and the expression of the C/EBPα and PPARγ transcription factors. For the combination of both herbal extracts a significantly greater inhibitory effect in the expression of both adipogenic transcription factors was observed. Moreover, treatment with 30 µg/mL pear pomace extract and 60 µg/mL of *G. cambogia* during differentiation significantly decreased FAS expression and in combination further suppression of FAS expression occurred (Sharma *et al*., 2018). Kang *et al*., (2013) reported that 8 days exposure to 0.001 – 1% *G. cambogia* significantly supressed the adipogenic differentiation of 3T3-L1 preadipocytes. Addition of *G. cambogia* extract also inhibited the expression of PPARγ, C/EBPα and aP2. The researchers postulated that *G. cambogia* extract supressed the adipogenic differentiation of 3T3-L1 adipocytes, through the regulation of multiple genes involved in adipogenesis such as PPARγ, C/EPBα and aP2 (Kang *et al*., 2013). Parjapath *et al*. (2018) showed that treatment with 0.5 and 1 µM (0.104 and 0.208 µg/mL) HCA calcium

salt (<95% purity) in 3T3-L1 preadipocytes resulted in increased lipid deposition in a dose-dependent manner but a significant reduction in lipid deposition was observed at concentrations greater than 10 µM. Treatment with HCA moderately improved glucose uptake in 3T3-L1 preadipocytes. Furthermore, HCA increased the expression of PPARγ gene by 1.4-fold and 2.9-fold during the early and late stages of differentiation, respectively. In this study it was concluded that HCA decreases circulating lipids through elevated levels of adipogenic specific genes, through PPARs activation, thereby augmenting adiposity and associated complications (Parjapath *et al*., 2018). The lack of evidence between this study and previous studies could be related to the use of mixture of herbal extract, duration of exposure during differentiation, purity of HCA and concentration used.

Although the effects of ephedrine as a weight loss supplement has been extensively studied over that past decades, there are still no available studies done on determining the effects of ephedrine during differentiation of preadipocytes. Therefore, studies are still required as ephedrine is still an active ingredient in many OTC weight loss supplements (Boozer *et al*., 2001). Although in the present study, at the concentrations evaluated there was no reduction in lipid accumulation when added during the differentiation process.

4.6 Conclusion

Findings from the present study do not support the efficacy of CLA, L-carnitine and HCA related to the reduction of the levels of preformed lipid or the differentiation of adipocytes. No effect was observed for ephedrine. The inability of CLA to reduce lipid droplets in adipocytes or to inhibit the differentiation of preadipocytes might be related to its lack of ability to activate lipid storage pathways through upregulation of the transcription factor PPARγ and enzymes or proteins involved in differentiation of adipocytes such as adipophilin, aP2, LPL and GPDH activity. The non-efficacy of dietary L-carnitine is associated with its inability to stimulate the β-oxidation of fatty acids in the adipocytes as the endogenous β-oxidation activity is sufficient. The non-efficacy of HCA on lipid content of adipocytes is associated with increased triglyceride levels and total cholesterol content. All weight loss compounds at the highest concentration evaluated was not cytotoxic, however at a higher concentration of 500 µg/mL toxicity was observed for ephedrine and CLA (Chapter 3). Lack of effect may be related to the cell line, reagents used for differentiation as well as the sensitivity of the markers used to evaluate change and effect.

Chapter 5: The effect of ephedrine, CLA, L-carnitine and HCA on erythrocytes and whole blood

5.1 Introduction

Obesity is widely associated with development of MS including T2DM, arterial hypertension and hypercholesterolemia. There is still contradictory evidence regarding the effect of obesity on erythrocytes deformability due to the variability in the different stages of obesity, comorbidities and different methodologies used for evaluation (Tomaiuolo, 2014). Several studies have demonstrated that there is a strong link between obesity and disordered haemostasis/haemostatic disturbances in the blood coagulation system (De Pergola & Pannacciulli, 2002). Dysregulation of metabolic homeostasis (which includes, platelet aggregation, coagulation and fibrinolysis) linked to obesity is known to result in insulin resistance, dyslipidaemia, alterations in the blood pressure regulation, and elevated risk of T2DM, CVD, chronic kidney disease and cancer. Two characteristics of thrombosis is chronic inflammation and reduced fibrinolysis (Blokhin & Lentz, 2013). Chronic inflammation can lead to eryptosis of erythrocytes and pathological changes to coagulation (Page *et al*., 2017). Furthermore, chronic inflammation also results in the changes in the structure of plasma protein fibrinogen resulting in abnormal plasma clot formation (Kell & Pretorius, 2015; Pretorius *et al*., 2016).

Currently, there are no obesity tailored therapeutic strategies available for prevention and treatment of thrombosis. Weight loss drugs can have an adverse effect on blood haemostasis with MS and can increase the risk for thrombosis. The current available information on anti-thrombotic drugs were done on non-obese patients (Blokhin & Lentz, 2013). The use of some natural products for treatment of thrombotic diseases in clinics is common (Chen *et al*., 2015). To our knowledge, no studies have been done to evaluate the effects of CLA, L-carnitine and HCA on blood haemostasis. Therefore, it is essential to determine the effects of weight loss products on blood haemostasis as these weight loss compounds may promote thrombosis or inhibit clotting.

The aim of this chapter was to determine if ephedrine, CLA, L-carnitine and HCA causes haemolysis, alters the morphology of human erythrocytes and adversely affects fibrin network formation.

The specific objectives for this chapter are:

1. To determine the effect of 30 min, 24 and 48 h exposure to low and high concentrations of ephedrine, CLA, L-carnitine and HCA on erythrocytes evaluated with the haemolysis assay.
2. Using whole blood, evaluate the effect of ephedrine, CLA, L-carnitine and HCA on erythrocyte morphology evaluated with SEM.
3. Using whole blood, evaluate the effect of ephedrine, CLA, L-carnitine and HCA on fibrin network formation evaluated with SEM.

5.2 Materials

5.2.1 Reagents, equipment and disposable plastic ware

The same weight loss samples and reagents were used as described in chapter 3 and 4. Additional reagents included: ethanol (EtOH) and hexamethyldisilazane (HMDS) was purchased from Merck, SA. Osmium tetroxide and sodium dodecyl sulphate (SDS) were purchased from Sigma-Aldrich Company, Atlasville, SA. Human thrombin (20 U/mL) was obtained from the South African National Blood Service.

Equipment and disposable glassware used were the same as indicated in chapter 3. Additional glassware included: glass cover slips purchased from LASEC, SA. Additional equipment included a Zeiss Ultra Plus FEG-SEM and Zeiss Crossbeam 540 FEG SEM (Munich, Germany).

5.2.2. Blood samples

Following informed consent (Research Ethics Committee – 415/2017), a volume of approximately 5 mL of venous blood was collected from five healthy male volunteers above the age of eighteen years old, that did not smoke or use any chronic medication. The blood was collected by a trained phlebotomist into 5 mL vacuum extraction blood tube containing 3.2% sodium citrate.

5.2.3 Laboratory facilities

The laboratory facilities used in this part of this study was the same as indicated in chapter 3 and 4.

5.3 Methods

5.3.1 Toxicity: Haemolysis assay

The haemolysis assay is used to measure the toxicity of compounds, where alterations to the cell membrane results in the leakage of the cell content including haemoglobin (Hb). Spectrometric quantification of Hb, is a measure of the extent of cell damage (Evans *et al*., 2013). Erythrocytes are the major cellular component of human blood and laboratory-based toxicity studies are therefore a relevant indication of possible systemic toxicity.

The collected whole blood was centrifuged at 2500 ×g for 10 min and the plasma and buffy coat were aspirated. The aspirated plasma was replaced with isoPBS (0.137 M NaCl, 3 mM KCl, 1.9 mM NaH_2PO_4, 8.1 mM $NaHPO_4$, pH 7.4) and the cell suspension was mixed. The erythrocytes were then diluted to 50% with isoPBS and were kept at 4°C for a maximum of 4 days. Before each experiment, the erythrocytes where washed with isoPBS by centrifugation at 1185 ×g for 3 min and then the removed supernatant was replaced with fresh isoPBS. Before use, the erythrocytes were diluted to 5% using isoPBS. For investigation of haemolytic activity, the erythrocyte suspension was incubated with 10 µL of each concentration of the weight-loss supplement (low and high concentrations of ephedrine, CLA, L-carnitine and HCA) at 37°C for 30 min, 24 and 48 h. For the negative control, 10 µL of PBS was added to the erythrocyte suspension and incubated for the same time intervals. The positive control, 100% haemolysis was generated by incubating the erythrocytes with 2% (w/v) SDS. After incubation, the samples were vortexed, mixed and centrifuged at 1185 ×g for 3 min. A volume of 70 µL of the supernatant of each sample was transferred to a 96-well plate and the absorbance of each supernatant was measured at a wavelength of 540 nm using a FLUOstar OPTIMA plate reader. The data was expressed as percentage of haemolytic activity of each sample relative to the untreated control erythrocytes using the following formula:

$$\%\,\boldsymbol{Haemolysis} = \left(\frac{\boldsymbol{A_{sample}} - \boldsymbol{A_{0\%}}}{\boldsymbol{A_{100\%}} - \boldsymbol{A_{0\%}}}\right) \times \mathbf{100}$$

Where $\mathbf{A_{sample}}$ is the absorbance of erythrocytes exposed to different concentrations of the weight loss supplements, $\mathbf{A_{0\%}}$ is the absorbance of erythrocytes exposed to PBS (0% haemolysis) and $\mathbf{A_{100\%}}$ is the absorbance of erythrocytes exposed to SDS (100% haemolysis).

5.3.2 Ultrastructural effects: Scanning electron microscopy

Blood clotting results in the formation of a dense network of fibrin fibres. The formed fibrin network aggregates the erythrocytes, platelets and other blood components to create a plug

in the injured blood vessel to prevent blood leakage during repair of the blood vessel (Carlson, 2019).

Scanning electron microscopy (SEM), provides information on cellular morphology and is widely used in toxicity studies to investigate the effects on general cell morphology and includes changes to the morphology and integrity of the cell membrane. Biological material is fixed and then coated with carbon. For SEM a beam of electrons collimated into a small spot on the specimen surface and the interaction between the electrons and the sample creates a variety of signals. The produced signals are then detected and displayed on a cathode ray tube screen (Kammlott, 1971).

For the investigation of morphological changes in erythrocytes and fibrin networks, 90 µL of whole blood was incubated with 10 µL of ephedrine, CLA, L-carnitine and HCA at concentrations of 10 and 100 µg/mL at 37°C for 30 min. Blood smears (whole blood only) were made on 10 mm round glass cover slips, with and without the addition of 5 µL of thrombin (20 U/mL). The coverslips were allowed to partially dry and were placed in the 24-well plates containing 0.075 mM sodium potassium phosphate ($NaPO_4$) buffer solution (pH=7.4) for 20 min. The samples were fixed in a 2.5% glutaraldehyde/formaldehyde (GA/FA) solution for 30 min and then washed three times in $NaPO_4$ buffer solution with 5 min intervals between washes. The samples were secondary fixed in 1% osmium tetroxide for 30 min and were then washed again 3 times with the same buffer, 5 min for each wash. The samples were then dehydrated by using an increasing serial dehydration step with 30%, 50%, 70%, 90% and 100% (3 times) EtOH, each for 5 min. The 100% EtOH was removed and a 100% HMDS was added, for 30 min for drying. Approximately 2 drops of HMDS were added on the cover slips and the samples were left to dry. Once the samples were dry, the cover slips were then mounted on aluminium stubs, coated with carbon and viewed with the Zeiss Ultra Plus FEG-SEM and Zeiss Crossbeam 540 FEG-SEM.

5.3.3 Data management and statistical analysis

For the haemolysis assay, blood of 5 different individuals was used. Each experiment was done in triplicate for each volunteer representing an individual experiment. The statistical methods used were the same as described in chapter 3 and 4.

SEM generates qualitative data and provides visual and descriptive information. The images (Figures 5.2 and 5.3) are a representative of 5 experiments in the various exposure groups.

5.4 Results

5.4.1 Haemolytic effects of ephedrine, CLA, L-carnitine and HCA

The haemolysis assay was used to evaluate the haemolytic activity of the three commonly used weight loss dietary supplements (CLA, L-carnitine and HCA) with ephedrine as a weight loss control (Figure 5.1). No haemolytic activity was observed for all the samples after 30 min incubation at all tested concentrations. The long-term (24 and 48 h) haemolytic activity of these weight loss dietary supplements was also assessed and the results revealed that none of the weight loss supplements induced significant haemolytic activity. No significant difference in haemolytic activity was observed between ephedrine and the tested weight loss supplements. HCA showed a significant membrane stability at the highest concentration of 500 µg/mL after 48 h exposure.

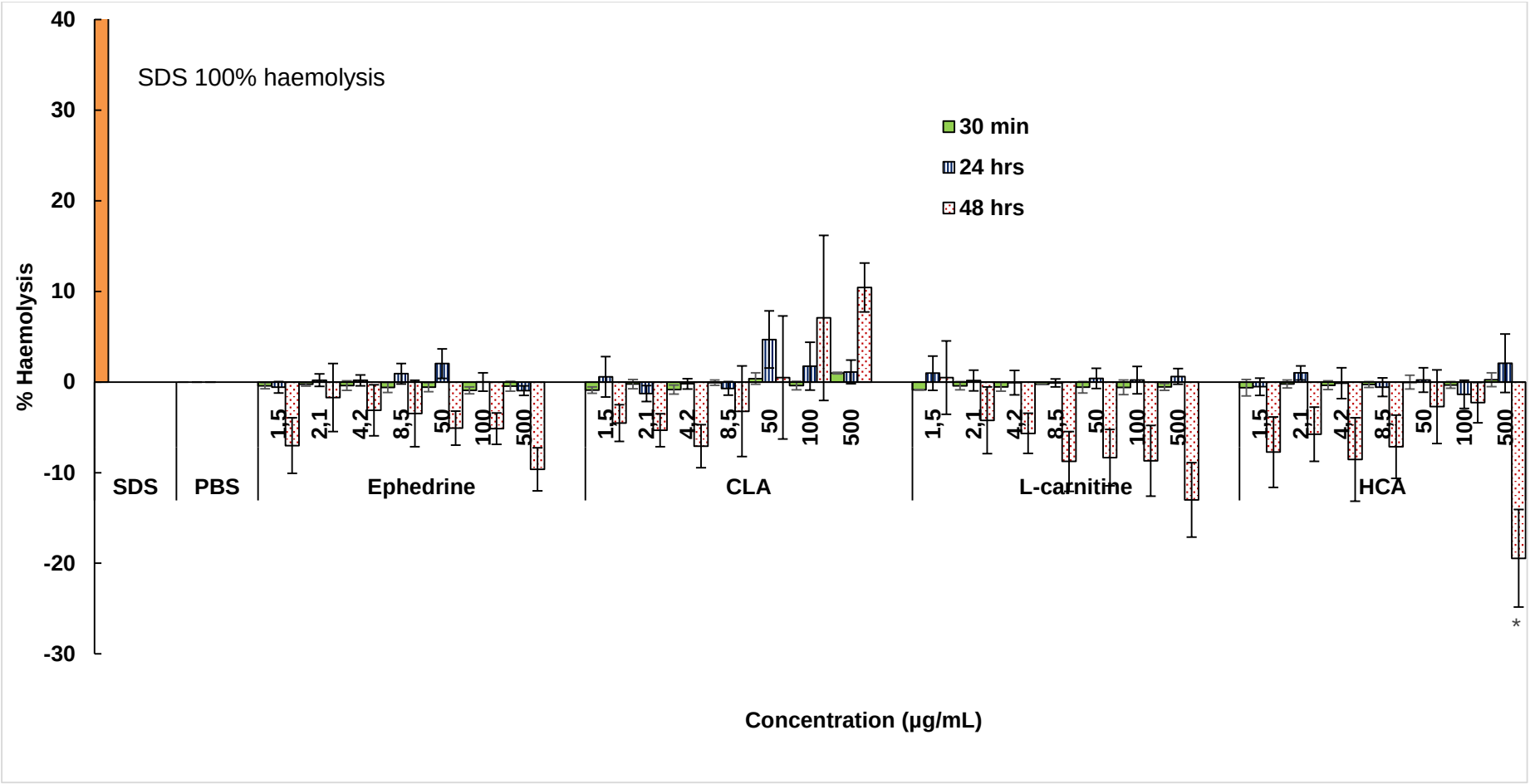
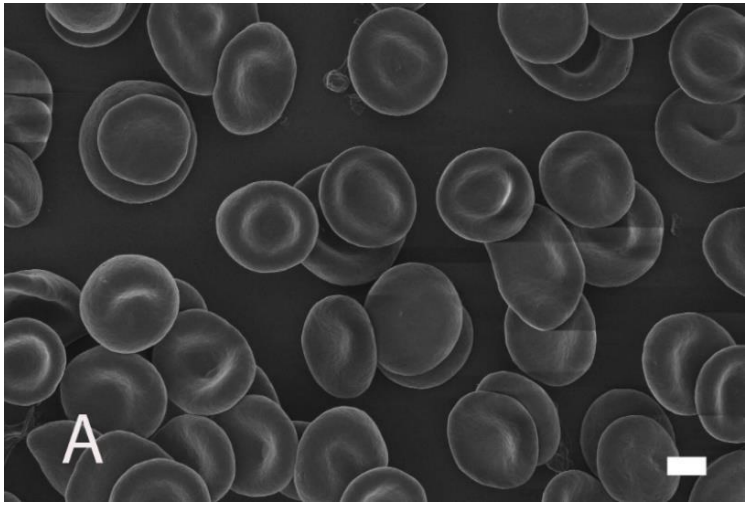


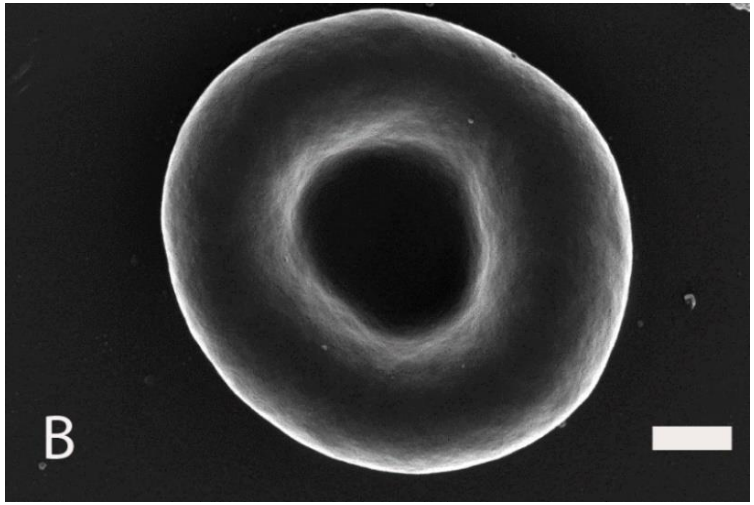
Figure 5.1: Haemolytic effect of low (1,5 – 8,5 µg/mL) and high (50 – 500 µg/mL) concentrations ephedrine, conjugated linoleic acid, L-carnitine and hydroxycitric acid on human erythrocytes following 30 min (bold colours), 24 h (striped) and 48 h (dotted) exposure with SDS as control (100%) haemolysis. Data presented are means ± SEM of the five independent experiments performed in duplicates. For all weight loss compounds, differences between SDS and each dosage was significant ($p<0.05$) and no differences were observed between the time intervals for each group, except for HCA at 500 µg/mL between 24 and 48 h. No differences were observed between concentrations for each group, except for HCA 50 and 100 µg/mL compared to 500 µg/mL.

5.4.2 Determination of ultrastructural changes on erythrocytes and fibrin network

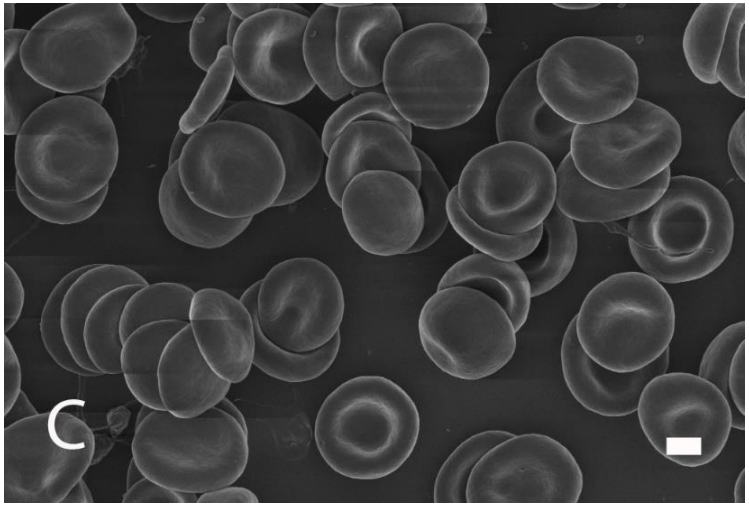
The effect of weight loss dietary supplements on ultrastructure of erythrocytes was assessed using SEM (Figure 5.2). Figure 5.2A & B shows erythrocytes from untreated whole blood that served as the control group. The erythrocytes of the control group retained the biconcave discoid form and smooth surface membrane typical of control erythrocytes. Exposure to OTC weight loss dietary supplements did not alter the morphology, appearance and surface of the erythrocytes after 30 min exposure (Figure 5.2C - R). No differences were observed in the morphology of erythrocytes treated with ephedrine and the tested weight loss supplements.

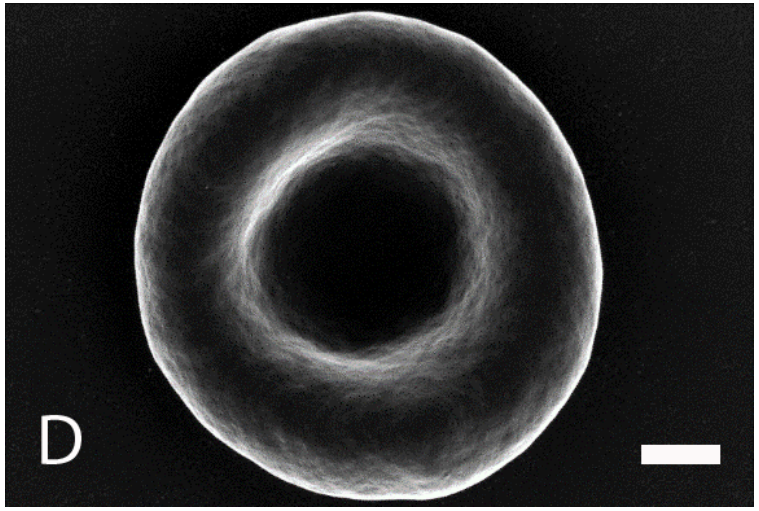
Control



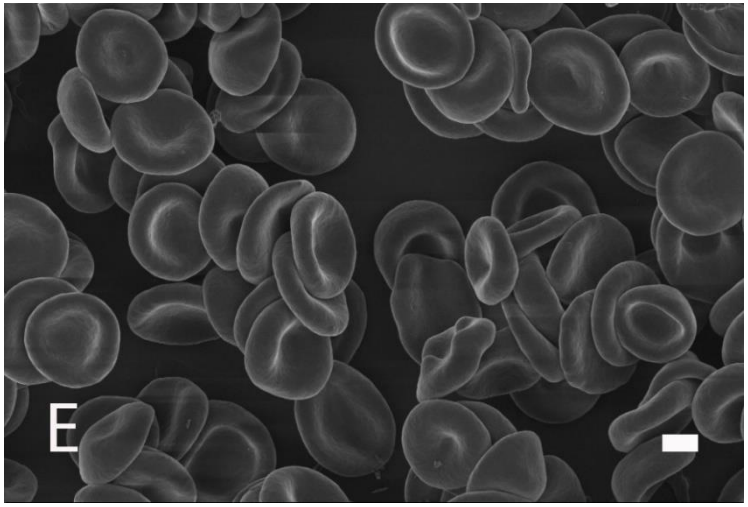


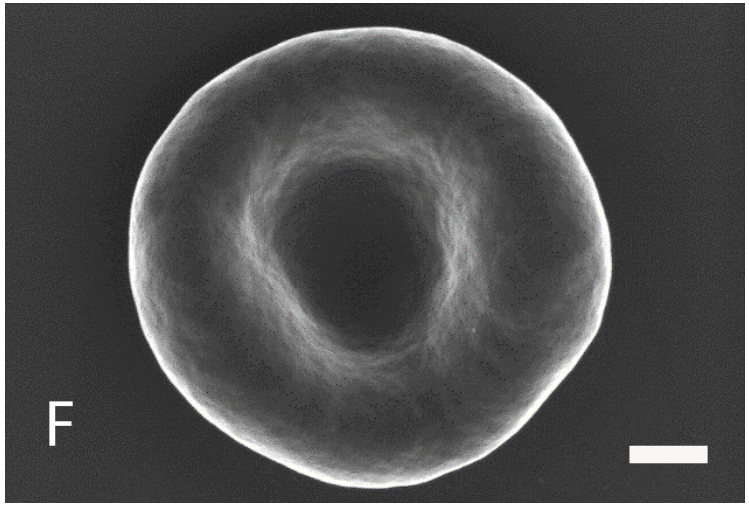
Ephedrine: 10 µg/mL



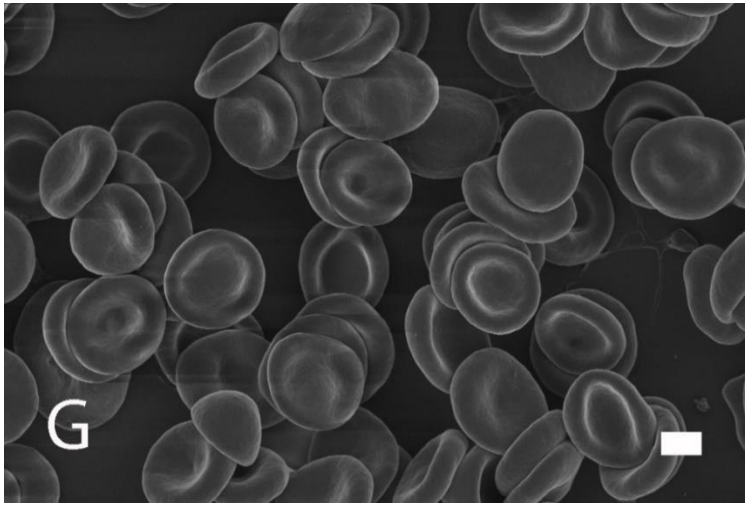


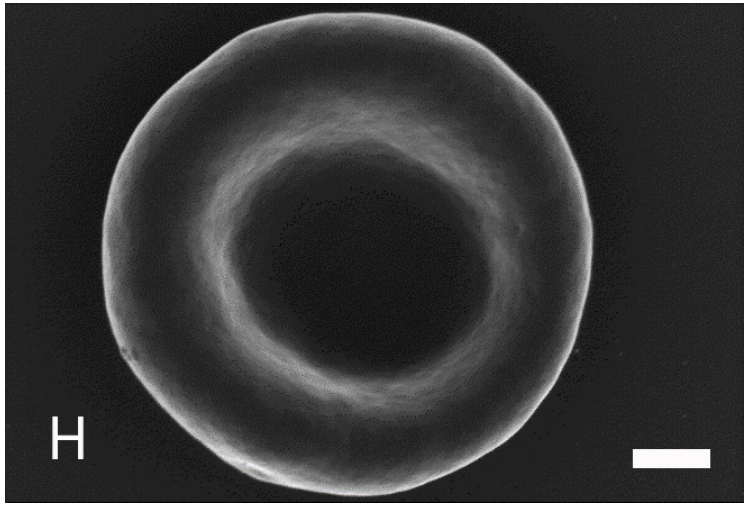
Ephedrine: 100 µg/mL



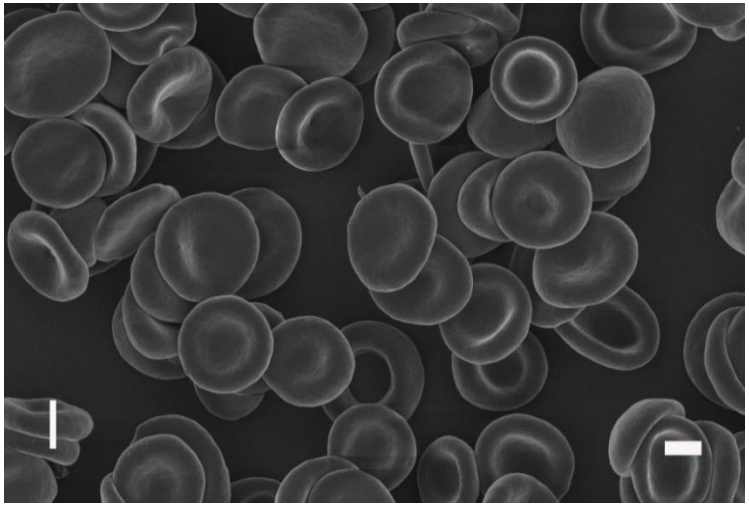


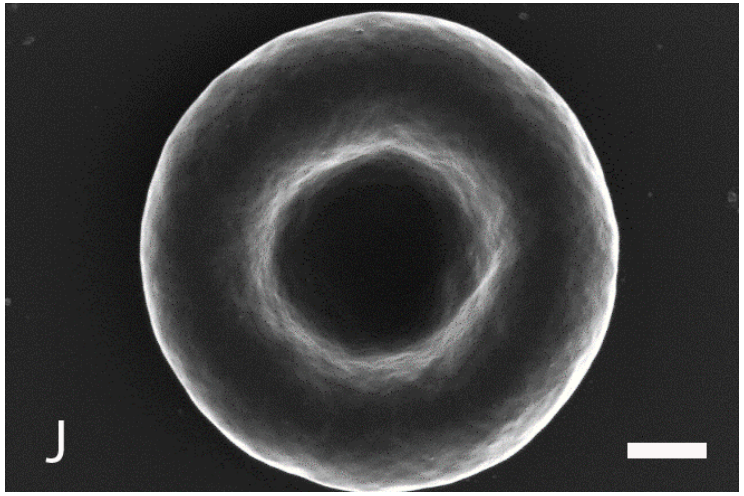
CLA: 10 µg/mL



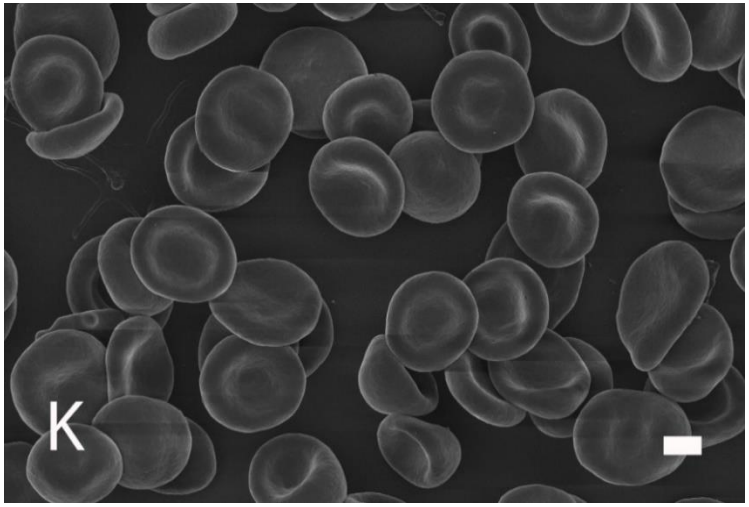


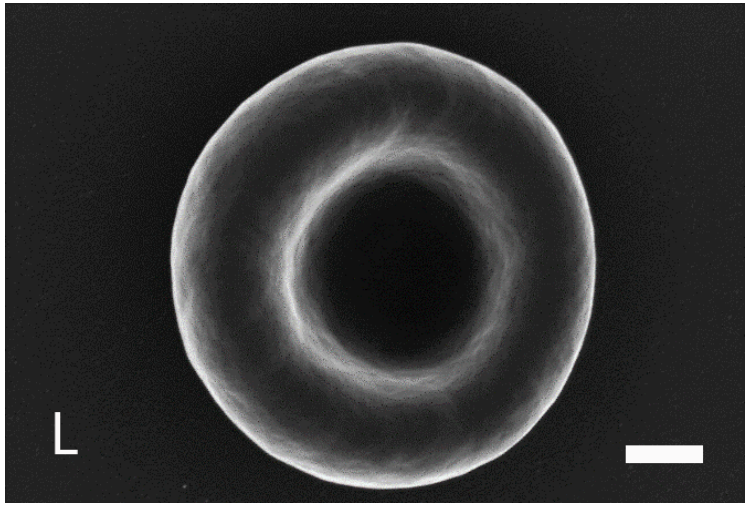
CLA: 100 µg/mL



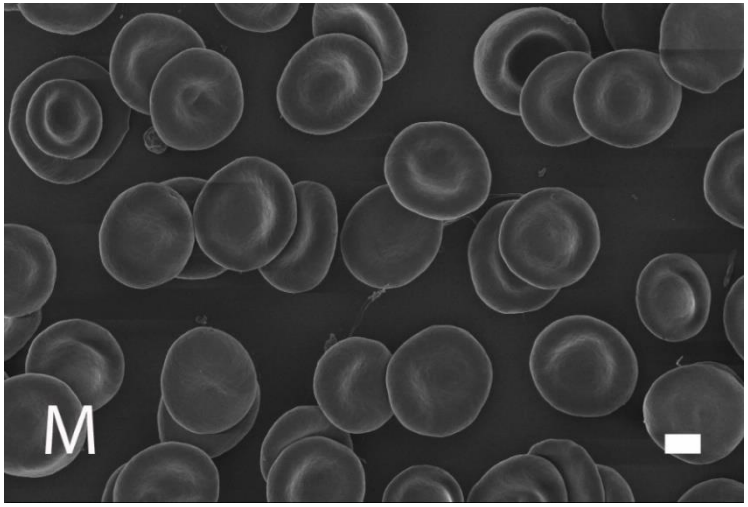


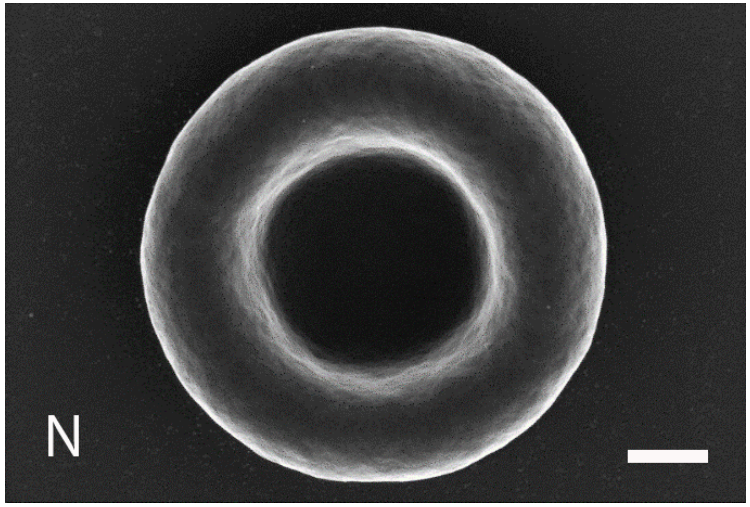
L-carnitine: 10 µg/mL



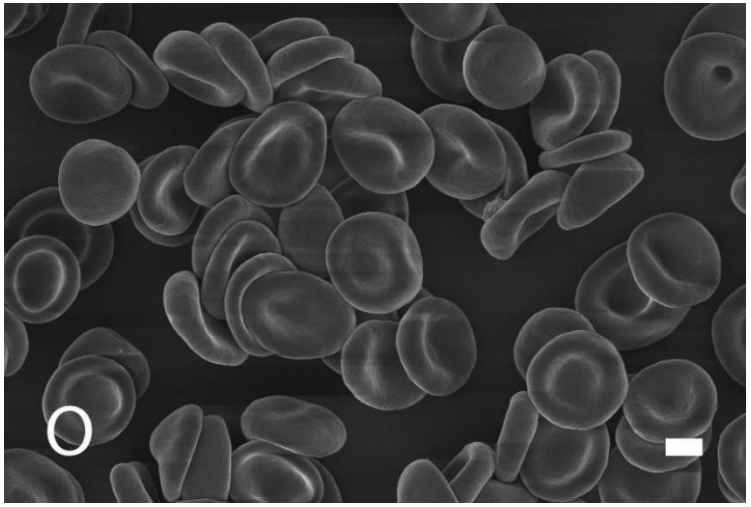


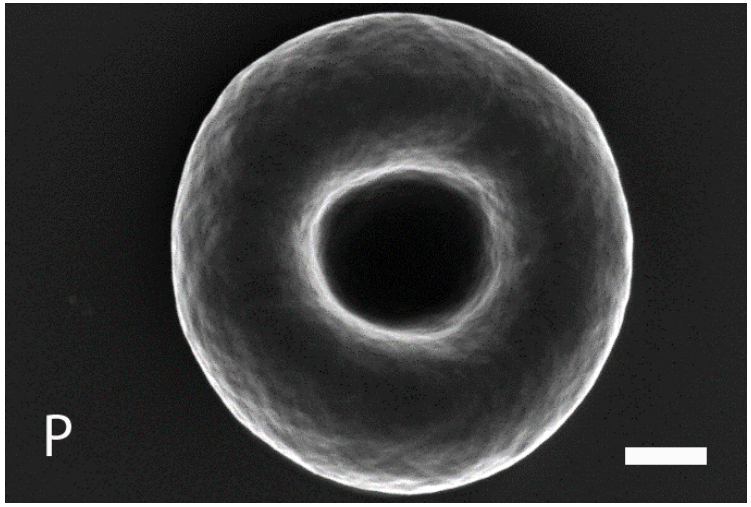
L-carnitine: 100 µg/mL





HCA: 10 µg/mL





HCA: 100 µg/mL

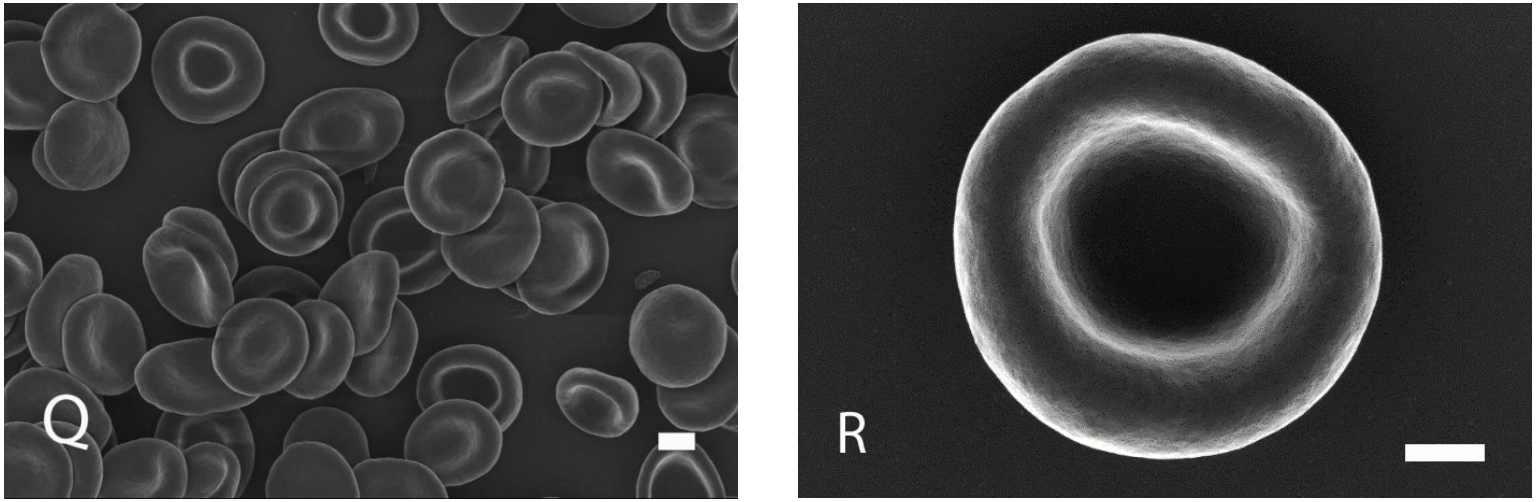
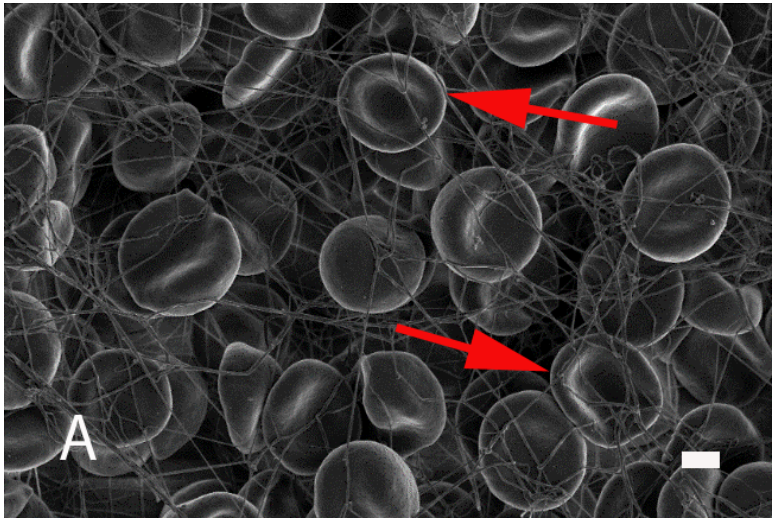


Figure 5.2: Representative SEM micrographs of erythrocytes from the control and weight loss dietary supplements treated group. **Fig. A & B**: control with normal biconcave disk shape of erythrocytes. **Fig C - F:** ephedrine, **E – J:** CLA, **K – N:** L-carnitine and **O – R:** HCA showing unchanged morphology after exposure to weight loss dietary supplements. (**Scale bars**: **B, D, F, H, J, L, N, P &R:** 1 µm; **A, C, E, G, I, K, M, O & Q:** 2 µm).

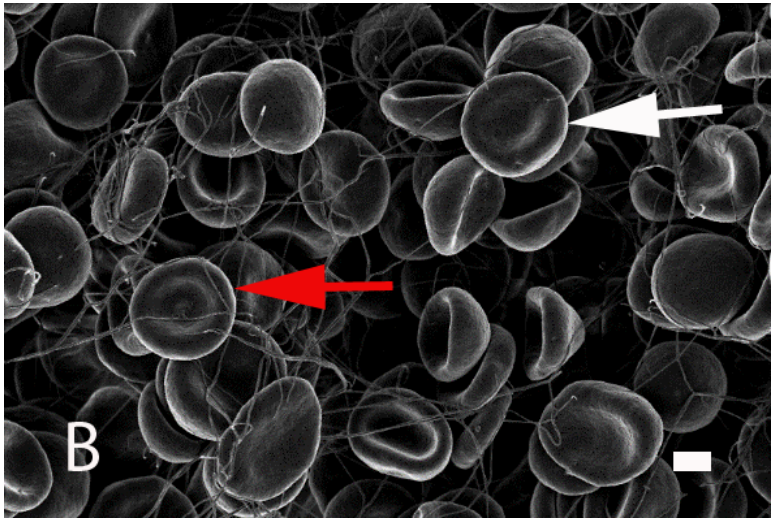
The effect of the investigated OTC weight loss dietary supplements on the fibrin structures formed in the presence of erythrocytes was evaluated using SEM (Figure 5.3). Figure 5.3A represents the control group, which shows normal erythrocytes with minimal interaction with the fibrin structures. Treatment with weight loss dietary supplements did not alter the morphology of erythrocytes and fibrin networks (Figure 5.3B – I). No differences were observed between erythrocytes treated with the control, ephedrine and the investigated weight loss supplements.

Control

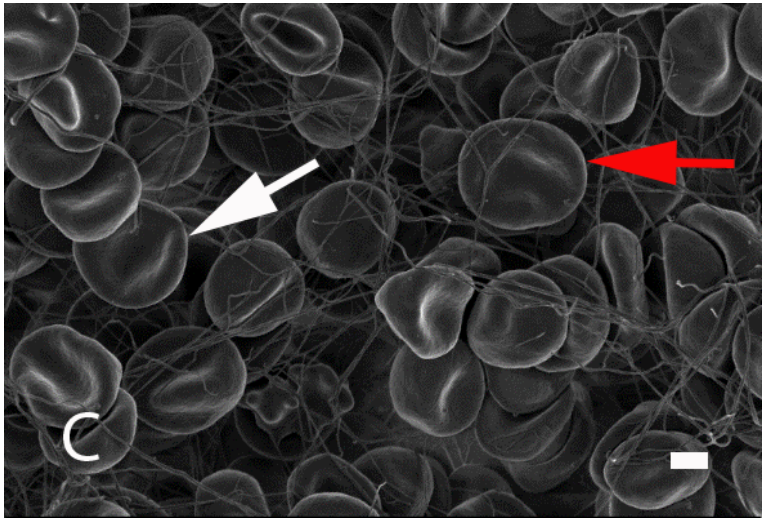


Ephedrine

10 µg/mL

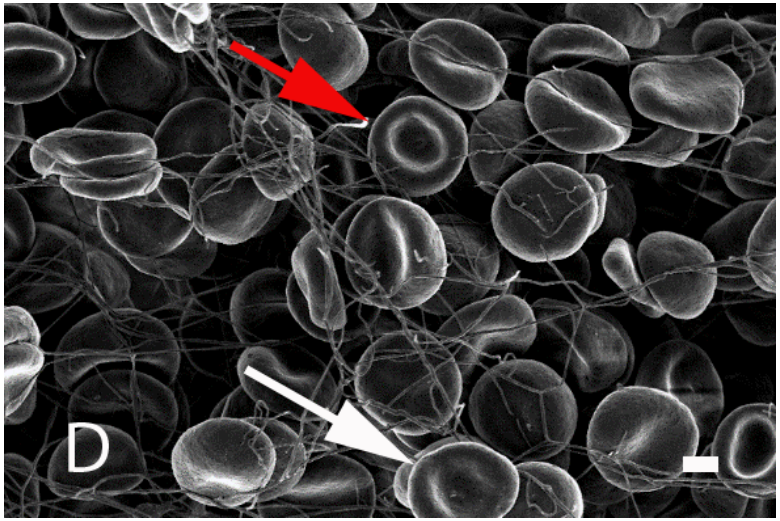


100 µg/mL

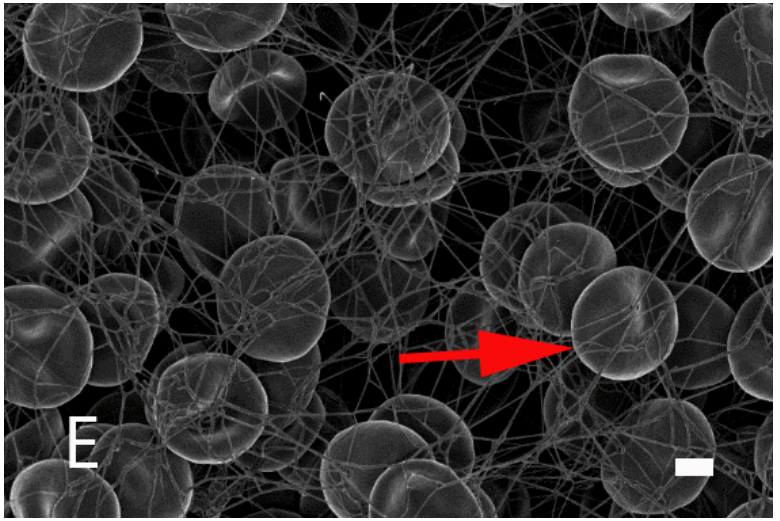


CLA

10 µg/mL

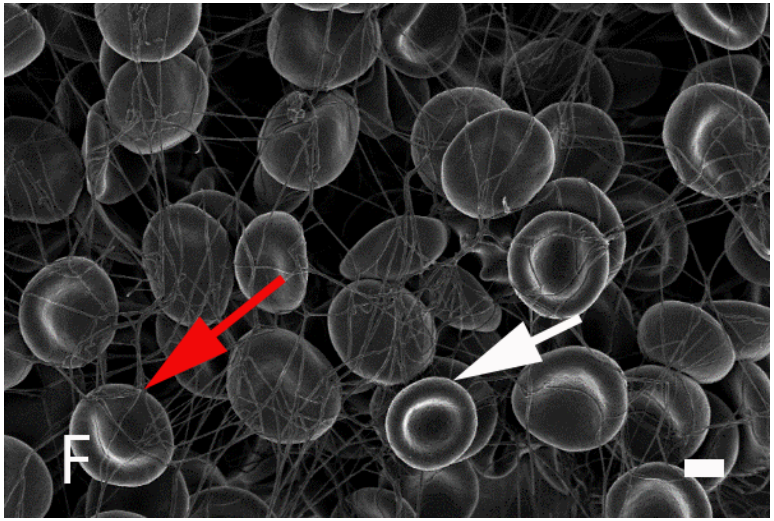


100 µg/mL

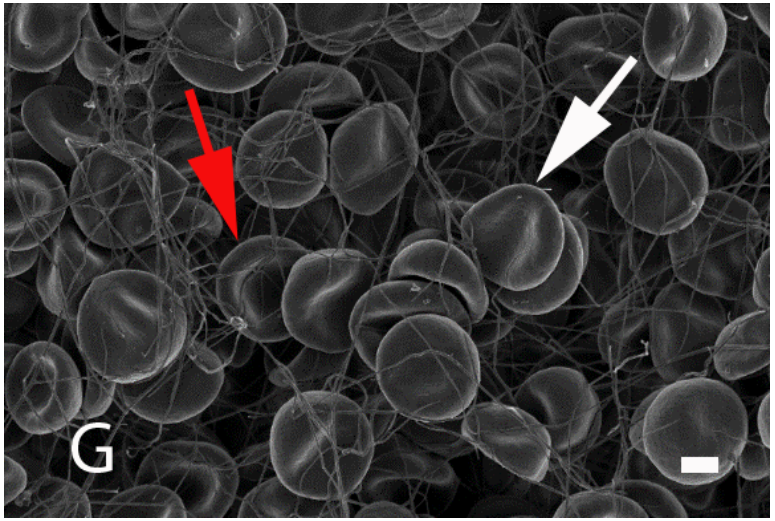


L-carnitine

10 µg/mL



100 µg/mL



HCA

10 µg/mL **100 µg/mL**

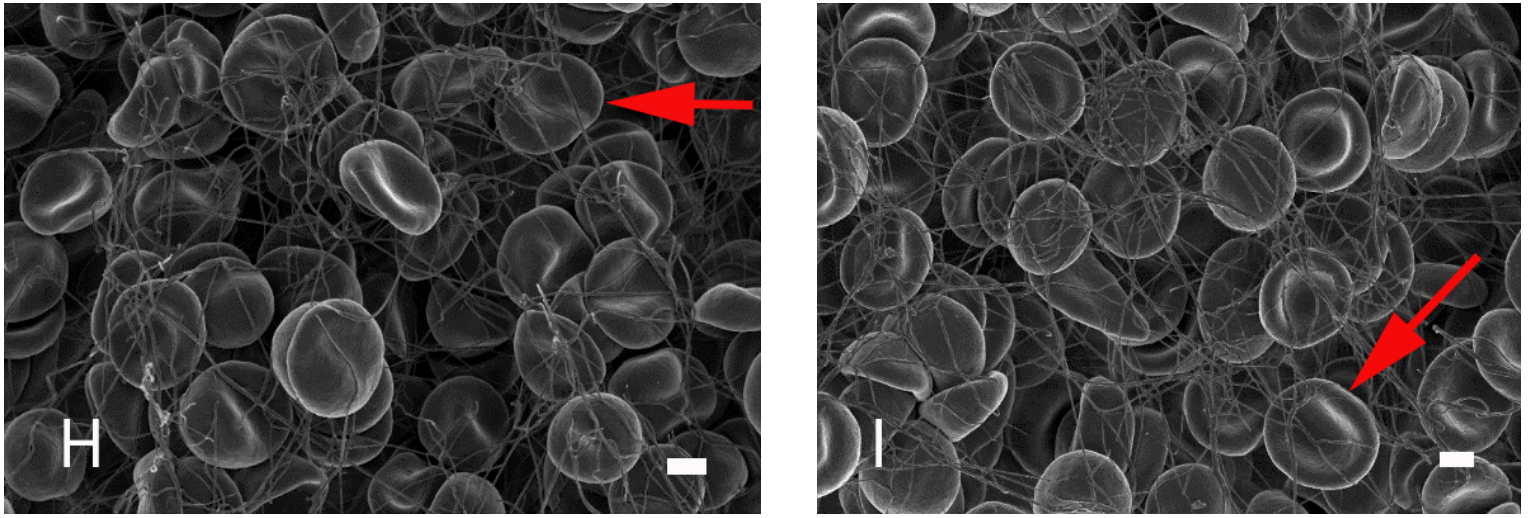


Figure 5.3: Scanning electron micrographs of whole blood with added thrombin from the control and weight loss dietary supplements treated group. **Fig. A:** control with normal morphology of erythrocytes with minimal interaction with fibrin network. **Fig B & C:** ephedrine, **D & E:** CLA, **F & G:** L-carnitine and **H & I:** HCA with no ultrastructural changes. **White arrows:** Normal erythrocytes; **Red arrow:** Interactions between erythrocytes and fibrin network. (**Scale bar**: 2 µm).

5.5 Discussion

5.5.1 Determination of haemolytic activity on erythrocytes

Erythrocytes are the most common cell type in the human body. Erythrocytes have a typical biconcave disc shape with an average diameter of 7.8 µm, a thickness of 2.5 µm in periphery, 1 µm in the centre and a volume of 85 – 90 μm^3 (Gothoskar, 2004) and allows the flexible movement of these cells through the narrow capillaries, which have a diameter of no more than 3 – 5 µm (Gothoskar, 2004, Tomaiuolo, 2014). Erythrocytes lack a nucleus and other organelles and the plasma membrane encloses cytoplasm rich in Hb which is essential for respiration. Erythrocytes generate their energy anaerobically due to the lack of mitochondria and do not utilize the O_2 that is transported (Gothoskar, 2004). The average life span of erythrocytes is 120 days and aged erythrocytes are removed from circulation by the spleen and liver. Following absorption of drugs/compounds into blood, due to their high levels in blood, erythrocytes are often one of the initial targets of toxicity. Therefore, mammalian erythrocytes are considered one of the best models for the rapid evaluation of cytotoxicity, where measurement of released Hb from damaged erythrocytes is an indicator of damage. In this study, the haemolysis assay was employed to assess the cytotoxic effect of the three commonly used weight loss dietary supplements, CLA, L-carnitine and HCA using ephedrine as control.

CLA did not show any significant haemolytic activity after 3, 24 and 48 h exposure to increasing concentrations of CLA. After 48 h exposure a slight but still insignificant increase in haemolytic activity was observed which could be related to an increase in ROS production observed in undifferentiated 3T3-L1 cells. ROS causes oxidative damage to cell membranes thereby altering the permeability (Blokhin & Lentz, 2013). Specifically, H_2O_2 can cross the erythrocyte plasma membrane and directly oxidize Hb to ferryl Hb, which is unable to transport oxygen (Rao *et al*., 1994). Yuan *et al*., (2015) reported that in Sprague-Dawley rats treated with linoleic acid (similar structure to CLA) at concentrations of 60 - 90 mM (16 826.4 – 25 239.6 µg/mL) for 24, 48 and 72 h a dose-dependent significant changes in both erythrocyte and Hb levels were observed compared to the control. The rats fed with 90 mM linoleic acid died within five minutes of administration and showed symptoms such as dyspnea, cyanosis, haemoptysis labiali convulsion and pulmonary embolism, which were considered the main cause of death. The acute induced erythrocyte damage in linoleic acid fed rats was associated with decreased GPx and SOD and H_2O_2 concentrations (Yuan *et al*., 2015).

Erythrocytes contain significant amounts of L-carnitine and its esters (Cooper *et al*., 1988) and L-carnitine uptake and storage by erythrocytes is linked to lower haemolytic activity and

increased levels of erythrocyte-ATP (Arduini *et al*., 1997). In the present study it was also found that L-carnitine did not cause the haemolysis of human erythrocytes. Toptas *et al*., (2006) reported that oral administration of 100 mg/kg/day improved erythrocytes deformability and these improvements of mechanical properties may be related to its antioxidant and membrane stability action (Toptas *et al*., 2006). L-carnitine was found to protect tissue against lead acetate (Ozsoy *et al*., 2011). Wistar albino rats were treated with 0.5 mg/kg lead acetate and 0.5 mg/kg L-carnitine once daily for 60 days. Lead acetate reduced erythrocyte levels, whereas the addition of L-carnitine in the diet improved erythrocyte levels and this protective effect was due to the antioxidant activity of L-carnitine (Ozsoy *et al*., 2011). Wanic-Kossoivska *et al*., (2007) observed a significant improvement in blood morphology parameters (Hb and erythrocytes cell membrane stabilization) as a result of treatment with L-carnitine 4×250 mg/day and erythropoietin for 6 weeks alone and in combination in patients on haemodialysis. The beneficial role of L-carnitine was thought to be due to its effects on membrane metabolic pathways that stabilized the plasma membrane of erythrocytes (Wanic-Kossowska *et al*., 2007). In contrast, Soumya *et al*., (2015) investigated the potential protective effect of L-carnitine at concentrations of 10 – 60 mM (1612 – 9672 µg/mL) on the erythrocytes from Wistar rats stored for a period of 20 days at 4°C. Haemolysis increased indicating that L-carnitine could not completely protect lipids and proteins from oxidative stress. Furthermore, a significant increase in SOD and MDA levels initially with a significant decrease in these levels towards the end of storage. Furthermore, the levels of CAT and GPx were lower in the experimental group compared to the control group during storage (Soumya *et al*., 2016).

Several previous studies have been conducted evaluating the effect of HCA on haematological parameters such as the RBC count and Hb levels. Although, HCA is the active ingredient of the *Garcinia* species, other phytochemicals of *Garcinia* species found in the fruit rinds and seeds have been investigated, more research is still required to elucidate the effect of HCA on erythrocytes. In the present study, 3, 24 and 48 h exposure to increasing concentrations of HCA did not cause erythrocyte haemolysis.

The beneficial effects of *G. cambogia* seed extract was reported in a study by Oluyemi *et al*., (2007) where treatment of Wistar rats with 200 and 400 mg/kg/day of *G. cambogia* seed extract for 5 weeks caused a significant increase in erythrocyte levels compared with the control group. In this study, it was concluded that *G. cambogia* seed extract had haematologically enhancing effects through its ability to trigger erythropoiesis (Oluyemi *et al*., 2007). A study by Ghosh & Mukherje, (2017) showed insignificant haemolysis in human erythrocytes after treatment with *G. cambogia* (containing 50.9% HCA) at concentrations of 10 – 100 µg/mL for 3 and 24 h. The protective effect of was linked to the high flavonoid content

of *G. cambogia* (Ghosh & Mukherjee, 2017). In another study by Correcirc *et al*., (2014), treatment with *G. cambogia* at concentrations of 125 and 250 µg/mL did not cause human erythrocyte haemolysis. Alabi *et al*., (2017) showed that oral administration of *G. kola* (garcinia species with HCA as active ingredient) extract at concentrations of 100 and 200 mg/kg/day in male Wistar rats for 28 days caused a significant increase in RBC count with a subsequent increase in Hb when compared to the control and this beneficial effect was attributed to the antioxidant capacity of the *G. kola* extract (Okoko, 2009).

There is still insufficient research conducted in determining the cytotoxic effect of ephedrine on erythrocytes. In the current study, incubation with increasing concentrations of ephedrine for 3, 24 and 48 h did not cause erythrocyte haemolysis. Similarly, a study conducted by Hyuga *et al*., (2016) showed no significant difference in haematological parameters (RBC count, haematocrit, haemoglobin concentration, platelets count and white blood count) between mice treated with 6 g of ephedrine alkaloids-free ephedra herb extract once per day for 14 days. The beneficial effect of the non-alkaloidal ephedra herb extract was associated with other active ingredients of ephedrine, including presence of proanthocyanidins. At the dosages used, no cytotoxicity was observed at the concentrations evaluated for ephedrine, CLA, L-carnitine and HCA.

5.5.2 Determination of ultrastructural changes of erythrocytes and fibrin networks

Fibrin, a fibrous fibre, is the main structural component of a blood clot (Van Kempen *et al*., 2014). Fibrinogen is a glycoprotein that is converted by thrombin to form fibrin strands thereby forming fibrin networks which stabilize the blood clot and form a secondary haemostatic plug (Palta *et al*., 2014). The haemostatic function of whole blood involves a balance between coagulation and fibrinolytic cascades, which is predominately affected by erythrocytes (Wohner *et al*., 2011). There is a strong link between obesity and disordered haemostasis/haemostatic disturbances in the coagulation and fibrinolytic cascades. Therefore, it is important to determine the effect of weight loss dietary supplements on erythrocytes and fibrin networks as the observed effect may be protective or promote blood clot formation. This study revealed that treatment with weight loss dietary supplements (ephedrine, CLA, L-carnitine and HCA) for 30 min did not cause ultrastructural changes to the erythrocytes and the fibrin network. Erythrocyte morphology was similar to that observed in Figure 5.2.

Despite extensive research conducted in determining the effect of CLA, there are few studies that show the ultrastructure of erythrocytes and fibrin networks exposed to CLA. Benito *et al*.,

(2001) revealed that the consumption of 3.9 g/d of CLA for 93 days by healthy human females did not have any effect on prothrombin time, activated levels of thromboplastin time and levels of antithrombin III compared to the control group. Furthermore, administration of CLA showed no significant physiological changes in blood coagulation and platelet function in healthy adult females. Following short-term exposure, CLA did not exhibit anti-thrombotic properties in humans (Benito *et al*., 2001). A study by Burdge *et al*., (2005) demonstrated oral administration of 1, 2 and 4 capsule(s) of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA isomers by healthy human males for 8 week periods resulted in the incorporation of the CLA isomers into the lipids of the erythrocyte plasma membrane and partially replacing specific saturated and polyunsaturated fatty acids (Burdge *et al*., 2005). Sato *et al*., (2011) found that ingestion of 2.2 g/d *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA isomers for a 3 week period by healthy humans resulted in increased rates of CLA retention in erythrocytes compared to other cells, suggesting that CLA in erythrocytes is not easily eliminated and this may be due to the lack of fatty acid metabolism in erythrocytes (Sato *et al*., 2011). In a randomized, double-blind, placebo-controlled trial conducted by Moloney *et al*., (2004) oral administration of 3.0 g/d cis-9, *trans*-11 and *trans*-10, *cis*-12 CLA (1:1) isomers in 32 humans with stable, diet-controlled T2DM for 8 weeks resulted in a significant reduction in plasma fibrinogen levels to normal ranges compared to the control group. The positive effect of CLA on fibrinogen concentrations would reduce the risk of factors of CVD (Mullen *et al*., 2004). All the above studies show that a beneficial effect is only observed after several weeks of treatment and therefore, few of these beneficial effects would not be observed in *in vitro* studies.

To our knowledge there are no studies conducted on investigating the effect of L-carnitine on the ultrastructure of erythrocytes and fibrin networks. The positive effect of L-carnitine was reported in a study by Arduini *et al*., (1990), where exposure to 20 – 300 µM (3.22 – 48.36) L-carnitine, increased erythrocyte membrane stability through specific interaction with one or more cytoskeleton proteins. Haemodialysis patients have an increased risk of blood coagulation and fibrin clot formation (Nikolaos *et al*., 2000). In these patients Nikolaos *et al*., (2000), found that supplementation with 30 mg/kg of L-carnitine measured every 3 months for 120 months resulted in a significant reduction in erythrocytes deformability compared to the control group. Limited literature is available on the effects of L-carnitine on blood coagulation. Hakeshzadeh *et al*., (2010) showed that haemodialysis patients that received 1000 mg/d of L-carnitine orally for 12 weeks had significant reduction in coagulation factors V, VII, IX and plasma fibrinogen concentration compared to placebo group. The authors suggested that L-carnitine may have a protective effect against CVD in these patients (Hakeshzadeh *et al*., 2010). In the present study, L-carnitine at the evaluated levels was found not to be cytotoxic.

Likewise, the effects of HCA on erythrocytes and fibrin networks is unknown. However, some studies have investigated coagulation factors and haematological parameters including blood viscosity that may affect the ultrastructure of erythrocytes and fibrin networks. Al-Gareeb *et al*., (2011) demonstrated that in healthy male volunteers, dosages of 100 and 500 mg of *G. cambogia* twice daily for a period of 2 weeks, significantly reduced blood viscosity and plasma fibrinogen levels compared the control group. The reduction in plasma fibrinogen levels were linked to anti-inflammatory properties of HCA (Al-Gareeb *et al*., 2013). Alkuraishy and Al-Gareeb, (2012) showed that patients with peripheral vascular diseases that ingested 300 mg of aspirin plus 500 mg *G. cambogia* daily for 2 weeks had significant reduction in plasma fibrinogen and blood viscosity. The protective effect of *G. cambogia* was related to its antioxidant properties and anti-inflammatory properties (Al-Gareeb *et al*., 2013) of the proanthocyanidins and not HCA which, as found in the present study does not have antioxidant activity.

Ephedra and its associated alkaloids have been associated with severe cardiovascular system events (Haller & Benowitz, 2000). Ephedra alkaloids are known to stimulate the sympathetic system by increasing platelet reactivity that may contribute to a pro-thrombotic state (Ramot & Nyska, 2007). Therefore, determination of the effect of ephedrine on the ultrastructure of erythrocytes and fibrin networks is very important. There are no studies conducted on studying the effect of ephedrine on the ultrastructure of erythrocytes and fibrin networks. Flordal *et al*., (1992) studied the effect of ephedrine on coagulation and on fibrinolysis. The study demonstrated that consumption of ephedrine by six healthy volunteers resulted in a significant prolonged bleeding time compared to the untreated control. Ephedrine consumption further activated fibrinolysis. There was no change in platelet count, factor VIII and von Willebrand factor. The authors suggested that ephedrine could be used in procedures that have increased risk of blood loss and venous thromboembolism (Flordal *et al*., 1992). More studies are still required in determining the effect of ephedrine on the ultrastructure of erythrocytes and fibrin networks. Short-term exposure of blood to ephedrine did not alter erythrocyte morphology or fibrin network formation.

5.6 Conclusion

Increasing concentrations of ephedrine, CLA, L-carnitine and HCA following 3, 24 and 48 h exposure did not cause human erythrocyte haemolysis. Exposure of human whole blood to these weight loss compounds for 30 min did not cause changes to erythrocyte morphology and the structure of the formed fibrin network. Therefore, under the experimental conditions used in the present study, ephedrine, CLA, L-carnitine and HCA does not adversely affect

blood haemostasis. The concentrations used are high and does not represent blood levels following absorption, which would be much lower. These weight loss compounds may have beneficial effects although these effects such as antioxidant activity and effects on erythrocyte rigidity should be investigated in future studies.

Chapter 6: Concluding discussion

Obesity is a growing epidemic concern globally. An estimate of at least two-thirds of the adult population globally are following different strategies for weight loss or to prevent weight gain (Boucher *et al*., 2001). Many people tend to adopt diets that contain less fat for weight management, and a few use the first-line therapy such as lifestyle and behavioural modification for weight loss. However, there is poor compliance to these weight loss strategies (Boucher *et al*., 2001). Many obese patients tend to use alternative or complimentary therapy such as OTC weight loss dietary supplements, yoga, acupuncture, homeopathy and hypnotherapy. The use of OTC weight loss dietary supplements is most commonly used as these products are readily available, easy to obtain and can in some instances be bought over the internet (Erdogan & Kurtuncu, 2018).

Previous studies have reviewed the molecular mechanisms, quality, safety and efficacy of the most commonly used weight loss dietary supplements (Saper *et al*., 2004, Vaughan Roger, 2014, Ríos-Hoyo & Gutiérrez-Salmeán, 2016). However, the use of some OTC weight loss dietary supplements is not recommended for use as there is still insufficient or conflicting data regarding the quality, safety and efficacy (Saper *et al*., 2004).

Therefore, the aim of this study was to provide evidence regarding the effect and safety of ephedrine (weight loss control) and three common weight loss OTC dietary supplements, CLA, L-carnitine and HCA with specific focus on the antioxidant properties, cell viability, adipogenicity and haemostatic effects.

The concentrations of CLA, L-carnitine and HCA were selected to include levels recommended by the manufactures of these products. Two concentration ranges were used to represent low (15 – 85 µg/mL) and high (500 – 5000 µg/mL) concentrations. The effect of ephedrine was evaluated over the same concentration range.

Levels of chemical and cellular antioxidant activity were determined using the ORAC and DCFH-DA assays, respectively. L-carnitine and HCA did not have antioxidant activity. However, HCA (only at high concentrations) but not L-carnitine protected 3T3-L1 preadipocytes against oxidative damage. Neither, L-carnitine nor HCA caused oxidative damage. Even at high concentrations no cytotoxic effects with and without AAPH was observed in the mouse fibroblasts, L929 and 3T3-L1 cell lines. In contrast, CLA was found to have chemical antioxidant activity at 25 – 250 µg/mL, however CLA induced oxidative damage at these concentrations in 3T3-L1 preadipocytes. No cellular antioxidant or oxidative effects

were observed for ephedrine and CLA, however at 250 µg/mL, both weight loss compounds were cytotoxic with the L929 cell line being more sensitive. Of the weight loss compounds evaluated, although CLA showed antioxidant activity (ORAC assay), this did not translate into cellular protection. Of concern is that CLA is marketed as a product with antioxidant properties. The gold standard for the evaluation of antioxidant activity is the ORAC assay (Pisoschi *et al*., 2016), and very few products are ever evaluated in a cellular model, and by doing so this adverse effect was identified. At high concentrations, CLA is as cytotoxic as ephedrine while L-carnitine and HCA, have no effects.

All compounds at 10 µg/mL and 100 µg/mL did not alter lipid levels in differentiated adipocytes or did not reduce lipid accumulation in differentiating adipocytes. At these concentrations no toxicity was observed in differentiated adipocytes although in L929 and undifferentiated 3T3-L1 preadipocytes toxicity was observed at higher concentrations. Although several studies have identified that CLA, L-carnitine and HCA reduce lipid accumulation in adipocytes (Simon *et al*., 2005; den Hartigh *et al*., 2013; Siegner *et al*., 2014; Ramaiah *et al*., 2017), the lack of effect in the present cell line may be related to differences in the cell lines such as the passage of the cell line, reagents used for differentiation as well as the extent to which the associated biochemical pathways are activated.

Ephedrine, CLA, L-carnitine and HCA at increasing concentrations following 3, 24 and 48 h exposure did not cause human erythrocyte haemolysis. Exposure of human whole blood to the weight loss compounds for 30 min did not cause changes to erythrocyte morphology and the structure of the fibrin network that formed. Findings were that ephedrine, CLA, L-carnitine and HCA does not adversely affect blood haemostasis. High concentrations of each weight loss compound were used and does not necessary represent blood levels following absorption, which would be much lower. It can therefore be concluded that in healthy individuals, these weight loss compounds will not adversely affect erythrocytes and blood clotting.

Limitations and recommendations

Obesity is a multifactorial disorder where genetic factors play a significant role. Obesity has been linked with more than 300 genes in humans that regulates transcription factors for adipocyte differentiation such as PPARγ and C/EPBs and enzymes or proteins such as aP2, LPL and GPDH. This study only evaluated the effects of ephedrine, CLA, L-carnitine and HCA using general parameters of effect such as changes in cell viability and lipid content. More detailed evaluation of the effects on specific markers such as transcription factors, enzymes

and proteins may provide more sensitive information on the effects and targets of these weight loss products. Methods such as real time polymerase chain reaction (qPCR), can be used to quantify changes in gene expression such as effects on the adipogenic transcription factors. Enzymatic analysis will provide evidence regarding the effect of these weight loss dietary supplements on the antioxidant enzymes such as CAT, SOD and GPx as well as the lipogenic enzymes such as LPL, ACC, FAS and SCD.

There are various mechanisms by which compounds affect the cell viability of cultured cells that eventually lead to cell death by necrosis or apoptosis. This study did not investigate the mechanism of reduced cell viability by ephedrine and CLA seen at higher doses in both cell lines. Therefore, assays such as the TUNEL assay with flow cytometry and AO/PI staining could be used to distinguish if the cell death occur by necrosis and apoptosis. Further, apoptotic markers such as Bax, p53, cytochrome c, caspase-3, caspase-9 and TNFα using enzyme linked immune-absorption assays (ELISA) could also be evaluated.

The effect of CLA isomers (*cis*-9, *trans*-11 and *trans*-10, *cis*-12) alone has been previously investigated. This study did not assess the safety and efficacy of these isomers alone *in vitro,* as the *cis*-9, *trans*-11 could antagonize the anti-adipogenic effect of *trans*-10, *cis*-12 vice-verse or one isomer can cause a significant cytotoxicity at higher dose resulting in inaccurate interpretation of the data. Therefore, the effect of CLA isomers should be evaluated independently as it would provide accurate data regarding the safety and efficacy of these isomers. The increase in reactive oxygen species (ROS) production could be caused by one of the CLA isomers. Therefore, the effect of the CLA isomers alone on ROS production should be assessed using both the chemical and cellular antioxidant-based assays.

The blood used for the evaluation on erythrocyte function and morphology was collected from healthy individuals, however these products are often used by patients that are obese and have T2DM. Future toxicity studies can be done in both populations, where whole blood from these patients can be used to determine if ephedrine, CLA, L-carnitine or HCA causes erythrocyte haemolysis or adversely affects blood coagulation. In studies with *Garcinia* extracts and the present study shows that HCA does not have any beneficial effects it would be worthwhile in future studies to evaluate the proanthrocyanidins and other flavonoids in *Garcinia* to determine if they have any weight loss properties.

In conclusion, at the concentrations evaluated ephedrine and CLA showed some toxicity while no toxicity, adverse or beneficial effects were observed for L-carnitine and HCA. This indicates that although not toxic, these OTC weight loss compounds have limited beneficial effects.

Chapter 7: References

Abdoli N, Azarmi Y & Eghbal M. 2015. Mitigation of statins-induced cytotoxicity and mitochondrial dysfunction by L-carnitine in freshly-isolated rat hepatocytes. Research in Pharmaceutical Sciences 10(2): 143-150.

Achenef M & Arifah A. 2012. Cytotoxic effects of conjugated linoleic acids on human breast cancer cells (MCF7). Academic Journal Cancer Research 5(1): 11-16.

Alabi QK, Akomolafe RO, Olukiran OS, Nafiu AO, Omole JG, Adefisayo AM & Oladele AA. 2017. Assessment of haematological and biochemical effects of kolaviron in male Wistar rats. British Journal of Pharmaceutical Research 16(3): 1-14.

Adams RL & Bird RJ. 2009. Coagulation cascade and therapeutics update: relevance to nephrology. Part 1: overview of coagulation, thrombophilias and history of anticoagulants. Nephrology 14(5): 462-470.

Alam MN, Bristi NJ & Rafiquzzaman M. 2013. Review on *in vivo* and *in vitro* methods evaluation of antioxidant activity. Saudi Pharmaceutical Journal 21(2): 143-152.

Al-Gareeb AIA & Al-kuraishy HM. 2011. Effects of *Garcinia cambogia* on blood viscosity: a randomized, double-blind, placebo-controlled study. Kufa Medical Journal 14(1): 161-165.

Ali A & Crowther N. 2009. Factors predisposing to obesity: a review of the literature. Journal of Endocrinology, Metabolism and Diabetes of South Africa 14(2): 81-84.

Ali AT & Crowther NJ. 2010. Factors predisposing to obesity: a review of the literature. South African Family Practice 52(3): 193-197.

Ali YM, Kadir AA, Ahmad Z, Yaakub H, Zakaria ZA & Hakim Abdullah MN. 2012. Free radical scavenging activity of conjugated linoleic acid as single or mixed isomers. Pharmaceutical Biology 50(6): 712-719.

Al-kuraishy HM, Al-Mgoter BSN & Al-Gareeb AIA. 2013. Dual effects of aspirin and *Garcinia cambogia* on human blood viscosity. Zanco Journal of Medical Sciences 17(3): 514-517.

Arduini A, Rossi M, Mancinelli G, Belfiglio M, Scurti R, Radatti GL & Shohet SB. 1990. Effect of L-carnitine and acetyl-L-carnitine on the human erythrocyte membrane stability and deformability. Life Sciences 47(26): 2395-2400.

Arterburn DE, Crane PK, Veenstra DL. 2004. The efficacy and safety of sibutramine for weight loss: a systemic review. Archives International Medical 164(1): 994-1003.

Al-Rimawi F, Abu-Lafi S, Abbadi J, Alamarneh AA, Sawahreh RA & Odeh I. 2017. Analysis of phenolic and flavonoids of wild Ephedra alata plant extracts by LC/PDA and LC/MS and their antioxidant activity. African Journal of Traditional, Complementary, and Alternative Medicines 14(2): 130-141.

Amin KA, Kamel HH & Eltawab MAA. 2011. Protective effect of *Garcinia* against renal oxidative stress and biomarkers induced by high fat and sucrose diet. Lipids in Health and Disease 10(1): 6-12.

Andreoli, MF Illesca FG, González MA & Bernal CA. 2010. Conjugated linoleic acid reduces hepatic steatosis and restores liver triacylglycerol secretion and the fatty acid profile during protein repletion in rats. Lipids 45:1035–1045.

Arduini A, Holme S, Sweeney J, Dottori S, Sciarroni A & Calvani M. 1997. Addition of L-carnitine to additive solution-suspended red cells stored at 4 degrees Celsius reduces *in vitro* haemolysis and improves *in vivo* viability. Transfusion 37(2): 166-174.

Armstrong M, Lambert M, Sharwood K & Lambert E. 2006. Obesity and overweight in South African primary school children - the health of the nation study. South African Medical Journal 96(5): 439-444.

Baraldi FG, Vicentini TM, Teodoro BG, Dalalio FM, Dechandt CRP, Prado LMR, Cardoso FC, Uyemura SA, Alberici LC. 2016. The combination of conjugated linoleic acid (CLA) and extra virgin olive oil increases mitochondrial and body metabolism and prevents CLA-associated insulin resistance and liver hypertrophy in C57Bl/6. The Journal of Nutritional Biochemistry 28: 147-154.

Barile Frank AFA. 1997. Continuous cell lines as a model for drug toxicity assessment. *In Vitro* Methods in Pharmaceutical Research 1: 33-39.

Basiricò L, Morera P, Dipasquale D, Tröscher A & Bernabucci U. 2017. Comparison between conjugated linoleic acid and essential fatty acids in preventing oxidative stress in bovine mammary epithelial cells. Journal of Dairy Science 100(3): 2299-2309.

Benito P, Nelson G, Kelley D, Bartolini G, Schmidt P & Simon V. 2001. The effect of conjugated linoleic acid on platelet function, platelet fatty acid composition, and blood coagulation in humans. Lipids 36(3): 221-227.

Benjamin S, Prakasan P, Sreedharan S, Wright AG, Spener F. 2015. Pros and cons of CLA consumption: an insight from clinical evidences. Nutrition & Metabolism 12(4): 1-20.

Bernas T & Dobrucki J. 2002a. Mitochondrial and nonmitochondrial reduction of MTT: interaction of MTT with TMRE, JC-1, and NAO mitochondrial fluorescent probes. Cytometry 47(4): 236-242.

Bernas T & Dobrucki J. 2002b. Mitochondrial and nonmitochondrial reduction of MTT: interaction of MTT with TMRE, JC-1, and NAO mitochondrial fluorescent probes. Cytometry Part A 47(4): 236-242.

Blokhin IO & Lentz SR. 2013. Mechanisms of thrombosis in obesity. Current Opinion in Haematology 20(5): 437-444.

Boozer C, Nasser J, Heymsfield S, Wang V, Chen G & Solomon J. 2001. An herbal supplement containing Ma Huang-Guarana for weight loss: a randomized, double-blind trial. International Journal of Obesity 25(3): 316-324.

Boucher JL, Shafer KJ & Chaffin JA. 2001. Weight loss, diets, and supplements: does anything work? Diabetes Spectrum 14(3): 169-175.

Boschi F, Rizzatti V, Zamboni M & Sbarbati A. 2014. Lipid droplets fusion in adipocyte differentiated 3T3-L1 cells: a Monte Carlo simulation. Experimental Cell Research 321(2): 201-2018.

Brandsch C & Eder K. 2002. Effect of L-carnitine on weight loss and body composition of rats fed a hypocaloric diet. Annals of Nutrition and Metabolism 46(5): 205-210.

Boyacioglu M, Turgut H, Akgullu C, Eryilmaz U, Kum C, Onbasili OA. 2014. The effect of L-carnitine on oxidative stress responses of experimental contrast-induced nephropathy in rats. The Journal of Veterinary Medical Science 76(1): 1-8.

Burdge GC, Derrick PR, Russell JJ, Tricon S, Kew S, Banerjee T, Grimble RF, Williams CM, Yaqoob P & Calder PC. 2005. Incorporation of *cis*-9, *trans*-11 or *trans*-10, *cis*-12 conjugated linoleic acid into human erythrocytes *in vivo*. Nutrition Research 25(1): 13-19.

Buys AV, Van Rooy M-J, Soma P, Van Papendorp D, Lipinski B & Pretorius E. 2013. Changes in red blood cell membrane structure in type 2 diabetes: a scanning electron and atomic force microscopy study. Cardiovascular Diabetology 12(1): 25-31.

Canbolat EP, Sağsöz N, Noyan V, Yücel A & Kısa Ü. 2017. Effects of L-carnitine on oxidative stress parameters in oophorectomized rats. Alexandria Journal of Medicine 53(1): 55-60.

Carey AL, Formosa MF, Van Every B, Bertovic D, Eikelis N, Lambert GW, Kalff V, Duffy SJ, Cherk MH, Kingwell BA. 2013. Ephedrine activates brown adipose tissue in lean but not obese humans. Diabetologia 56(1): 147-155.

Carlson BM. 2019. Chapter 10: The circulatory system. The human body: Linking structure and Function. Academic Press 271-301.

Cavaliere H, Floriano I & Medeiros-Neto G. 2001. Gastrointestinal side effects of orlistat may be prevented by concomitant prescription of natural fibers (psyllium mucilloid). International Journal of Obesity & Related Metabolic Disorders 25(7): 1-10.

Cedikova M, Kripnerová M, Dvorakova J, Pitule P, Grundmanova M, Babuska V, Mullerova D & Kuncova J.2016. Mitochondria in white, brown and beige adipocytes. Stem Cells International: 1(1): 1-11.

Cha YS. 2008. Effects of L-carnitine on obesity, diabetes, and as an ergogenic aid. Asia Pacific Journal of Clinical Nutrition 17(S1): 306-308.

Chen C, Yang F, Zhang Q, Wang F, Hu Y, Xia Z. 2015. Natural products for antithrombosis. Evidence-Based Complementary and Alternative Medicine 1(1): 1-17.

Chinnadurai KHK, Kanwal AK, Tyagi CS & Ross P. 2013. High conjugated linoleic acid enriched ghee (clarified butter) increases the antioxidant and antiatherogenic potency in female Wistar rats. Lipids Health Disorder 12:121-124.

Christianson JL, Nicoloro S, Straubhaar J & Czech MP. 2008. Stearoyl-CoA desaturase 2 is required for peroxisome proliferator-activated receptor γ expression and adipogenesis in cultured 3T3-L1 cells. Journal of Biological Chemistry 283(5): 2906-2916.

Cigolini M, Targher G, Andreis IB, Tonoli M, Agostino G & De Sandre G. 1996. Visceral fat accumulation and its relation to plasma haemostatic factors in healthy men. Arteriosclerosis, Thrombosis, and Vascular Biology 16(3): 368-374.

Clouatre DL & Preuss HG. 2013. Hydroxycitric acid does not promote inflammation or liver toxicity. World Journal of Gastroenterology 19(44): 8160-8162.

Cois A & Day C. 2015. Obesity trends and risk factors in the South African adult population. BMC Obesity 2(1): 42-51.

Cooper MB, Forte CA & Jones DA. 1988. Carnitine and acetylcarnitine in red blood cells. Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism 959(2): 100-105.

Correcirc AD, Prete PSC, Cesar PHS & de Moura Oliveira CH. 2014. Pharmacotoxic evaluation of extracts of medicinal plants used in the treatment of obesity. African Journal Pharmacy and Pharmacology 8(43): 1103-1109.

Cui J, Bai Y, Lin C. 2017. Influences of different intensities exercise combined with conjugated linoleic acid on resting metabolic rate of adolescent rats. Sichuan Sports Science 1: 8-16.

Cusack S, Jewell C & Cashman K. 2005. The effect of conjugated linoleic acid on the viability and metabolism of human osteoblast-like cells. Prostaglandins, Leukotrienes and Essential Fatty Acids 72(1): 29-39.

Darbre PD. 2017. Endocrine disruptors and obesity. Current Obesity Reports 6(1): 18-27.

Dare S, Mackay DF, Pell JP. 2015. Relationship between smoking and obesity: a cross-sectional study of 499, 504 middle-aged adults in the UK general population. PloS One 10(4): 1-12.

Davie EW, Fujikawa K & Kisiel W. 1991. The coagulation cascade: initiation, maintenance, and regulation. Biochemistry 30(43): 10363-10370.

De Meyer SF, Deckmyn H & Vanhoorelbeke K. 2009. von Willebrand factor to the rescue. Blood 113(21): 5049-5057.

Den Hartigh LJ, Han CY, Wang S, Omer M, Chait A. 2013. 10E, 12Z-conjugated linoleic acid impairs adipocyte triglyceride storage by enhancing fatty acid oxidation, lipolysis and mitochondrial reactive oxygen species. Journal of Lipid Research 1(1): 1-40.

De Pauw A, Tejerina S, Raes M, Keijer J & Arnould T. 2009. Mitochondrial dysfunction in adipocyte differentiation and systemic metabolic alterations. The American Journal of Pathology 175(3): 927-939.

De Pergola G, De Mitrio V, Giorgino F, Sciaraffia M, Minenna A, Di Bari L, Pannacciulli N & Giorgino R. 1997a. Increase in both pro-thrombotic and anti-thrombotic factors in obese premenopausal women: relationship with body fat distribution. International Journal of Obesity 21(7): 527-535.

De Pergola G, De Mitrio V, Sciaraffia M, Pannaccuilli N, Minenna A, Giorgino F, Petronelli M, Laudadio E & Giorgino R. 1997b. Lower androgenicity is associated with higher plasma levels of prothrombotic factors irrespective of age, obesity, body fat distribution, and related metabolic parameters in men. Metabolism 46(11): 1287-1293.

De Pergola G & Pannacciulli N 2002. Coagulation and fibrinolysis abnormalities in obesity. Journal of Endocrinological Investigation 25(10): 899-904.

Den Hartigh LJ, Han CY, Wang S, Omer M & Chait A. 2013. 10E, 12Z-conjugated linoleic acid impairs adipocyte triglyceride storage by enhancing fatty acid oxidation, lipolysis, and mitochondrial reactive oxygen species. Journal of Lipid Research 1(1): 1-40.

Diepvens K, Westerterp KR & Westerterp-Plantenga MS. 2007. Obesity and thermogenesis related to the consumption of caffeine, ephedrine, capsaicin, and green tea. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 292(1): 77-85.

Ding ST, McNeel RL & Mersmann HJ. 2000. Conjugated linoleic acid increases the differentiation of porcine adipocytes *in vitro*. Nutrition Research 20(11): 1569-1580.

Dragunow M, Cameron R, Narayan P & O'Carroll S. 2007. Image-based high-throughput quantification of cellular fat accumulation. Journal of Biomolecular Screening 12(7): 999-1005.

Dwyer J, Coates P & Smith M. 2018. Dietary supplements: regulatory challenges and research resources. Nutrients 10(1): 1-24.

Egras AM, Hamilton WR, Lenz TL & Monaghan MS. 2010. An evidence-based review of fat modifying supplemental weight loss products. Journal of Obesity 1(1): 1-7.

Endres LK, Straub H, McKinney C, Plunkett B, Minkovitz CS, Schetter CD, Ramey S, Wang C, Hobel C, Raju T. 2015. Postpartum weight retention risk factors and relationship to obesity at one year. American Journal Obstetrics and Gynaecology 125(1): 144-152.

Erdogan Z & Kurtuncu M. 2018. Use of dietary supplements for weight loss in obese patients in Zonguldak, Turkey. African Journal of Traditional, Complementary and Alternative Medicines. 15(1): 59-65.

Evans BC, Nelson CE, Shann SY, Beavers KR, Kim AJ, Li H, Nelson HM, Giorgio TD & Duvall CL. 2013. *Ex vivo* red blood cell haemolysis assay for the evaluation of pH-responsive endosomolytic agents for cytosolic delivery of biomacromolecular drugs. Journal of Visualized Experiments 73: 1-5.

Farahzadi R, Mesbah-Namin SA, Zarghami N & Fathi E. 2016. L-carnitine effectively induces hTERT gene expression of human adipose tissue-derived mesenchymal stem cells obtained from the aged subjects. International Journal of Stem Cells 9(1): 107-114.

Fagali N & Catala A. 2008. Antioxidant activity of conjugated linoleic acid isomers, linoleic acid and its methyl ester determined by photoemission and DPPH techniques. Biophysical Chemistry 137(1): 56-62.

Feng S, Reuss L & Wang Y. 2016. Potential natural products in the inhibition of adipogenesis through regulation of PPARγ expression and/or its transcriptional activity. Molecules 21(10): 1-19.

Filozof C, Pinilla F & Fernández-Cruz A. 2004. Smoking cessation and weight gain. Obesity Reviews 5(2): 95-103.

Flordal PA & Svensson J. 1992. Haemostatic effects of ephedrine. Thrombosis Research 68(3): 295-302.

Fritz IB. 1959. Action of carnitine on long chain fatty acid oxidation by liver. American Journal of Physiology 197(2): 297-304.

Fontanarosa PB, Rennie D & Deangelis CD. 2003. The need for regulation of dietary supplements—lessons from ephedra. Journal of the American Medical Association 289(12): 1568-1570.

Food & Drug Administration. 1997. Dietary supplements containing ephedrine alkaloids: proposed rule. Federal Register (National Archives Records Service, Office of the Federal Register) 62(017): 30678-30685.

Fukushima K. 2004. Bioactivity of Ephedra: integrating cytotoxicity assessment with real-time biosensing 1(1): 1-67.

Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M & Shimomura I. 2017. Increased oxidative stress in obesity and its impact on metabolic syndrome. The Journal of Clinical Investigation 114(12): 1752-1761.

Ganapathy T. 2019. Excessive gestational weight retention and weight gain in postpartum: perception of women. Indian Journal of Health Sciences and Biomedical Research 12(1): 28-34.

Gerlach G, Loeber S, Herpertz S. 2016. Personality disorders and obesity: a systemic review. Obesity Reviews 17(8): 961-723.

Ghosh I & Mukherjee A. 2017. *In vitro* cyto-genotoxicity of hydroxycitric acid: a weight-loss dietary supplement. Journal of Exploratory Research in Pharmacology 2(2): 41-48.

Gothoskar AV. 2004. Resealed erythrocytes: a review. Pharmaceutical Technology 28(3): 140-155.

Goudarzvand M, Afraei S, Yaslianifard S, Ghiasy S, Sadri G, Kalvandi M, Alinia T, Mohebbi A, Yazdani R & Azarian SK. 2016. Hydroxycitric acid ameliorates inflammation and oxidative stress in mouse models of multiple sclerosis. Neural Regeneration Research 11(10): 1610-1616.

Gul R, Jan SU, Faridullah S, Sherani S & Jahan N. 2017. Preliminary phytochemical screening, quantitative analysis of alkaloids, and antioxidant activity of crude plant extracts from ephedra intermedia Indigenous to balochistan. The Scientific World Journal: 1(1): 1-7.

Gülcin İ. 2006. Antioxidant and antiradical activities of L-carnitine. Life Sciences 78(8): 803-811.

Ha Y, Grimm N & Pariza M. 1987. Anticarcinogens from fried ground beef: heat-altered derivatives of linoleic acid. Carcinogenesis 8(12): 1881-1887.

Hakeshzadeh F, Tabibi H, Ahmadinejad M, Malakoutian T & Hedayati M. 2010. Effects of L-carnitine supplement on plasma coagulation and anticoagulation factors in haemodialysis patients. Renal Failure 32(9): 1109-1114.

Haller CA & Benowitz NL. 2000. Adverse cardiovascular and central nervous system events associated with dietary supplements containing ephedra alkaloids. New England Journal of Medicine 34(25): 1833-1838.

Haller CA, Jacob P, Benowitz NL. 2005. Short-term metabolic and hemodynamic effects of ephedra and guarana combinations. Clinical Pharmacology & Therapeutics 77(6): 560-571.

Hardy OT, Czech MP & Corvera S. 2012. What causes the insulin resistance underlying obesity? Current Opinion in Endocrinology, Diabetes and Obesity 19(2): 81-87.

Hassan A, Hawa S, Fry JR, Bakar A & Fadzelly M. 2013. Phytochemicals content, antioxidant activity and acetylcholinesterase inhibition properties of indigenous *Garcinia parvifolia* fruit. Biomedical Research International 1(1): 1-8.

Hayamizu K, Ishii Y, Kaneko I, Shen M, Okuhara Y, Shigematsu N, Tomi H, Furuse M, Yoshino G & Shimasaki H. 2003. Effects of *Garcinia cambogia* (hydroxycitric acid) on visceral fat accumulation: a double-blind, randomized, placebo-controlled trial. Current Therapeutic Research 64(8): 551-567.

Hyuga S, Hyuga M, Oshima N, Maruyama T, Kamakura H, Yamashita T, Yoshimura M, Amakura Y, Hakamatsuka T, Odaguchi H, Goda Y & Hanawa T. 2016. Ephedrine alkaloids-free ephedra herb extract: a safer alternative to ephedra with comparable analgesic, anticancer, and anti-influenza activities. Journal of Natural Medicines 70(3): 571-583.

Howard BV, Van Horn L, Manson JE, Stefanick ML, Wassertheil-Smoller S, Kuller LH, LaCroix AZ, Langer RD & Lesser NL. 2006. Low-fat dietary pattern and risk of cardiovascular disease: the women's health initiative randomized controlled dietary modification trial. Journal of the American Medical Association 295(6): 655-666.

Hu S, Wang L, Yang D, Li L, Togo J, Wu Y, Liu Q, Li B, Li M, Wang G. 2018. Dietary fat, but not protein of carbohydrate, regulates energy intake and causes adiposity in mice. Cell Metabolism 28(3): 415-431.

Iga T, Satoh T, Yamamoto S, Fukui K, Song S, Choi K, Roh S & Sasaki S. 2009. Differential action of trans-10, *cis*-12 conjugated linoleic acid on adipocyte differentiation of ovine and 3T3-L1 preadipocytes. Asian-Australasian Journal of Animal Sciences 22(11): 1566-1573.

Jain S & Singh SN. 2015. Effect of L-carnitine supplementation on nutritional status and physical performance under calorie restriction. Indian Journal of Clinical Biochemistry 30(2): 187-193.

Jang A, Kim D, Sung KS, Jung S, Kim HJ & Jo C. 2014. The effect of dietary α-lipoic acid, betaine, L-carnitine, and swimming on the obesity of mice induced by a high-fat diet. Food & Function 5(8): 1966-1974.

Jena B, Jayaprakasha G, Singh R & Sakariah K. 2002. Chemistry and biochemistry of (−)-hydroxycitric acid from *Garcinia*. Journal of Agricultural and Food Chemistry 50(1): 10-22.

Kadidrareddy RH, Mathakala V & Palempalli UMD. 2016. Therapeutic activity of conjugated linoleic acids synthesized by lactobacillus plantarum. International Journal of Pharmacological Biological Science 7(4): 215-223.

Kallassy H, Fayyad-Kazan M, Makki R, El-Makhour Y, Rammal H, Leger DY, Sol V, Fayyad-Kazan H, Liagre B & Badran B. 2017. Chemical composition and antioxidant, anti-Inflammatory, and antiproliferative activities of lebanese ephedra campylopoda plant. Medical Science Monitor Basic Research 23: 313-325.

Kammlott G. 1971. Some aspects of scanning electron microscopy. Surface Science 25(1): 120-146.

Kang ES, Ham SA, Hwang JS, Lee CK & Seo HG. 2013. Effects of *Garcinia cambogia* extract on the adipogenic differentiation and lipotoxicity. Korean Journal for Food Science of Animal Resources 33(3): 411-416.

Kang MJ. 2018. The adiposity rebound in the 21st century children: meaning for what? Korean Journal of Pediatrics 61(12): 375-380.

Karlic H & Lohninger A. 2004. Supplementation of L-carnitine in athletes: does it make sense? Nutrition 20(7): 709-715.

Kaufman A, Augustson EM & Patrick H. 2012. Unraveling the relationship between smoking and weight: the role of sedentary behaviour. Journal of Obesity 1(1): 1-11.

Keaney JF, Larson MG, Vasan RS, Wilson PW, Lipinska I, Corey D, Massaro JM, Sutherland P, Vita JA & Benjamin EJ. 2003. Obesity and systemic oxidative stress. Arteriosclerosis, Thrombosis, and Vascular Biology 23(3): 434-439.

Kennedy A, Martinez K, Schmidt S, Mandrup S, Lapoint K & Mcintosh M. 2010. Anti-obesity mechanisms of action of conjugated linoleic acid. The Journal of Nutritional Biochemistry 21(3): 171-179.

Khan A, Jan G, Khan A, Gul Jan F, Bahadur A & Danish M. 2017. *In vitro* antioxidant and antimicrobial activities of ephedra gerardiana (root and stem) crude extract and fractions. Evidence-Based Complementary and Alternative Medicine 1-7.

Kim HJ, Park JM, Kim JA & Ko BP. 2008. Effect of herbal ephedra sinica and evodia rutaecarpa on body composition and resting metabolic rate: a randomized, double-blind clinical trial in Korean premenopausal women. Journal of Acupuncture and Meridian Studies 1(2): 128-138.

Kim YJ, Choi M, Park YB, Kim SR, Lee M, Jung UJ. 2013. *Garcinia cambogia* attenuates diet-induced adiposity but exacerbates hepatic collagen accumulation and inflammation. World Journal of Gastroenterology 19(29): 4689-4701.

Kolodziejczyk J, Saluk-Juszczak J & Wachowicz B. 2011. L-Carnitine protects plasma components against oxidative alterations. Nutrition 27(6): 693-699.

Kong S, Ding C, Huang L, Bai Y, Xiao T, Guo J & Su Z. 2017. The effects of COST on the differentiation of 3T3-L1 preadipocytes and the mechanism of action. Saudi Journal of Biological Sciences 24(2): 251-255.

Lahmann P, Lissner L, Gullberg B & Berglund G. 2000. Sociodemographic factors associated with long-term weight gain, current body fatness and central adiposity in Swedish women. International Journal of Obesity 24(6): 685-694.

Laitinen J, Power C & Järvelin MR. 2001. Family social class, maternal body mass index, childhood body mass index, and age at menarche as predictors of adult obesity. The American Journal of Clinical Nutrition 74(3): 287-294.

Lawlor DA & Chaturvedi N. 2016. Treatment and prevention of obesity-are there critical periods for intervention? Oxford University Press 35: 3-9.

Le Lay S, Simard G, Martinez MC & Andriantsitohaina R. 2014. Oxidative stress and metabolic pathologies: from an adipocentric point of view. Oxidative medicine and cellular longevity 1(1): 1-18.

Lee AY, Jang Y, Hong SH, Chang SH, Park S, Kim S, Kang KS, Kim JE & Cho MH. 2017. Ephedrine-induced mitophagy via oxidative stress in human hepatic stellate cells. The Journal of Toxicological Sciences 42(4): 461-473.

Lehnen TE, Da Silva MR, Camacho A, Marcadenti A & Lehnen AM. 2015. A review on effects of conjugated linoleic fatty acid (CLA) upon body composition and energetic metabolism. Journal of the International Society of Sports Nutrition 12(1): 36-46.

Leung YH & Liu RH. 2000. *Trans*-10, *cis*-12-conjugated linoleic acid isomer exhibits stronger oxyradical scavenging capacity than *cis*-9, *trans*-11-conjugated linoleic acid isomer. Journal of Agricultural and Food Chemistry 48(11): 5469-5475.

Lui RH & Finley J. 2005. Potential cell culture models for antioxidant research. Journal of Agriculture and Food Chemistry 53(10): 4311-4314.

Li L, Peng M, Ge C, Yu L & Ma H. 2017. (-)-Hydroxycitric acid reduced lipid droplets accumulation via decreasing acetyl-CoA supply and accelerating energy metabolism in cultured primary chicken hepatocytes. Cellular Physiology and Biochemistry 43: 812-831.

Lipowicz A. 2003. Effect of husbands' education on fatness of wives. American Journal of Human Biology 15(1): 1-7.

Liu RH & Finley J. 2005. Potential cell culture models for antioxidant research. Journal of Agricultural and Food Chemistry 53(10): 4311-4314.

Liu AG, Smith SR, Fujioka K & Greenway FL. 2013. The effect of leptin, caffeine/ephedrine, and their combination upon visceral fat mass and weight loss. Obesity 21(10): 1991-1996.

Longo N, Frigeni M & Pasquali M. 2016. Carnitine transport and fatty acid oxidation. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 1863(10): 2422-2435.

López-Alarcón C & Denicola A. 2013. Evaluating the antioxidant capacity of natural products: a review on chemical and cellular-based assays. Analytica Chimica Acta 763: 1-10.

Maddux BA, See W, Lawrence JC, Goldfine AL, Goldfine ID & Evans JL. 2001. Protection against oxidative stress—induced insulin resistance in rat L6 muscle cells by micromolar concentrations of α-lipoic acid. Diabetes 50(20): 404-410.

Mahmoud GS & Amer AS. 2013. Effect of natural extract of hydroxycitric acid on lipid profile, liver and testes of adult male rats. Asian Academy of Management Journal 11(1): 162-182.

Malchow-Møller A, Larsen S, Hey H, Stokholm KH, Juhl E & Quaade F. 1981. Ephedrine as an anorectic: the story of the 'Elsinore pill'. International Journal of Obesity 5(2): 183-187.

Malik MAA, Ali MF, Muhammad NA. 2019. The use of weight loss products among overweight and obese patients in Malaysia. Malaysian Journal of Medicine and Health Sciences 15: 23-30.

Marcadenti A & Coelho RCLA 2015. Dietary Antioxidant and Oxidative Stress: Interaction between vitamins and genetics. Journal of Nutrition Health Food Science 3: 1-7.

Manna P & Jain SK. 2015. Obesity, oxidative stress, adipose tissue dysfunction, and the associated health risks causes and therapeutic strategies. Metabolic Syndrome and Related Disorders 13(10): 423-444.

Márquez F, Babio N, Bulló M & Salas-Salvadó J 2012. Evaluation of the safety and efficacy of hydroxycitric acid or *Garcinia cambogia* extracts in humans. Critical Reviews in Food Science and Nutrition 52: 585-594.

Maslak E, Buczek, E, Szumny A, Szczepnski, W, Franczyk-Zarow M, Kopec A, Chlopicki S, Leszczynska T, Kostogrys RB. 2015. Individual CLA isomers, c9t11 and t10c12, prevent excess liver glycogen storage and inhibit lipogenic genes expression induced by high-fructose diet in rats. BioMed Research International 1(1): 1-10.

Marseglia L, Manti S, D'angelo G, Nicotera A, Parisi E, Di Rosa G, Gitto E & Arrigo T. 2015. Oxidative Stress in Obesity: A Critical Component in Human Diseases. International Journal of Molecular Sciences 16(1)**:** 378-400.

Martikainen PT & Marmot MG. 1999. Socioeconomic differences in weight gain and determinants and consequences of coronary risk factors. The American Journal of Clinical Nutrition, 69(4): 719-726.

Martins SV, Pires VM, Madeira AP, Nascimento M, Alfaia CM, Castro MF, Soveral G, Prates JA & Lopes PA. 2017. Novel anti-adipogenic properties of the individual *trans*8, *cis*10 conjugated linoleic acid (CLA) isomer in 3T3-L1 adipocytes. European Journal of Lipid Science and Technology 119(2): 1600042-1600052.

Meadus WJ, Vahmani, P, Duff P, Zantinge JL, Turner TD, Dugan MER. 2017. Oxidation and apoptosis are induced by CLA-t10c12 similar to DHA in 3T3 adipocyte cells. Functional Foods in Health & Disease 7(2): 149-167.

Mehlem A, Hagberg CE, Muhl L, Eriksson U & Falkevall A. 2013. Imaging of neutral lipids by oil red O for analyzing the metabolic status in health and disease. Nature Protocols 8(6): 1149-1154.

Melaku A, Kadir AA, Othman F, Meng GY & Sazili AQ. 2012. Cytotoxic effects of conjugated linoleic acids on human hepatoma cancer cells (HepG2). Journal of Molecular Pathophysiology 1(1): 43-48.

Mertens I & Van Gaal L. 2002. Obesity, haemostasis and the fibrinolytic system. Obesity Reviews 3(2): 85-101.

Meydani M, Hasan ST. 2010. Dietary polyphenols and obesity. Nutrients 2: 737-751.

McMurray F, Patten DA & Harper ME. 2016. Reactive oxygen species and oxidative stress in obesity—recent findings and empirical approaches. Obesity 24(11): 2301-2310.

McNeel RL, Smith EB & Mersmann HJ. 2003. Isomers of conjugated linoleic acid modulate human preadipocyte differentiation. *In Vitro* Cellular & Developmental Biology Animal 39(8-9): 375-382.

McTaggart JS, Lee S, Iberl M, Church C, Cox RD, Ashcroft FM. 2011. FTO is expressed in neurons throughout the brain and its expression is unaltered by fasting. PLoS One 6(11): 27968-27968.

Micale V, Drago F, Elling CE, Wotjak CT. 2019. The cannabiod CB1 antagonist TM38837 with limited penetrance to the brain lacks fear-promoting effects in mice. Frontiers in Pharmacology 10: 207-211.

Miller JR, Siripurkpong P, Hawes J, Majdalawieh A, Ro H-S & Mcleod RS. 2008. The *trans*-10, *cis*-12 isomer of conjugated linoleic acid decreases adiponectin assembly by PPARγ-dependent and PPARγ-independent mechanisms. Journal of Lipid Research 49(3): 550-562.

Miller SC. 2004. Safety concerns regarding ephedrine-type alkaloid-containing dietary supplements. Military medicine 169(2): 87-91.

Millington GW. 2007. The role of proopiomelanocortin (POMC) neurones in feeding behaviour. Nutrition & Metabolism 4(1): 18-34.

Minakshi GC, Vasanth K, Tanupriya K, Kumar RM, Agrawal A, Dubey GP. 2015. Phytochemical evaluation and antioxidant potential of *Garcinia indica* fruits on H_2O_2 induced oxidative stress in THP-1 cell line. International Journal of Pharmacology 11(7): 672-680.

Mobarak H, Fathi E, Farahzadi R, Zarghami N & Javanmardi S. 2017. L-carnitine significantly decreased aging of rat adipose tissue-derived mesenchymal stem cells. Veterinary Research Communications 41(1): 41-47.

Mohamed GA, Ibrahim SR, Elkhayat ES & El Dine RS. 2014. Natural anti-obesity agents. Bulletin of Faculty of Pharmacy, Cairo University 52(2): 269-284.

Moosavi M, Rezaei M, Kalantari H, Behfar A & Varnaseri G. 2016. L-carnitine protects rat hepatocytes from oxidative stress induced by T-2 toxin. Drug and Chemical Toxicology 39(4): 445-450.

Mullen A, Moloney F, Nolan JJ, Yeow T-P & Roche HM. 2004. Conjugated linoleic acid supplementation, insulin sensitivity, and lipoprotein metabolism in patients with type 2 diabetes mellitus. The American Journal of Clinical Nutrition 80(4): 887-895.

Ness-Abramof R & Apovian CM. 2005. Drug-induced weight gain. Drugs of Today 41(8): 547-550.

Nieuwdorp M, Stroes ES, Meijers JC & Büller H. 2005. Hypercoagulability in the metabolic syndrome. Current Opinion in Pharmacology 5(2): 155-159.

Nikolaos S, George A, Telemachos T, Maria S, Yannis M & Konstantinos M. 2000. Effect of L-carnitine supplementation on red blood cells deformability in haemodialysis patients. Renal Failure 22(1): 73-80.

Nisha V, Priyanka A, Anusree S & Raghu K. 2014. (-)-Hydroxycitric acid attenuates endoplasmic reticulum stress-mediated alterations in 3T3-L1 adipocytes by protecting mitochondria and downregulating inflammatory markers. Free Radical Research 48(11): 1386-1396.

O'Connell TL. 2004. An overview of obesity and weight loss surgery. Clinical Diabetes 22(3): 115-120.

Oberholzer HM, Van Der Schoor C & Bester MJ. 2015. Sibutramine, a serotonin–norepinephrine reuptake inhibitor, causes fibrosis in rats. Environmental Toxicology and Pharmacology 40(1): 71-76.

Ohia SE, Awe SO, Leday AM, Opere CA & Bagchi D. 2001. Effect of hydroxycitric acid on serotonin release from isolated rat brain cortex. Research Communications in Molecular Pathology and Pharmacology 109(3-4): 210-216.

Okoko T. 2009. *In vitro* antioxidant and free radical scavenging activities of *Garcinia kola* seeds. Food and Chemical Toxicology 47(10): 2620-2623.

Oluyemi KA, Omotuyi IO, Jimoh OR, Adesanya OA, Saalu CL & Josiah SJ. 2007. Erythropoietic and anti-obesity effects of *Garcinia cambogia* (bitter kola) in Wistar rats. Biotechnology and applied biochemistry 46(1): 69-72.

Ou B, Huang D, Hampsch-Woodill M, Flanagan JA & Deemer EK. 2002. Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study. Journal of Agricultural and Food Chemistry 50(11): 3122-3128.

Ozsoy S, Ozsoy B, Ozyildiz Z & Aytekin I. 2011. Protective effect of L-carnitine on experimental lead toxicity in rats: a clinical, histopathological and immunohistochemical study. Biotechnic & Histochemistry 86(6): 436-443.

Page MJ, Bester J, Pretorius E. 2018. Interleukin-12 and its procaugulant effect on erythrocytes, platelets and fibrin(ogen): lesser known side of inflammation. British Journal Haematology 180(1): 110-117.

Palta S, Saroa R & Palta A. 2014. Overview of the coagulation system. Indian Journal of Anaesthesia 58(5): 515-520.

Pamukcu Gunaydin G, Dogan NO, Levent S & Kurtoglu Celik G. 2015. Herbal weight loss pill overdose: sibutramine hidden in pepper pill. Case Reports in Emergency Medicine: 1-4.

Parjapath R, Sali VK, Kannan SM, Kurapati A & Vasanthi HR 2018. Hydroxycitric acid-induced activation of peroxisome proliferator-activated receptors in 3T3-L1 adipocyte cells. Pharmacognosy Research 10(3): 275-281.

Pavelescu LA. 2015. On reactive oxygen species measurements in living systems. Journal of Medicine and Life 8(1): 38-42.

Peng ML, Han J, Li LL, Ma HT. 2018. Metabolomics reveals the mechanism of (−)-hydroxycitric acid promotion of protein synthesis and inhibition of fatty acid synthesis in broiler chickens. Animal 12(4): 774-783.

Pisoschi AM, Pop A, Cimpeanu C, Predoi G. 2016. Antioxidant capacity determination in plants and plant-derived products: a review. Oxidative Medicine and Cellular Longevity 1(1): 1-36.

Pignatelli P, Lenti L, Sanguigni V, Frati G, Simeoni I, Gazzaniga P, Pulcinelli FM & Violi F. 2003. Carnitine inhibits arachidonic acid turnover, platelet function, and oxidative stress. American Journal of Physiology Heart and Circulatory Physiology 284(1): H41-H48.

Pittler MH & Ernst E. 2004. Dietary supplements for body-weight reduction: a systematic review. The American Journal of Clinical Nutrition 79(4): 529-536.

Pooyandjoo M, Nouhi M, Shab-Bidar S, Djafarian K & Olyaeemanesh A. 2016. The effect of (L-) carnitine on weight loss in adults: a systematic review and meta-analysis of randomized controlled trials. Obesity Reviews 17(10): 970-976.

Priyadhardhini A, Ahalya SP, Vaishnavi P, Pavithra S & Rosario AR. 2019. A review on benefits and toxicity of orlistat therapy. Drug Invention Today 12(3): 1-10.

Puoane T, Steyn K, Bradshaw D, Laubscher R, Fourie J, Lambert V & Mbananga N. 2002. Obesity in South Africa: the South African demographic and health survey. Obesity 10(10): 1038-1048.

Qi R, Yang F, Huang J, Peng H, Liu Y & Liu Z. 2014. Supplementation with conjugated linoleic acid decreases pig back fat deposition by inducing adipocyte apoptosis. BMC Veterinary Research 10(1): 141-148.

Raatz S, Conrad Z, Johnson L, Picklo M, Jahns L. 2017. Relationship of the reported intakes of fats and fatty acids to body weight in US adults. Nutrients 9(5): 438-450.

Radakovic M, Djelic N, Plecas-Solarovic B, Spremo-Potparevic B, Zivkovic L & Bajic V. 2011. Evaluation of the effects of ephedrine on human lymphocytes in the comet assay. Acta Veterinaria 61(4): 363-371.

Rainer L & Heiss CJ. 2004. Conjugated linoleic acid: health implications and effects on body composition. Journal of the Academy of Nutrition and Dietetics 104(6): 963-968.

Ramiah KS, Deepak B, Naveen B, Panneerselvam C. 2003. Effects of levocarnitine on mitochondrial antioxidant systems and oxidative stress in aged rats. Drugs R&D 4: 141–147.

Ramiah KS, Meng GY, Sheau Wei T, Swee Keong Y, Ebrahimi M. 2014. Dietary conjugated linoleic acid supplementation leads to downregulation of PPAR transcription in broiler chickens and reduction of adipocyte cellularity. Peroxisome Proliferator-Activated Receptor Research 1: 1-11.

Ramiah KS, Meng GY, Keong YS, Ebrahimi M & Tan SW. 2017. The effects of conjugated linoleic acid isomers on the morphological changes in adipose tissue and adipogenic genes expressions on primary adipose tissue. Italian Journal of Animal Science 16(2): 253-258.

Ramot Y & Nyska A. 2007. Drug-induced thrombosis—experimental, clinical, and mechanistic considerations. Toxicologic Pathology 35(2): 208-225.

Ribeiro AS, Pina FLC, Dodero SR, Silva DR, Schoenfeld BJ, Junior PS, Fernandes RR *et al*. 2016. Effect of conjugated linoleic acid associated with aerobic exercise on body fat and lipid profile in obese women: a randomized, double-blinded, and placebo-controlled trial. International Journal of Sport Nutrition and Exercise Metabolism 26(2): 135-144.

Rios-Hoyo A & Gutierrez-Salmean G. 2016. New dietary supplements for obesity: What we currently know. Current Obesity Reports 5(2): 262-270.

Rossignoli CP, Dechandt CRP, Souza AO, Sampaio IH, Vicentini TM, Teodoro BG, Neto MPC, Ferrari GD, Couto-Lima CA, Alberici LC. 2018. Effects of intermittent dietary supplementation with conjugated linoleic acid and fish oil (EPA/DHA) on body metabolism and mitochondrial energetics in mice. The Journal Nutritional Biochemistry 60: 16-23.

Roy S, Shah H, Rink C, Khanna S, Bagchi D, Bagchi M & Sen CK. 2007. Transcriptome of primary adipocytes from obese women in response to a novel hydroxycitric acid–based dietary supplement. DNA and Cell Biology 26(9): 627-639.

Rudich A, Tirosh A, Potashnik R, Hemi R, Kanety H & Bashan N. 1998. Prolonged oxidative stress impairs insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. Diabetes 47(10): 1562-1569.

Ruiz-Ojeda F, Rupérez A, Gomez-Llorente C, Gil A & Aguilera C. 2016a. Cell models and their application for studying adipogenic differentiation in relation to obesity: A review. International journal of molecular sciences 17(7): 1040-1066.

Samad F & Ruf W. 2013. Inflammation, obesity, and thrombosis. Blood 122(20): 3415-3422.

Saper RB, Eisenberg DM & Phillips RS. 2004. Common dietary supplements for weight loss. American Family Physician 70: 1731-1740.

Sato K, Shinohara N, Honma T, Ito J, Arai T, Nosaka N, Aoyama T, Tsuduki T & Ikeda I. 2011. The change in conjugated linoleic acid concentration in blood of Japanese fed a conjugated linoleic acid diet. Journal of Nutritional Science and Vitaminology 57(5): 364-371.

Satory DL & Smith SB. 1999. Conjugated linoleic acid inhibits proliferation but stimulates lipid filling of murine 3T3-L1 preadipocytes. The Journal of Nutrition 129(1): 92-97.

Savini I, Catani MV, Evangelista D, Gasperi V & Avigliano L. 2013. Obesity-associated oxidative stress: strategies finalized to improve redox state. International Journal of Molecular Sciences 14(5): 10497-10538.

Scott MA, Nguyen VT, Levi B & James AW. 2011. Current methods of adipogenic differentiation of mesenchymal stem cells. Stem Cells and Development 20(10): 1793-1804.

Serra D, Mera P, Malandrino MI. 2013. Mitochondrial fatty acid oxidation in obesity. Antioxidant Redox Signal 9: 269–284.

Shahmirzadi FE, Ghavamzadeh S & Zamani T. 2019. The effect of conjugated linoleic acid supplementation on body composition, serum insulin and leptin on obese adults. Archives of Iranian Medicine 22(5): 255-261.

Sharma A, Joseph G & Singh R. 2014. Antioxidant and anti-platelet aggregation properties of bark extracts of Garcinia pedunculata and *Garcinia cowa.* Journal of Food Science and Technology 51(8): 1626-1631.

Sharma K, Kang S, Gong D, Oh SH, Park EY, Oak MH & Yi E. 2018. Combination of *Garcinia cambogia* extract and pear pomace extract additively suppresses adipogenesis and enhances lipolysis in 3T3-L1 cells. Pharmacognosy Magazine 14(54): 220-228.

Shekelle PG, Hardy ML, Morton SC, Maglione M, Mojica WA, Suttorp MJ, Rhodes SL, Jungvig L, Gagné J. 2003. Efficacy and safety of ephedra and ephedrine for weight loss and athletic performance: a meta-analysis. JAMA 289(12): 1537-1545.

Shen P, Kershaw JC, Yue Y, Wang O, Kim K, McClements DJ, Park Y. 2018. Effects of conjugated linoleic acid (CLA) on fat accumulation, activity, and proteomics analysis in Caenorhabditis elegans 249: 193-201.

Shrivastava A & Johnston ME. 2010. Weight-gain in psychiatric treatment: risks, implications, and strategies for prevention and management. Mens Sana Monographs 8(1): 53-59.

Siegner R, Heuser S, Holtzmann U, Söhle J, Schepky A, Raschke T, Stäb F, Wenck H & Winnefeld M. 2010. Lotus leaf extract and L-carnitine influence different processes during the adipocyte life cycle. Nutrition & Metabolism 7(1): 66-72.

Simon E, Macarulla M, Fernandez-Quintela A, Rodriguez V & Portillo M. 2005. Body fat-lowering effect of conjugated linoleic acid is not due to increased lipolysis. Journal of Physiology and Biochemistry 61(2): 363-369.

Skaal L & Pengpid S. 2011. Obesity and health problems among South African healthcare workers: do healthcare workers take care of themselves? South African Family Practice 53(6): 563-567.

Slocum N, Durrant JR, Bailey D, Yoon L, Jordan H, Barton J, Brown RH, Clifton L, Milliken T & Harrington W. 2013. Responses of brown adipose tissue to diet-induced obesity, exercise, dietary restriction and ephedrine treatment. Experimental and Toxicologic Pathology 65(5): 549-557.

So MH, Iris M & Li ET. 2009. Dietary fat concentration influences the effects of *trans*-10, *cis*-12 conjugated linoleic acid on temporal patterns of energy intake and hypothalamic expression of appetite-controlling genes in mice. The Journal of Nutrition 139(1): 145-151.

Sobal J & Stunkard AJ. 1989. Socioeconomic status and obesity: a review of the literature. Psychological Bulletin 105(2): 260-268.

Solal. 2019. Wellness warehouse, accessed 9 June 2019, https://www.wellnesswarehouse.com/brands/solal/

Solarska K, Lewińska A, Karowicz-Bilińska A & Bartosz G. 2010. The antioxidant properties of carnitine *in vitro*. Cellular & Molecular Biology Letters 15(1): 90-95.

Soumya R, Carl H & Vani R. 2016. L-carnitine as a potential additive in blood storage solutions: a study on erythrocytes. Indian Journal of Haematology and Blood Transfusion 32(3): 328-334.

Surai PF. 2015. Antioxidant action of carnitine: molecular mechanisms and practical applications. EC Veterinary Science 2(1): 66-84.

Terpstra AHM, Javadi M, Beynen AC, Kocsis S, Lankhorst AE, Lemmens AG, Mohede ICM. 2003. Dietary conjugated linoleic acids as free fatty acids and triacylglycerols similarly affect body composition and energy balance in mice. The Journal of Nutrition 133(10): 3181-3186.

Terpstra AHM. 2004. Effect of conjugated linoleic acid on body composition and plasma lipids in human: an overview of the literature. The American Journal of Clinical Nutrition 79(3): 352- 361.

Thiyam G. 2011. Mechanisms of lipid droplet formation by conjugated linoleic acid (CLA) isomers and its effects on cell viability 1-160.

Tobias DK, Chen M, Manson JE, Ludwig DS, Wilett W, Hu FB. 2015. Effect of low-fat diet interventions versus other diet interventions on long-term weight changes in adults: a systematic review and meta-analysis. The Lancet Diabetes & Endocrinology 3(12): 968-979.

Tomaiuolo G. 2014. Biomechanical properties of red blood cells in health and disease towards microfluidics. Biomicrofluidics 8(5): 51501-51505.

Toptas B, Baykal A, Yesilipek A, Isbir M, Kupesiz A, Yalcin O & Baskurt O. 2006. L-carnitine deficiency and red blood cell mechanical impairment in β-thalassemia major. Clinical Hemorheology and Microcirculation 35(3): 349-357.

Toubro S, Astrup A, Breum L & Quaade F. 1993. Safety and efficacy of long-term treatment with ephedrine, caffeine and an ephedrine/caffeine mixture. International journal of obesity and related metabolic disorders. Journal of the International Association for the Study of Obesity 17: S69-S72.

Tung CH, Han MS & Qi J. 2017. Total control of fat cells from adipogenesis to apoptosis using a xanthene analog. PloS One 12(6): e0179158-e0179162.

Turk MW, Yang K, Sereika SM, Ewing LJ, Burke LE. 2009. Randomized clinical trials of weight-loss maintenance: a review. Journal Cardiovascular Nursing 24(1): 58-80.

Ueno Y, Koike M, Shimada Y, Shimura H, Hira K, Tanaka R, Uchiyama Y, Hattori N, Urabe T. 2015. L-carnitine enhances axonal plasticity and improves white-matter lesions after chronic hypoperfusion in rat brain. Journal of Cerebral Blood Flow Metabolism 35:382–391.

Van Der Schoor C. 2015. An *in vivo* and *in ovo* evaluation of the toxicity of sibutramine: 1-198.

Van Der Schoor C, Oberholzer HM, Bester MJ & Van Rooy MJ. 2014. The effect of sibutramine, a serotonin-norepinephrine reuptake inhibitor, on platelets and fibrin networks of male Sprague-Dawley rats: a descriptive study. Ultrastructural Pathology 38(6): 399-405.

Van Kempen TH, Bogaerds AC, Peters GW & Van De Vosse FN. 2014. A constitutive model for a maturing fibrin network. Biophysical Journal 107(2): 504-513.

Van Meerloo J, Kaspers GJ & Cloos J. 2011. Cell sensitivity assays: the MTT assay. Cancer Cell Culture: Methods and Protocols: 237-245.

Varalakshmi K, Sangeetha C, Samee U, Irum G, Lakshmi H & Prachi S. 2011. *In vitro* safety assessment of the effect of five medicinal plants on human peripheral lymphocytes. Tropical Journal of Pharmaceutical Research 10(1): 33-40.

Vaughan Roger AR. 2014. Effects of commercially available dietary supplements on resting energy expenditure: a brief report. ISRN Nutrition 1(1): 1-7.

Wang H & Joseph JA. 1999. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radical Biology and Medicine 27(5): 612-616.

Wang P, Renes J, Bouwman F, Bunschoten A, Mariman E & Keijer J. 2007. Absence of an adipogenic effect of rosiglitazone on mature 3T3-L1 adipocytes: increase of lipid catabolism and reduction of adipokine expression. Diabetologia 50(3): 654-665.

Wang QA, Tao C, Gupta RK & Scherer PE. 2013. Tracking adipogenesis during white adipose tissue development, expansion and regeneration. Nature medicine 19(10): 1338-1342.

Wang Q, Ju X, Chen Y, Dong X, Luo S, Liu H & Zhang D. 2017. L-carnitine exerts a cytoprotective effect against H_2O_2-induced oxidative stress in the fathead minnow muscle cell line. Aquaculture Research 48(3): 941-954.

Wanic-Kossowska M, Kazmierski M, Pawliczak E & Kobelski M. 2007. Combined therapy with L-carnitine and erythropoietin of anaemia in chronic kidney failure patients undergoing haemodialysis. Polish Archives of Internal Medicine 117(1-2): 14-19.

Watanabe T, Tsujino I, Konno S, Ito YM, Takashina C, Sato T, Isada A, Ohira H, Ohtsuka Y & Fukutomi Y. 2016. Association between smoking status and obesity in a nationwide survey of Japanese adults. PloS One 11(3): 1-14.

Watson JA & Lowenstein JM. 1970. Citrate and the conversion of carbohydrate into fat fatty acid synthesis by a combination of cytoplasm and mitochondria. Journal of Biological Chemistry 245(22): 5993-6002.

Watras AC, Buchholz AC, Close RN, Zhang Z, Schoeller DA. 2007. The role of conjugated linoleic acid in reducing body fat and preventing holiday weight gain. International Journal of Obesity 31(1): 481-487.

Werber J, Wang YJ, Milligan M, Li X & Ji JA. 2011. Analysis of 2, 2′-azobis (2-amidinopropane) dihydrochloride degradation and hydrolysis in aqueous solutions. Journal of Pharmaceutical Sciences 100(8): 3307-3315.

Weigle DS. 2003. Pharmacological therapy of obesity: Past, present and future. The Journal of Clinical Endocrinology and Metabolism 88(6): 2462-2469.

Wohner N, Sótonyi P, Machovich R, Szabó L, Tenekedjiev K, Silva MM, Longstaff C & Kolev K. 2011. Lytic resistance of fibrin containing red blood cells. Arteriosclerosis, Thrombosis, and Vascular Biology 31(10): 2306-2313.

Wlodarczyk M & Nowicka G. 2019. Obesity, DNA damage, and development of obesity-related diseases. International Journal of Molecular Sciences 20: 1-18.

Yamaguchi F, Saito M, Ariga T, Yoshimura Y & Nakazawa H. 2000. Free radical scavenging activity and antiulcer activity of garcinol from *Garcinia indica* fruit rind. Journal of Agricultural and Food Chemistry 48(6): 2320-2325.

Yeganeh A, Taylor CG, Poole J, Tworek L, Zahradka P. 2016. *Trans*-10, *cis*-12 conjugated linoleic acid inhibits 3T3-L1 adipocyte adipogenesis by elevating β-catenin levels. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids 1861(4): 363-370.

Yang L, Xian D, Xiong X, Lai R, Song J & Zhong J. 2018. Proanthocyanidins against oxidative stress: from molecular mechanisms to clinical applications. BioMed Research International 1(1): 11-16.

Ye J, Li J, Yu Y, Wei Q, Deng W, Yu L. 2010. L-carnitine attenuates oxidant injury in HK-2 cells via ROS-mitochondria pathway. Regulatory Peptides 161: 1-3.

Yin X, Lanza IR, Swain JM, Sarr MG, Nair KS & Jensen MD. 2014. Adipocyte mitochondrial function is reduced in human obesity independent of fat cell size. The Journal of Clinical Endocrinology & Metabolism 99(2): E209-E216.

Yu L, Haley S, Perret J, Harris M, Wilson J & Qian M. 2002. Free radical scavenging properties of wheat extracts. Journal of Agricultural and Food Chemistry 50(6): 1619-1624.

Yu HS, Do SH, Jeong WI, Yang HJ, Yuan DW, Hong IH, Lee HR, Lee IS, Kim YK & Choi MS. 2008. Cytotoxic effects of the conjugated linoleic acid isomers t10c12, c9t11-CLA and mixed form on rat hepatic stellate cells and CCL4-induced hepatic fibrosis. The Journal of Nutritional Biochemistry 19(3): 175-183.

Yu J, Ye J, Liu X, Han Y & Wang C. 2011. Protective effect of L-carnitine against H_2O_2-induced neurotoxicity in neuroblastoma (SH-SY5Y) cells. Neurological Research 33(7): 708-716.

Yuan T, Fan WB, Cong Y, Xu HD, Li CJ, Meng J, Bao NR & Zhao JN. 2015. Linoleic acid induces red blood cells and haemoglobin damage via oxidative mechanism. International Journal of Clinical and Experimental Pathology 8(5): 5044-5050.

Yun HS, Do SH, Jeong WI, Yang HJ, Yuan DW, Hong IH, Lee HR, Lee IS, Kim YK & Choi MS. 2008. Cytotoxic effects of the conjugated linoleic acid isomers t10c12, c9t11-CLA and mixed form on rat hepatic stellate cells and CCl4-induced hepatic fibrosis. The Journal of Nutritional Biochemistry 19(3): 175-183.

Zafar MI, Mills KE, Zheng J, Peng MM, Ye X, Chen LL. 2019. Low glycaemic index diets as an intervention for obesity: a systematic review and meta-analysis. Obesity Reviews 20(2): 290-315.

Zanotti J, Capp E, Wender MCO. 2015. Factors associated with postpartum weight retention in a Brazilian cohort. Revista Brasileira de Ginecologia e Obstetricia 37(4): 164-171.

Zebisch K, Voigt V, Wabitsch M & Brandsch M. 2012. Protocol for effective differentiation of 3T3-L1 cells to adipocytes. Analytical Biochemistry 425(1): 88-90.

Zhai JJ, Liu ZL, Li JM, Chen JP, Jiang L, Wang DM, Yuan J, Shen JG, Yang DP & Chen JQ. 2010. Different mechanisms of *cis*-9, *trans*-11-and *trans*-10, *cis*-12 conjugated linoleic acid affecting lipid metabolism in 3T3-L1 cells. The Journal of Nutritional Biochemistry 21(11): 1099-1105.

Zhang X, Wang B, Long F, Yang Z, Yu S, Wang Y, Wei X, Jing L, Liu G. 2008. Effect of dietary conjugated linoleic acid (CLA) on the growth and lipid metabolism of geese and fatty acid composition of the tissues. South African Journal of Animal Science 38(1): 12-20.

Zhang T, Zhang Li, Ke, Bin, Sun J, Liu T, Huang Y, Chen X, Liu M, Li F, Luo D. 2019. L-carnitine ameliorated weight loss in fasting therapy: A propensity score-matched study. Complementary Therapies in Medicine 44: 162-165.

Zhou YT, Wang ZW, Higa M, Newgard CB & Unger RH. 1999. Reversing adipocyte differentiation: implications for treatment of obesity. Proceedings of the National Academy of Sciences 96(5): 2391-2395.

Appendix

Ethical clearance

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

28/09/2017

Approval Certificate
New Application

Ethics Reference No: 415/2017

Title: In vitro toxicity of weight loss supplements, conjugated linoleic acid, levo-carnitine and hydroxycitric acid

Dear Bonisiwe Georginah Chiloane

The **New Application** as supported by documents specified in your cover letter dated 19/09/2017 for your research received on the 19/09/2017, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 27/09/2017.

Please note the following about your ethics approval:
- Ethics Approval is valid for 2 years
- Please remember to use your protocol number (**415/2017**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

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

☎ 012 356 3084 deepeka.behari@up.ac.za / fhsethics@up.ac.za http://www.up.ac.za/healthethics
✉ Private Bag X323, Arcadia, 0007 - Tswelopele Building, Level 4, Room 60, Gezina, Pretoria

Consent form

PICD Nr

PATIENT OR PARTICIPANT'S INFORMATION & INFORMED CONSENT DOCUMENT

STUDY TITLE: *In vitro* toxicity of weight loss supplements, conjugated linoleic acid, Levo-carnitine and hydroxycitric acid

SPONSOR: University of Pretoria

Principal Investigators: Bonisiwe Georginah Chiloane (Principle investigator), Prof HM Oberholzer (Supervisor)

Institution: University of Pretoria

DAYTIME AND AFTER HOURS TELEPHONE NUMBER(S):

Daytime numbers: Ms Chiloane 074 049 1812, Prof HM Oberholzer 012 319 2533

Afterhours: Ms Chiloane 074 049 1812

DATE AND TIME OF FIRST INFORMED CONSENT DISCUSSION:

				:
dd	**mm**	**y**		**Time**

Dear Mr. / Mrs. ..... date of consent procedure …...../…...../….....

1) INTRODUCTION

You are invited to volunteer for a research study. This information leaflet is to help you to decide if you would like to participate. Before you agree to take part in this study you should fully understand what is involved. If you have any questions, which are not fully explained in this leaflet, do not hesitate to ask the investigator. You should not agree to take part unless you are completely happy about all the procedures involved. In the best interests of your health, it is strongly recommended that you discuss with or inform your personal doctor of your possible participation in this study, wherever possible.

2) THE NATURE AND PURPOSE OF THIS STUDY

You are invited to take part in a research study. The red blood cells (also called erythrocytes) are the main type of cells found in the blood. These cells can be isolated in the laboratory and be used to determine the effect of different compounds on cell functioning and structure. The aim of this study is to determine the effect weight loss supplements (conjugated linoleic acid, L-carnitine and hydroxycitric acid) on the structure of red blood cells. The effect of these weight loss supplements will also be further investigated on the blood clotting system (also known as coagulation system) by using various laboratory techniques.

3) EXPLANATION OF PROCEDURES TO BE FOLLOWED

This study involves answering some questions regarding your health status.

Question	**Answer**	
	Yes	**No**
Are you older than 18?		
Are you taking any medication?		
Do you smoke?		

If you are older than 18, are not taking any medication and are a non-smoker and you agree to be in this study, we would like to collect one small tube of blood. The blood will be drawn by putting a needle into a vein in your arm. The tube of blood will not be labelled with your name, but a number will be assigned. This is done to ensure anonymity. In the laboratory the blood components will be separated from each other and the red blood cells will be exposed to different concentrations of the weight loss supplements. The effect of these weight loss supplements will then be accessed on the structure of the red blood cells and on the coagulation system.

Any blood left over after these measurements will be destroyed as biohazardous material.

4) RISK AND DISCOMFORT INVOLVED

The only possible risk and discomfort involved is the taking of blood from a vein. Small bruising or mild soreness at the puncture site may be experienced for several days. Any risk is minimized using pre-packaged sterilized equipment and careful attention to proper technique. The University of Pretoria has limited insurance for research related injuries.

5) POSSIBLE BENEFITS OF THIS STUDY

Although you will not benefit directly from the study, the result of this study will provide evidence about the safety and efficacy of weight loss supplements in humans.

6) WHAT ARE YOUR RIGHTS AS A PARTICIPANT

Your participation in this study is entirely voluntary. You can refuse to participate or stop at any time during the study without giving any reason. Your withdrawal will not affect you in any way.

7) HAS THE STUDY RECEIVED ETHICAL APPROVAL?

This Protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, telephone numbers 012 356 3084 / 012 356 3085 and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2013), which deals with the recommendations guiding doctors in biomedical research involving human/subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

8) INFORMATION AND CONTACT PERSON

Any questions regarding the study can be directed to Ms Bonisiwe Georginah Chiloane on 074 049 1812 or Prof HM Oberholzer on 012 319 2533.

9) CONFIDENTIALITY

All records obtained whilst in this study will be regarded as confidential. Results will be published or presented in such a way that volunteers remain unidentifiable.

10) CONSENT TO PARTICIPATE IN THIS STUDY

I have read or had read to me in a language that I understand the above information before signing this consent form. The content and meaning of this information have been explained to me. I have been given opportunity to ask questions and am satisfied that they have been

answered satisfactorily. I understand that if I do not participate it will not alter my management in any way. I hereby volunteer to take part in this study.

I have received a signed copy of this informed consent agreement.

.....	.....
Patient name	Date
.....	.....
Patient signature	Date
.....	.....
Investigator's name	Date
.....	.....
Investigator's signature	Date
.....	.....
Witness name and signature	Date