IN VITRO & *IN VIVO* ANTI-ADIPOGENIC EFFECTS OF A STANDARDIZED QUASSINOID COMPOSITION FROM *EURYCOMA LONGIFOLIA*

DHARVIND BALAN

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2019

IN VITRO & *IN VIVO* ANTI-ADIPOGENIC EFFECTS OF A STANDARDIZED QUASSINOID COMPOSITION FROM *EURYCOMA LONGIFOLIA*

DHARVIND BALAN

DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF MEDICAL SCIENCE

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2019

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: DHARVIND BALAN

Matric No: MGN150037

Name of Degree: MASTER OF MEDICAL SCIENCE

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

In vitro & *in vivo* anti-adipogenic effects of a standardized quassinoid composition from *Eurycoma longifolia*.

Field of Study: Pharmacology

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date:

Subscribed and solemnly declared before,

Witness's Signature Date:

Name:

Designation:

IN VITRO & *IN VIVO* ANTI-ADIPOGENIC EFFECTS OF A STANDARDIZED QUASSINOID COMPOSITION FROM *EURYCOMA LONGIFOLIA*

ABSTRACT

Dysregulation of adipocyte metabolism often leads to metabolic dysfunction and obesity. Comorbidities of obesity up-surge the medical risk factors in modern days society. *Eurycoma longifolia* Jack (EL) is an herbal medicinal plant native to South-East Asian countries, widely known as 'Tongkat Ali.' Bioactive compounds of EL were previously shown to reduce omentum fat mass and oestradiol-induced fatty uterine adhesion in rats. However, the exact role of EL in the modulation of adipogenesis remains to be investigated further. Therefore, this study sought to investigate the effects of a standardized quassinoids composition of EL (SQEL) on the adipogenesis of 3T3-L1 pre-adipocyte cells and in high-fat-diet-induced obese C57BL/6J mice. The 3T3-L1 cells were induced to differentiate and treated with various concentrations of SQEL (0-100 μg/mL) for 8 days. Oil Red O staining and intracellular triglyceride quantification assay were performed to determine the effect of SQEL on adipogenic differentiation and cytosolic triglyceride accumulation in 3T3-L1 cells. In addition, the effect of SQEL on lipolysis was also examined by quantifying glycerol released by mature adipocytes treated with SQEL at various concentrations. The mRNA and protein expressions of adipogenic markers were measured using qRT-PCR and immunoblot analysis, respectively. Similarly, C57BL/6J mice were fed with normal and high-fat diet and given 5 mg/kg and 10 mg/kg (i.p) of SQEL for 12 weeks. Weekly food intake and body weight were measured throughout the treatment period. Oral glucose tolerance and intraperitoneal insulin tolerance tests were conducted on the 12th week of the study. At the end of treatment, blood serum was collected for measurement of serum lipids. Epididymal and perirenal fat pads and liver were excised, weighed and used for

histological studies. SQEL significantly inhibited adipogenesis and reduced intracellular accumulation of lipids in adipocytes. Moreover, the mRNA expression of major adipogenic transcription factors (PPARγ and C/EBPα) and markers (FAS, LPL and KLF15) were reduced while Sirt1 and FoxO3 proteins were upregulated during the initial stage of differentiation. On the other hand, SQEL also increased lipolysis by elevating glycerol production and reducing intracellular triglycerides in mature adipocytes. In addition, SQEL suppressed body weight gain and decreased epididymal and perirenal fat pad mass in both the normal and high-fat diet fed mice. Moreover, accumulation of fat in the liver and size of adipose tissue were found reduced with SQEL treatment in high-fat diet mice. SQEL treatment also induced energy expenditure in adipose tissue of mice on HFD by elevating the expression of thermogenic genes, AMPK and PGC-1α. Furthermore, SQEL also improved glucose intolerance and decreased elevated total cholesterol and triglyceride in mice fed with high-fat diet. These novel findings suggest that SQEL has an anti-adipogenic effect and could be explored as an alternative pharmacologic agent inhibiting cellular differentiation of pre-adipocytes for the prevention of obesity.

Keywords: *Eurycoma longifolia*; Adipogenesis; Energy homeostasis; Lipolysis; Obesity

KESAN-KESAN *IN VITRO* & *IN VIVO* ANTI-ADIPOGENIK KOMPOSISI QUASSINOID YANG DISERAGAMKAN DARIPADA *EURYCOMA LONGIFOLIA*

ABSTRAK

Masalah regulasi metabolisma adiposit menyebabkan disfungsi metabolik dan obesiti. Obesiti pula meningkatkan risiko masalah kesihatan di kalangan masyarakat moden. *Eurycoma longifolia* Jack (EL) ialah tumbuhan ubatan herba dikenali sebagai Tongkat Ali yang kebayakanya terdapat di negara-negara Asia Tenggara. Dalam kajian terdahulu, sebatian bioaktif daripada EL didapati boleh mengurangkan jisim lemak omentum dan pemendapan rahim berlemak teraruh daripada estradiol dalam tikus. Walaubagaimanapun, fungsi EL di dalam modulasi adipogenesis masih belum dikaji. Oleh sebab itu, kajian ini dilakukan untuk mengkaji kesan komposisi quassinoids yang diseragamkan (SQEL) dalam adipogenesis dengan menggunakan sel-sel pra-adiposit 3T3-L1 dan model tikus obes C57BL/6J yang diinduksikan oleh diet berlemak tinggi. Sel-sel 3T3-L1 telah didorong untuk diferensiasi dan dirawat dengan SQEL dalam pelbagai kepekatan (0-100 μg/mL) selama 8 hari. Ujian pewarnaan Oil Red O dan pengkuantitian trigliserida intraselular telah dilakukan untuk mengenalpasti kesan SQEL keatas proses adipogenesis dalam sel-sel 3T3-L1. Tambahan pula, kesan SQEL dalam adipolisis juga diperiksa dengan mengukur kandungan gliserol yang dihasilkan oleh sel-sel adiposit matang yang dirawat dengan SQEL dalam pelbagai kepekatan. Selain itu, profil mRNA dan protein berkaitan dengan proses adipogenik juga diukur dengan mengunakan kaedah reaksi rantai polimerase transkripsi membalik dan analisis imunoblot. Tikus C57BL/6J diberi diet biasa dan diet berlemak tinggi dan juga diberikan 5 mg/kg dan 10 mg/kg (i.p) SQEL selama 12 minggu. Pengambilan makanan dan berat badan diukur setiap minggu sepanjang tempoh rawatan. Ujian toleransi glukosa oral dan toleransi insulin intraperitoneum dijalankan pada minggu ke-12. Pada akhir rawatan,

serum darah dikumpulkan untuk pengukuran lipid serum. Pad lemak epididimal dan perirenal dan hati dikeluarkan, ditimbang dan digunakan untuk kajian histologi. Kajian ini membuktikan bahawa SQEL menghalang proses adipogenesis, mengurangkan pengumpulan lipid intraselular dan kandungan trigliserida dalam adiposit dengan ketara. Selain itu, expresi mRNA faktor transkripsi (PPARγ dan C/EBPα) dan penanda utama adipogenik (FAS, LPL dan KLF15) dikurangkan malah ekspresi protein Sirt1 dan FoxO3 ditingkatkan pada peringkat awal pembezaan sel pra-adiposit. Seterusnya, SQEL juga meningkatkan lipolisis dengan meningkatkan pengeluaran gliserol dan mengurangkan trigliserida intraselular dalam adiposit matang. Di samping itu, SQEL juga menindas peningkatan berat badan dan menurunkan jisim pad lemak epididimal dan perirenal di dalam kedua-dua model tikus yang diberi makan diet biasa dan berlemak tinggi. Selain itu, pengumpulan lemak di dalam hati dan saiz tisu adiposa dapat dikurangkan dengan rawatan SQEL dalam model tikus yang diberi diet berlemak tinggi. Rawatan SQEL juga menginduksi penggunaan tenaga dalam tisu adipos tikus yang diberi diet berlemak tinggi dengan meningkatkan ekspresi gen-gen termogenik, AMPK dan PGC-1α. Tambahan pula, SQEL membaikpulih intoleransi glukosa dan menurunkan jumlah kolesterol dan trigliserida dalam tikus yang diberi diet berlemak tinggi. Penemuan novel ini menunjukkan bahawa SQEL mempunyai kesan anti-adipogenik dan berpotensi sebagai ejen farmakologi alternatif yang menghalang pembezaan sel-sel pra-adiposit untuk pencegahan obesiti.

Kata kunci: *Eurycoma longifolia*; Adipogenesis; Homeostasis tenaga; Lipolisis; Obesiti

ACKNOWLEDGEMENTS

First and foremost, I would like to extend my gratitude to my supervisors, Associate Prof Dr Wong Pooi Fong and Dr Dharmani Devi Murugan for their valuable guidance, suggestions, advice, critics, understanding and supports in ensuring the completion of this research study successfully.

A special thanks go to Dr Umarani Subramaniam for motivating and sponsoring me to pursue my graduate studies in the University of Malaya.

I would also like to thank the Ministry of Agriculture and Agro-Based Industry, Malaysia for providing financial support to complete this study successfully.

My gratitude also goes to Prof. Chan Kit Lam for providing the standardized quassinoids composition and his valuable advice for the project.

I am thankful to the Department of Pharmacology and the Faculty of Medicine, University of Malaya for providing the infrastructure to accomplish this research work.

I would like to express my heartfelt thanks to my lab mates and colleagues, Darren, Kind Leng, Juliana, Kong, Claire, Celine, and Yi Lin, who helped me throughout this study.

Last but not least, my beloved family members and relatives for their motivation, moral support and confidence in me that pulled me up during my difficult times.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

↑	:	increase
↓	:	decrease
°C	:	degree Celsius
a.u	:	arbitrary unit
x g	:	gravitational force
kg/m^2	:	kilograms by the square to height in meters
AAALAC	:	Association for Assessment and Accreditation of Laboratory Animal Care International
Abs	:	Absorbance
ACC	:	Acetyl-CoA carboxylase
Akt	:	protein kinase B
AMM	:	adipocyte maintenance medium
AMP	:	adenosine monophosphate
AMPK	:	AMP-activated protein kinase
ANOVA	:	Analysis of variance
aP2	:	adipocyte protein 2
APC	:	adenomatous polyposis coli
ATCC	:	American Type Culture Collection
BAT	:	brown adipose tissue
BMI	:	body mass index
BSA	:	bovine serum albumin
BW	:	body weight
C/EBPα	:	CCAAT-enhancer-binding protein alpha
C/EBPβ	:	CCAAT-enhancer-binding protein beta
C/EBPδ	:	CCAAT-enhancer-binding protein delta
cDNA	:	complementary DNA
cm	:	centimeter
CO^2	:	carbon dioxide
CoA	:	co-enzyme A
CREB	:	cAMP response element-binding protein
DM	:	differentiation medium
DMEM	:	Dulbecco's Modified Eagle Medium

DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
DTT	:	Dithiothreitol
EL	:	*Eurycoma longifolia* Jack
ERK	:	extracellular signal-regulated kinases
et al	:	*ea alia* (and another people)
Eu	:	eurycomanone
FABPs	:	fatty-acid-binding proteins
FAS	:	fatty acid synthetase
FoxO	:	Forkhead box
FoxO3	:	Forkhead box O3
FZD	:	frizzled
g	:	gram
GLUT4	:	glucose transporter 4
GSK3β	:	glycogen synthase kinase 3-β
HCl	:	hydrochloric acid
HDL	:	high-density lipoprotein
HFD	:	high-fat diet
HPLC	:	high performance liquid chromatographic
i.p	:	intraperitoneal
IC50	:	half maximal inhibitory concentration
IPITT	:	intraperitoneal insulin tolerance test
IR	:	insulin receptor
IRS	:	insulin receptor substrate
Iso	:	isoproterenol
IU	:	international unit
JNKs	:	c-Jun amino-terminal kinases
kcal	:	kilocalorie
kg	:	kilogram
KLF15	:	Kruppel-like factor 15
L	:	liter
LD50	:	half-maximal lethal dose
LDL	:	low-density lipoprotein
LEF/TCF	:	lymphoid-enhancer-binding factor/T-cell-specific transcription factor

LPL	:	lipoprotein lipase
LRP5/6	:	low-density-lipoprotein-receptor-related protein-5 or -6
m	:	meter
M	:	molar
MAPK	:	mitogen-activated protein kinases
MDI	:	isobutyl-methyl-xanthine/dexamethasone/insulin
mg	:	milligram
mL	:	milliliter
mM	:	millimolar
mMol	:	millimolar
mRNA	:	messenger RNA
MSCs	:	mesenchymal stem cells
mTOR	:	mammalian target of rapamycin
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	:	Nicotinamide adenine dinucleotide phosphate
ND	:	normal diet
NDC	:	non-differentiated control
NF	:	nuclear factor
nM	:	nanomolar
nm	:	nanometer
NP-40	:	Nonidet P-40
o.g	:	oral garvage
OGTT	:	oral glucose tolerance test
p38	:	p38 MAPK
PAGE	:	Polyacrylamide Gel Electrophoresis
PBS	:	phosphate buffered saline
PCM	:	pre-adipocyte culture medium
PGC-1α	:	PPAR-γ coactivator 1α
PI3K	:	phosphoinositide 3-kinase
PKA	:	protein kinase A
PPARγ	:	peroxisome proliferator-activated receptor gamma
PPRE	:	peroxisome proliferator response elements
PRDM16	:	PR domain-containing16
qRT-PCR	:	quantitative real-time polymerase chain reaction
RIPA	:	Radioimmunoprecipitation assay

RNA	:	Ribonucleic acid
rpm	:	revolutions per minute
RXR	:	Retinoid X receptor alpha
SDS	:	Sodium Dodecyl Sulfate
SEM	:	standard error of mean
Sf	:	sulforaphane
Sirt1	:	Sirtuin 1
SMRT	:	silencing mediator of retinoid and thyroid hormone receptors
SQEL	:	standardized quassinoids composition of EL
TBS-T	:	Tris-buffered saline - tween 20
TSC2	:	tuberous sclerosis complex 2
TZDs	:	Thiazolidinediones
UCP1	:	uncoupling protein-1
v/v	:	volume/volume
VC	:	vehicle control
V	:	volt
w/v	:	weight/volume
w/w	:	weight/weight
WAT	:	white adipose tissue
µL	:	microliter
µM	:	micromolar
µm	:	micrometre

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Adipocytes are a crucial cellular constituent of fat tissues that play a key role in modulating energy homeostasis and lipid metabolism (Unger *et al.*, 2010). These fat cells are responsible for the accumulation of lipid molecules during energy excess and metabolizing it in the events of energy demand (Ali *et al.*, 2013). Adipogenesis is a cellular differentiation process whereby fibroblastic pre-adipocytes turn into adipocytes in the event of caloric overabundance (Hausman *et al.*, 2001; Sarjeant & Stephens, 2012). Uncontrolled adipogenesis leads to overgrowth of adipose tissues which then enhances buildup of the fatty tissue mass and eventually contributes to the development of obese condition (Fruhbeck *et al.*, 2001). The alarming rise in the prevalence of obesity is observed among people from both developed and developing nations (Zang *et al.*, 2013). In addition, obesity underpins the development of many other serious metabolic diseases such as type 2 diabetes mellitus, hypertension and cardiovascular diseases that possess higher mortality rate (He *et al.*, 2013). As adipogenesis facilitates the progression of obesity, various studies have focused on inhibiting adipocytic differentiation, downregulating lipogenesis and enhancing lipolysis as strategies in preventing the development of obesity (Dave *et al.*, 2012; He *et al.*, 2013; Inafuku *et al.*, 2013; Park *et al.*, 2012; Song *et al.*, 2013).

Interestingly, adipogenesis is a tightly regulated multi-step process which comprises the activation of several transcriptional factors. These transcription initiators are responsible for regulating the expression of adipogenesis-specific genes that lead to the development of adipocyte phenotype. Major adipogenic transcription factors such as CCAAT/enhancer-binding protein β (C/EBPβ), peroxisome proliferator-activated receptor γ (PPARγ), and CCAAT/enhancer-binding protein α (C/EBPα) play a crucial role in coordinating this complex cellular differentiation (Rosen & MacDougald, 2006;

White & Stephens, 2010). C/EBPβ is expressed upon adipogenic induction and switches on the promoters of the genes encoding PPARγ and C/EBPα which then activates the expression of adipogenic genes such as fatty acid binding protein (aP2), fatty acid synthetase (FAS) and lipoprotein lipase (LPL) that promotes the progression of adipocytic differentiation (Cristancho & Lazar, 2011; Farmer, 2006).

Earlier studies have demonstrated that PPARγ alone is enough for adipocyte differentiation (Tontonoz *et al.*, 1994) and its induction is significantly increased during adipogenesis development (Chawla *et al.*, 1994) suggesting that PPARγ functions as the lead regulator of pre-adipocyte differentiation and therefore, its down-regulation is considered important to suppress adipogenesis. Several lines of evidence suggest that PPARγ could be down-regulated via numerous signaling pathways. Interestingly, AMP-activated protein kinase (AMPK) pathway suppressed adipogenesis by down-regulating the expression of PPARγ (He *et al.*, 2013; Huang *et al.*, 2011). In addition, mitogen-activated protein kinases (MAPK) pathway is also involved in the regulation of adipogenesis by phosphorylating PPARγ, thereby inducing its degradation that suppresses the downstream expression of adipogenic genes (Bost *et al.*, 2005; Zang *et al.*, 2013). Furthermore, studies have found that phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathway (Park *et al.*, 2012) and Akt-tuberous sclerosis complex 2-mammalian target of rapamycin (Akt-TSC2-mTORC) pathway (Zhang *et al.*, 2009) also influence PPARγ expression via insulin signaling in adipogenesis.

Nevertheless, the activity of transcription factors is also regulated at numerous stages through protein acetylation by both acetylase and deacetylase. The histone deacetylase sirtuin 1 (Sirt1) possesses significant roles in a variety of biological processes including adipogenesis. It was reported that the suppression of PPARγ activity by Sirt1 impaired

adipogenesis and induced lipolysis in matured adipocytes (Picard *et al.*, 2004). Earlier evidence showed that overexpression of Sirt1 attenuates adipocyte differentiation by reducing the expression of PPARγ and C/EBPα in 3T3-L1 cells (Picard *et al.*, 2004). Similarly, reduced Sirt1 levels promote PPARγ acetylation and C/EBPα activity, which leads to adipocyte differentiation (Qu *et al.*, 2016). Conversely, the inhibitory effect of PPARγ on Sirt1 activity (Han *et al.*, 2010) and modulation of Sirt1 expression by C/EBPα during adipogenesis (Jin *et al.*, 2010) indicate Sirt1 could be regulated by adipogenic transcription factors as well. Hence, improving our understanding of the molecular mechanisms in adipogenesis could pave ways for the identification of therapeutic targets against metabolic diseases that are quickly becoming global concerns.

Health awareness programs promoting the practices of a healthy lifestyle, healthy dietary habits and increasing physical activities are highly advocated in the management of obesity. These together with pharmacological therapy may provide additional benefit in ameliorating comorbidities and promoting adherence to behaviour modifications, which in turn may improve physical functions for greater physical activity (Apovian *et al.*, 2015). Currently, several prescriptive anti-obesity drugs can be used to suppress appetite, reduce lipid absorption and enhance fat oxidation for weight management of obese individuals (Padwal & Majumdar, 2007). However, poor efficacy and serious side effects of those drugs have led to their withdrawal from the market leaving only a handful of drugs that can be consumed for the said purpose.

In recent years, the use of naturally-derived compounds for the pharmacological management of obesity has received much attention from the scientific community. Several flavonoids commonly present in herbal plants were reported to possess significant anti-adipogenic effects and these include luteolin (Kwon *et al.*, 2016), isorhamnetin

(Zhang *et al.,* 2016), butein (Wang *et al.,* 2017), delphinidin (Rahman *et al.,* 2016). Hence, exploring phytochemicals as an alternative source to identify and develop safe and effective anti-adipogenic agent for managing obesity remains an interesting option.

Eurycoma longifolia Jack (EL), typically known as 'Tongkat Ali' is a popular tropical plant classified under Simaroubaceae family and native to Southeast Asia countries. Its raw crude powder is widely used as an additive in canned energy drinks and coffee mixtures, as well as generally consumed as a health supplement in the form of capsules. The root of this plant is a popular traditional medication for fever after birth, boils, ulcer wound healing treatment and also acts as energy booster among men (Low *et al.*, 2013). Quassinoids which include the pure compound, eurycomanone found in EL were reported to have biological activities such as anti-plasmodial, anti-cancer, pro-fertility and aphrodisiac activities in males (Bhat & Karim, 2010). Moreover, the effects of EL in the role of adipogenesis were shown indirectly by the reduction of omentum fat mass (Solomon *et al.*, 2014) and its synergistic effect in combination studies with other bioactive compounds in body weight management (Talbott, 2007). However, to date, there is limited information on the effects of EL-derived quassinoids in adipogenesis including the mechanisms involved. Thus, the present study aims to investigate the potential lipid modulation effects of a standardized quassinoids enriched-fraction (SQEL) and eurycomanone of *Eurycoma longifolia* in adipocytes and in high-fat diet-induced obese mice.

CHAPTER 2: LITERATURE REVIEW

2.1 Adipocytes

2.1.1 Origin of Adipocytes

Adipocytes are the key cellular components of adipose tissue that function as energy storage depot, heat insulator and as a protective layer for many important body organs. They originate from pluripotent mesenchymal stem cells (MSCs) that are capable of developing into several cell types including adipocytes, myocytes, chondrocytes and osteocytes (Covas *et al.,* 2008; Lin *et al.,* 2010).

2.1.2 Adipogenesis

Adipogenesis is a developmental process in which mesenchymal stem cell differentiates into preadipocyte, which then differentiates to become adipocyte (Figure 2.1) (Ali *et al.*, 2013). This process involves a series of well-defined steps including commitment, recruitment and proliferation and terminal differentiation. During the commitment phase, the pluripotent stem cells are committed to differentiate towards adipocyte lineage; hence losing the potential to differentiate into other cell types (Otto & Lane, 2005). As a result, preadipocytes are formed. Next, the preadipocytes proliferate and expand their numbers, known as recruitment and proliferation phase will undergo growth arrest phase after which mitotic clonal expansion is initiated. During this process, the growth-arrested preadipocytes will undergo one more round of DNA replication and cell division which leads to the clonal expansion of the committed cells (Rangwala & Lazar, 2000). The DNA replication step at this stage is crucial to switch on key transcriptional factors which regulate the terminal differentiation of adipocytes.

Terminal differentiation is the final phase where preadipocytes acquire characteristics of matured adipocytes which are non-dividing, lipid-filled and morphologically round. The matured adipocytes actively secrete proteins for lipid synthesis and have enhanced

insulin sensitivity (Rosen & MacDougald, 2006). Henceforth, the adipocytes could perform many biological functions as an energy reserve and specialized endocrine cells. To date, adipogenesis is one of the most intensively studied developmental processes due to the availability of good experimental cell culture models of adipocyte differentiation (Ali *et al.*, 2013).

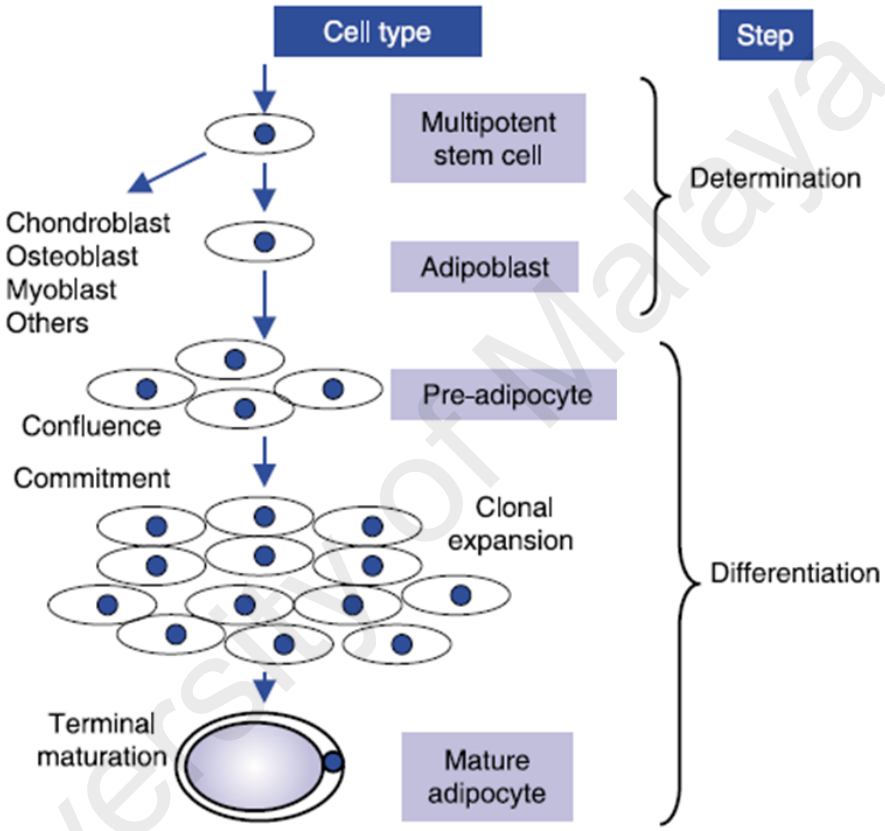

Figure 2.1: Determination and differentiation of adipocytes. Adapted and modified from (Fève, 2005).

2.1.3 *In vitro* Models of Adipogenesis

Several cellular models are used to study adipogenesis and adipocyte function *in vitro*. The first group of the cellular model system comprises of pluripotent fibroblasts such as 10T1/2, BALB/c-3T3, RCJ3.1, and CHEF/18 fibroblast cell lines that can differentiate

into myocytes, chondrocytes and adipocytes (Sarjeant & Stephens, 2012). However, low proliferative capacity and inconsistent differentiation potential during passaging limit the use of primary preadipocytes for the said purpose (Fève, 2005). Therefore, the alternative source of preadipocytes to study the mechanism of adipogenesis are now mainly derived from transgenic mice (Valet *et al.*, 2002). Fibroblast-like preadipocytes derived from these animal models including 3T3-L1, 3T3-F422A, 1246, Ob1771, TA1, and 30A5 that can differentiate into adipocytes are now commonly used to study adipogenesis in a laboratory setting (Sarjeant & Stephens, 2012).

Among these, 3T3-L1 and 3T3-F422A are well-characterized preadipocytes cell lines and widely used to study adipocyte biology *in vitro*. Todaro and Green first isolated 3T3 cells (Green & Meuth, 1974). These cells were obtained from 17 to 19-day-old Swiss 3T3 mouse embryos via clonal isolation (Green & Kehinde, 1979). They are highly preferred because of their capacity to develop a homogeneous mature adipocytes population which is biochemically and morphologically indistinguishable from adipocytes *in situ*. This cell line can be passaged with minimal fall in differentiation potential and is sensitive towards lipogenic and lipolytic drug treatment (Ali *et al.*, 2013; Sarjeant & Stephens, 2012). Moreover, the confluent fibroblastic 3T3 cells can undergo differentiation to become lipid-filled and round-shaped adipocytes when stimulated appropriately using hormonal inducer for *in vitro* study (Green & Kehinde, 1975).

2.1.4 Hormonal Inducers of Adipogenesis

Post-confluent preadipocytes require a suitable mixture of adipogenic inducing agents to initiate adipogenesis. There are various mixtures of adipogenesis hormonal inducers developed based on the origin of preadipocyte cell lines and the differentiation protocols (Gregoire *et al.*, 1998; Hwang *et al.*, 1997). Nevertheless, the standard adipogenesis differentiation inducer mixture that is widely in use is isobutyl-methyl-

xanthine/dexamethasone/insulin (MDI) which was discovered by (Student *et al.*, 1980). The isobutyl-methyl-xanthine is a non-competitive phosphodiesterase inhibitor which elevates intracellular cAMP level and activates protein kinase A by phosphorylation and eventually activates cAMP response element-binding protein (CREB) to initiate adipogenesis (Essayan, 2001; Farmer, 2006). Then, the phosphorylated CREB initiates the expression of adipogenic transcription factors (Birsoy *et al.*, 2008). In addition, dexamethasone is a well-known artificial glucocorticoid analogue that binds and activates glucocorticoid receptor and hence activates transcription factors of adipogenesis at the early stages of differentiation (Lee *et al.*, 1991). However, it may exert inhibitory effect if added at the later stage of the differentiation process, indicating that the effects of hormones are only required during the induction phase of adipogenesis (Caprio *et al.*, 2007). On top of that, the presence of insulin is utmost important throughout adipogenesis (induction and maintenance) since insulin signaling is crucial for proliferation and differentiation of preadipocytes (Ailhaud, 1982). Insulin is the peptide hormone that also suppresses lipolysis and support lipogenesis by promoting glucose uptake for the synthesis of triglycerides in adipocytes (Chen *et al.*, 2016).

2.2 Transcriptional Regulation of Adipogenesis

The differentiation of preadipocytes to adipocytes is highly regulated by a network of transcription factors that direct the expression of key adipogenic proteins responsible for the maturation of adipocytes (Farmer, 2006). The peroxisome proliferator-activated receptor γ (PPARγ), and the CCAAT/enhancer binding proteins (C/EBPs) such as C/EBPα, β and δ are the main regulators of adipogenesis. *In vitro* induction of adipogenesis by hormonal cocktail activates the expression of C/EBPβ and C/EBPδ (Ramji & Foka, 2002). C/EBPs tend to accumulate within 24 hours of adipogenesis induction and initiate mitotic clonal expansion. Subsequently, the glycogen synthase kinase-3β and mitogen-activated protein kinase (MAPK) phosphorylate and activate

C/EBPβ which then together with C/EBPδ induces the expression of PPARγ and C/EBPα (Tang *et al.*, 2005). PPARγ and C/EBPα are the two principal transcription factors that regulate the entire process of adipogenic differentiation in mammalian cells (Lefterova & Lazar, 2009). Following their expressions, both PPARγ and C/EBPα cross activate each other by a positive feedback mechanism and also transactivate a huge group of downstream target genes that determine the fate of adipocytes (Elberg *et al.*, 2000). The accumulated C/EBPα on the day 2 of differentiation stage is phosphorylated by cyclin D3 protein which leads to the inhibition of cell proliferation to facilitate the final differentiation stage of adipogenesis (Wang *et al.*, 2006). Figure 2.2 summarizes the transcriptional control of adipogenesis at different stages of preadipocyte differentiation.

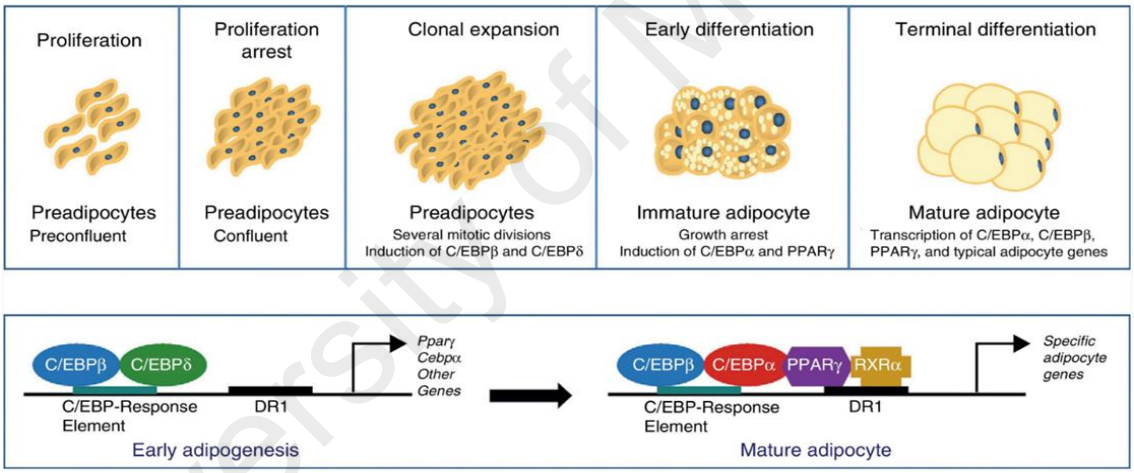

Figure 2.2: Differentiation from preadipocyte to mature adipocyte and the transcriptional control at different stages of adipogenesis. C/EBPβ together with C/EBPδ induces the expression of PPARγ and C/EBPα during the early phase of adipogenesis. Heterodimerization of RXR with PPARγ enables the DNA binding of PPARγ which then induces the expression of adipocyte-specific genes in matured adipocytes. Retinoid X receptor alpha; Adapted and modified from (Esteve, 2014).

2.2.1 Peroxisome Proliferator-Activated Receptor γ

Peroxisome Proliferator-Activated Receptor γ (PPARγ) is a member of ligand-activated transcription factors which binds to the target gene promoter to activate gene transcription of proteins involved in various biological processes including energy metabolism, cell proliferation and inflammation (Kersten, 2002). PPARγ forms a heterodimer with retinoid X receptor (RXR) which enables DNA binding at PPARγ target genes through interactions with PPARγ-response element (Tontonoz & Spiegelman, 2008).

PPARγ1 and PPARγ2 are the two protein isoforms of PPARγ which are expressed as a result of splicing and alternate promoter usage (Zhu *et al.*, 1995). Although both PPARγ1 and PPARγ2 are plentifully expressed in adipose tissues, inactivation of PPARγ2 in 3T3-L1 cells blocks adipogenic differentiation while PPARγ1 had no effect on adipogenesis (Ren *et al.*, 2002). Moreover, selective PPARγ2 deficiency impairs the development of white adipose tissue, reduces lipid accumulation and down-regulates the expression of adipogenic genes in mice (Zhang *et al.*, 2004). This suggests that PPARγ2, not PPARγ1, plays the regulatory role in adipogenesis. Emerging evidence has demonstrated that PPARγ is the master regulator of adipogenesis whereby its absence impairs the capability of preadipocytes to differentiate into adipocytes (Barak *et al.*, 1999; Rosen *et al.*, 1999). Rosen *et al*. further showed that ectopic expression of C/EBPα in embryonic fibroblast cells lacking PPARγ prevents the cells to differentiate (Rosen *et al.*, 2002) while Wu *et al*. showed overexpression of PPARγ2 in C/EBPα-null embryonic fibroblast cells restores adipogenesis (Wu *et al.*, 1999). These studies conclusively reveal that PPARγ2 is the significant transcriptional regulator of adipogenesis and that C/EBPα may co-regulate the expression of PPARγ2. In addition, the failure of PPARγ-deficient transgenic mice to form adipose tissue further confirms the necessity of PPARγ for the development of adipocytes both *in vitro* and *in vivo* (Barak *et al.*, 1999). Moreover,

deactivation of PPARγ in mature adipocytes induces insulin resistance through the downregulation of genes related to insulin signaling, lipolysis and free fatty acid intake (Gray *et al.*, 2006; Tamori *et al.*, 2002). In addition, it was shown PPARγ hyperactivity in mice impairs obesity-induced insulin resistance while PPARγ deletion, particularly in fat, muscle or liver, eventually leads to hyperlipidemia, hyperglycemia, or hyperinsulinemia (He *et al.*, 2003; Hevener *et al.*, 2003; Norris *et al.*, 2003). Similarly, human subjects with a dominant-negative mutation in the gene encoding PPARγ (*PPARG*) showed potential to develop lipodystrophy and insulin resistance (Agarwal & Garg, 2002).

The presence of a ligand is important for PPARγ to repress and activate the target genes. PPAR ligand includes fatty acids, prostaglandins and oxidized phospholipids (Kersten, 2002). Some naturally occurring PPAR agonists including 9, 10-dihydroxy-octadecenoic acid and 15-deoxy-D(12, 14)-PGJ(2) have been reported to promote adipogenesis while inhibiting osteogenesis (Lecka-Czernik *et al.*, 2002). Thiazolidinediones (TZDs) are well-known synthetic ligands of PPARγ and functions as insulin sensitizers in the treatment of type 2 diabetes. TZDs have demonstrated glucose-lowering and improvement of insulin sensitivity in insulin-resistant animal models and patients (Evans *et al.*, 2004). Additionally, rosiglitazone also stimulates adipogenesis in human mesenchymal stem cells (Benvenuti *et al.*, 2007).

2.2.2 CCAAT/Enhancer Binding Proteins

The CCAAT/Enhancer Binding Proteins (C/EBPs) belong to a family of basic leucine zipper transcription factors, where C/EBPα, β and δ are the members that are well established in adipogenesis. C/EBPs are not adipocyte-specific proteins. They are also expressed in other body tissues including liver, lung, adrenal gland and placenta (Birkenmeier *et al.*, 1989; Yeh *et al.*, 1995). Interestingly, C/EBPβ and δ have been found

expressed mainly during the early phase of adipogenesis while C/EBPα expression overrides in the later stage of differentiation (Cao *et al.*, 1991) (Figure 2.2). This is exemplified in embryonic fibroblasts isolated from C/EBPβ and δ deficient mice that are unable to differentiate upon hormonal induction and eventually fails to express PPARγ, C/EBPα and other adipogenic markers (Tanaka *et al.*, 1997). This suggests that *in vitro* adipocyte differentiation requires the activation of C/EBPs and PPARs that sequentially leads the transcriptional cascade for the maturation of adipocytes. However, an *in vivo* study has reported that transactivation of PPARγ and C/EBPα can take effect in the absence of C/EBPβ and δ while severely impairing the adipocyte development (Tanaka *et al.*, 1997). Therefore, it can be concluded that the presence of C/EBPβ and δ is crucial for complete maturation of adipocytes both *in vitro* and *in vivo*.

2.3 Adipogenic Proteins

As the positive feedback loop of PPARγ and C/EBPα kick-starts adipogenesis, several pro-adipogenic proteins such as fatty acid synthase (FAS), lipoprotein lipase (LPL), fatty-acid-binding proteins (FABPs) and Kruppel-like factor 15 (KLF15) are actuated for the establishment of matured adipocytes.

2.3.1 Fatty Acid Synthase

Fatty acid synthase (FAS) is the primary enzyme involved in *de novo* lipogenesis. FAS is responsible for the elongation of long-chain fatty acids for the synthesis of triglycerides via esterification with glycerol-3-phosphate (Ruderman *et al.*, 1999). Fatty acid synthesis takes place predominantly in the liver and adipose tissues. In adipocytes, FAS extends the short-chain malonyl CoA by condensation with acetyl-CoA to form long-chain fatty acids such as palmitoyl-CoA and stearoyl-CoA. Since FAS promotes fatty acid synthesis and fat accumulation, several studies have demonstrated its significant role in the development of adipocytes and obesity (Berndt *et al.*, 2007; Kovacs *et al.*, 2004; Kumar

et al., 2002). Therefore, this key lipid metabolizing enzyme found abundantly in adipose tissue is a potential drug target to attenuate excess fat storage. In fact, inhibition or downregulation of this protein has been shown to reduce adipocyte differentiation, lipid synthesis and activate fat oxidation and energy expenditure in a transgenic mouse model (Lenhard, 2011). For example, it was shown that FAS inhibition by a potent inhibitor of fatty acid synthase, C57 significantly reduces the body weight of obese mice by suppressing food intake (Kumar *et al.*, 2002). Likewise, it was shown that inhibition of FAS by another inhibitor of FAS, tannic acid in 3T3-L1 pre-adipocytes impairs adipogenesis, represses intracellular lipid accumulation and downregulates mRNA levels of FAS and PPARγ (Fan *et al.*, 2013).

2.3.2 Lipoprotein Lipase

Lipoprotein lipase (LPL) is a glycoprotein synthesized in parenchymal cells and is mainly found in adipose and muscle tissues (Bouvy-Liivrand *et al.*, 2014). LPL is an extracellular lipid-lysing enzyme that is responsible for the hydrolysis of lipids. This is essential to facilitate the rapid clearance of lipid load by releasing free fatty acids from circulating triglycerides for adipose and skeletal muscle tissue uptake. The lipolytic function of LPL is highly controlled by several cofactors. The apolipoprotein C-II in plasma activates while apolipoprotein C-III inhibits the lipolytic activity of LPL (Kinnunen *et al.*, 1977; Schoonjans *et al.*, 1999). Insulin has also been shown to regulate the activity of LPL during adipogenesis in 3T3-L1 cells (Semenkovich *et al.*, 1989). Interestingly, PPAR response element is found in human LPL promoter site and PPARγ agonist upregulates the mRNA level of LPL in 3T3-L1 preadipocytes suggesting that PPARγ could be the upstream regulator of this lipolytic enzyme (Schoonjans *et al.*, 1996). Furthermore, increased expression of PPARγ during adipogenesis also elevates the expression of LPL and promotes lipid accumulation in adipocytes (Naowaboot *et al.*, 2014). In addition, reduced LPL activity is associated with coronary artery disease while

the increased activity may lead to hypotriglyceridemia due to the imbalanced distribution of fatty acids (Schneider *et al.*, 2002).

The circulation of free fatty acids within the body could be altered by the variation in the tissue-specific expression pattern of LPL (Preiss-Landl *et al.*, 2002). LPL also has a gatekeeping role in directing the lipid deposition in other tissues (Greenwood, 1985). Free fatty acids are moved into adipose tissue for storage in the event of increased ratio of adipose tissue to muscle LPL activity which is common in obese individuals while the direction of the free fatty acid flow is reversed from the storage to oxidation in muscle if the above ratio is switched the other way around (Kern, 1997). Hence, the activity of LPL is boosted in muscles and fat tissues of individuals who are physically active and obese, respectively (Kern, 1997).

2.3.3 Fatty-Acid-Binding Proteins

Fatty-acid-binding proteins (FABPs) are carrier proteins with high affinity for saturated fatty acids, unsaturated fatty acids and eicosanoids. They facilitate the entry of fatty acids across the plasma membrane, distribute fatty acids within the cell, determine fatty acids storage compartments, regulate fatty acid metabolic enzyme activity and protect the enzymes and membrane from detergent-like effects of fatty acids (Vogel Hertzel & Bernlohr, 2000). FABPs are comprised of nine family members and expressed in various tissues including liver, intestine, heart, lungs, brain and testis. Among the nine members, FABP4 is predominantly expressed in adipocytes and macrophages. The FABP4 also known as 14-15 kDa size adipocyte lipid-binding protein (ALBP) and adipocyte protein 2 (aP2). It is estimated that 3% of the total soluble cellular protein is composed of aP2 in 3T3-L1 cells (Coe & Bernlohr, 1998). Chronic exposure to high extracellular lipids can elevate aP2 level in adipocytes, indicating that fatty acid exposure regulates aP2 expression (Veerkamp & Van Moerkerk, 1993). A previous research

finding has shown that FABP4-deficient mouse preadipocytes displays elevated PPARγ expression and enhances adipogenesis compared to wild-type cells (Garin-Shkolnik *et al.*, 2014). This group also demonstrated that FABP4 induces proteasomal degradation of PPARγ by stimulating ubiquitination and hence, suggests that FABP4 negatively regulates adipogenesis by downregulating PPARγ in adipocytes (Garin-Shkolnik *et al.*, 2014). FABPs also build up fatty acid pool in the cytoplasm to increase substrate availability for lipid metabolizing enzymes such as glycerol-3-phosphates or hormone-sensitive lipase (Haunerland & Spener, 2004). In addition, FABP4 also directly interact with hormone-sensitive lipase and participate in lipolysis by transporting the free fatty acids generated after hydrolysis of triglycerides (Shen *et al.*, 1999).

2.3.4 Kruppel-Like Factor 15

Kruppel-like factors (KLFs) are members of zinc-finger transcription factors which are crucial regulators of adipose tissue development. The presence of highly conserved Cys2/His2 zinc fingers in triplets is a unique feature of the KLF family (Bieker, 2001). The three zinc fingers at the carboxyl-terminal allow KLFs to bind at GC- and CACCC-boxes of DNA for transactivation (Turner & Crossley, 1999). Among all KLF member, KLF 15 is abundantly expressed in adipose, kidney, liver, heart and skeletal muscle tissues and is involved in adipogenesis (Uchida *et al.*, 2000). Mori and coworkers showed that the expression of KLF 15, which is a downstream effector of C/EBPβ or C/EBPδ increases at day 6 of adipogenesis in 3T3-L1cells (Mori *et al.*, 2005). However, inhibition of KLF 15 could impair PPARγ, C/EBPα, aP2, or glucose transporter 4 (GLUT4) expression while its overexpression could raise PPARγ during an earlier stage of differentiation (Wu & Wang, 2013). Apart from this, overexpression of KLF 15 in adipocytes could also initiate adipocyte maturation and modulates gluconeogenesis by increasing GLUT4 expression (Gray *et al.*, 2002). On the other hand, reduced KLF 15 expression was formerly observed in adipose tissue of diet-induced obese mice while

KLF15$^{-/-}$ mice are prevented from high fat diet-induced insulin resistance and also suffered from hypoglycemia after an overnight fast (Gray *et al.*, 2007; Sartoretto *et al.*, 2008). Therefore, these findings are suggestive of possible central role attributed to KLF15 in modulating glucose and lipid metabolism.

2.4 Signaling Pathways Involved in Adipogenesis

Adipogenic differentiation involves the commitment of numerous cellular signaling pathways to control PPARγ and C/EBPα, the key regulators of adipogenesis. Extensive studies on fat cell biology have unveiled several signaling pathways that play an important regulatory role in this differentiation process. These pathways include insulin signaling, adenosine monophosphate (AMP)-activated protein kinase (AMPK), mitogen-activated protein kinase (MAPK), Wnt signaling, and histone deacetylase Sirtuin 1 (Sirt1) signaling pathways.

2.4.1 Insulin Signaling Pathway

Insulin is required throughout preadipocytes differentiation and hence insulin signaling is crucial for adipogenesis. Although the regulation of glucose and lipid metabolism is the ultimate role of insulin, it also regulates the growth and differentiation of adipose tissue (Shaham *et al.*, 2008). Insulin performs its biological role by docking to the insulin receptor (IR), a tyrosine kinase that will be phosphorylated and activated upon insulin binding. Furthermore, insulin receptor substrate (IRS) proteins act as the bridging molecule connecting IR activation to downstream kinase cascades for the activation of several pathways including phosphatidylinositol 3-kinase PI3K and MAPK pathways (Fritsche *et al.*, 2008; Mi *et al.*, 2015)

It has been reported that the inhibition of insulin-dependent IR activation during the early phase of adipogenesis prevents the activation of PI3K/AKT pathway and blocks the differentiation in 3T3-L1 preadipocytes (Kwon *et al.*, 2012). Moreover, mice adipose

tissue lacking IR have reduced fat mass and levels of C/EBPα, further revealing the significance of insulin signaling to sustain the physiological functions of adipocytes (Blüher *et al.*, 2002). Similarly, in IRS-1and IRS-2 double-deficient mouse embryonic fibroblast cells, adipogenesis is severely impaired together with reduced expression of PPARγ, C/EBPα and its downstream adipogenic markers (LPL and FABP) (Miki *et al.*, 2001). Numerous antiadipogenic agents were reported to regulate the cellular differentiation of adipocytes via insulin-mediated signaling pathways, indicating the importance of IRS proteins in mediating insulin signaling that governs adipogenesis-associated downstream signaling pathways (Liu *et al.*, 2014; Mi *et al.*, 2015; Yoon *et al.*, 2013; Zang *et al.*, 2013).

2.4.2 AMP-activated Protein Kinase (AMPK) Pathway

AMP-activated protein kinase (AMPK) is an energy metabolic sensor and regulator that is involved in energy balance including food intake and calorie expenditure. Impairment of AMPK in energy homeostasis leads to metabolic disorders and cardiovascular diseases (Lage *et al.*, 2008). Elevated AMP: ATP ratio in the event of metabolic stress induced by a drop in cellular ATP level activates AMPK. In addition, AMPK is allosterically activated by AMP to monitor energy balance within the cell by restoring AMP: ATP ratio via modulating metabolic process that produces or consumes ATP in various body tissues (Kahn *et al.*, 2005).

Interestingly, AMPK is a key regulator of lipid metabolism. AMPK phosphorylates and modulates several regulatory enzymes participating in sterol and fatty acid syntheses such as hydroxymethyl-glutaryl-CoA reductase and acetyl-CoA carboxylase (ACC) (Hadie & Carling, 1997). The upregulation of phosphorylated AMPKα increases ACC activity and reduces PPARγ, C/EBPα and FAS expression and leads to the inhibition of adipogenesis (He *et al.*, 2013; Kim *et al.*, 2014). On the contrary, suppression of AMPKα

phosphorylation could also reduce the expression of adipogenic transcription factors and eventually impairs cellular differentiation in 3T3-L1 cells suggesting that AMPK plays contradicting roles in modulating adipogenesis (Choi *et al.*, 2015). Activated AMPK not only induces fatty oxidation but also inhibits fatty acid synthesis by reducing the malonyl-CoA levels during physical activities in muscles, liver and hypothalamus (Kahn *et al.*, 2005; López *et al.*, 2007). Moreover, AMPK also attenuates lipolysis by impairing protein kinase A (PKA)-mediated hormone-sensitive lipase (HSL) activation and phosphorylating HSL as observed previously in adipocytes (Dagon *et al.*, 2006; Sullivan *et al.*, 1994). However, cAMP-mediated activation of AMPK leads to activation of lipolysis in 3T3-L1 adipocytes (Yin *et al.*, 2003). Mice fed a high-fat diet lacking both AMPKα1 and AMPKα2 have increased fat mass as compared with its wild-type (Villena *et al.*, 2004; Zhang *et al.*, 2012). Interestingly, AMPK could favor adipogenesis via different signaling pathways such as extracellular signal-regulated kinase (ERK) pathway, Akt/mTOR signaling and Wnt/β-catenin pathway (Kim *et al.*, 2012; Pantovic *et al.*, 2013; Zhao *et al.*, 2011). In addition, AMPK signaling is modulated by several compounds such as apigenin, ursolic acid and indole-3-carbinol which are also reported to inhibit adipogenesis and stimulate lipolysis via 3T3-L1 cells (Choi *et al.*, 2014; He *et al.*, 2013).

2.4.3 Mitogen-Activated Protein Kinase (MAPK) Pathway

The extracellular signal-regulated kinases (ERKs) 1 and 2; c-Jun amino-terminal kinases (JNKs) 1, 2 and 3; and p38 MAPK (p38) α, β, γ, and δ are MAPKs mainly involved in coordinating intracellular signaling pathway in cellular proliferation and differentiation (Jeffrey *et al.*, 2007). Numerous studies have elucidated the role of MAPKs in adipogenesis. The involvement of ERK in adipocyte differentiation was first demonstrated through the overexpression of Ras, a potent activator of ERK pathway which leads to adipogenesis in 3T3-L1 preadipocyte cells (Benito *et al.*, 1991). Moreover,

Sale *et al*. have confirmed that ERKs are required for adipogenic differentiation of preadipocytes (Sale *et al.*, 1995). However, it is shown that ERK-mediated phosphorylation of PPARγ impairs its transcriptional activity and blocks adipogenesis, suggesting that ERK could play both positive and negative regulatory roles in modulating adipocyte differentiation (Camp & Tafuri, 1997). On the other hand, *in vivo* study with ERK1 deficient mice on the high-fat diet showed reduced adiposity and decreased insulin resistance (Bost *et al.*, 2005). Besides this, inhibition of p38MAPK during the early phase of adipogenesis could impair C/EBPβ phosphorylation and its transactivation activity leading to reduced adipose cell formation. On top of that, mice lacking JNK1 were prevented from HFD-induced weight gain and have improved insulin sensitivity while JNK phosphorylation inhibition promotes adipogenesis in 3T3-L1 cells (Hirosumi *et al.*, 2002; Liu *et al.*, 2014).

2.4.4 Wnt Signaling Pathway

Wnts are glycoproteins that bind to frizzled (FZD) transmembrane receptors to initiate a tightly preserved signaling pathway that possesses a crucial role in regulating several biological processes including cell proliferation and differentiation (Logan & Nusse, 2004). This signal transduction takes place in two ways; either through canonical (β-catenin dependent) or non-canonical (β-catenin independent) pathways (Strutt, 2003; Veeman *et al.*, 2003). In the canonical pathway, β-catenin is the main transcriptional activator for the transactivation of Wnt target genes. In the absence of Wnt, cytoplasmic β-catenin is engaged to a degradation complex composed of Axin and adenomatous polyposis coli (APC), which allows phosphorylation by casein kinase I and glycogen synthase kinase 3-β (GSK3β) and eventually leads to ubiquitination and proteasomal degradation. However, in the presence of Wnt, the binding of Wnt to FZD receptors and low-density-lipoprotein-receptor-related protein-5 or -6 (LRP5/6) co-receptors inactivates the degradation complex and subsequently induces hyperphosphorylation of

β-catenin. The activated β-catenin translocates to the nucleus and binds to lymphoid-enhancer-binding factor/T-cell-specific transcription factor (LEF/TCF) family of transcription factors to activate Wnt target genes (Christodoulides *et al.*, 2009).

Interestingly, Wnt signaling specifically through the canonical pathway has been reported to inhibit adipogenesis *in vitro*. Initiation of Wnt signaling leads to dysregulation of the cell cycle, blocked mitotic clonal expansion, impaired PPARγ and C/EBPα expression and eventually inhibits adipogenic differentiation in 3T3-L1 preadipocytes (Ross *et al.*, 2002). Moreover, disruption of intracellular Wnt signaling by overexpression of Axin or dominant-negative TCF4 in 3T3-L1 preadipocytes activates adipogenesis (Ross *et al.*, 2000). Besides this, the expression of a Wnt signaling activator, Wnt10b was found elevated in preadipocytes and declined upon induction of adipogenesis (Bennett *et al.*, 2002). However, ectopic expression of Wnt10b stabilizes β-catenin and impairs adipogenesis in 3T3-L1 preadipocytes (Krishnan *et al.*, 2006). On the other hand, FABP4-mediated Wnt10b expressing transgenic mice showed reduced total body fat, improved sensitivity towards glucose and insulin as well as prevented from HFD-induced adipose tissue accumulation (Longo *et al.*, 2004).

2.4.5 Sirtuin1 Signaling Pathway

Sirtuins (Sirts) are histone deacetylases, known to regulate numerous physiological processes including inflammation, cellular senescence, proliferation, and metabolism. Although seven mammalian sirtuin proteins (Sirt1-Sirt7) have been discovered, Sirt1 is the most well-studied human sirtuin protein (Chung *et al.*, 2010). Sirt1 is a nuclear protein that plays a crucial regulatory role in cellular metabolic and physiological processes by its deacetylase activity on histones and non-histone proteins which include nuclear factor (NF)-κB, FOXO3, p53, PPARγ, PPAR-γ coactivator 1α (PGC-1α), and endothelial nitric oxide synthase (eNOS) (Yang & Sauve, 2006). In addition, Sirt1 also modulates

adipogenesis and lipolysis. Sirt1 inhibits transactivation of PPARγ by interacting with nuclear receptor co-repressor and silencing mediator of retinoid and thyroid hormone receptors (SMRT), which then reduces adipogenesis and induces lipolysis (Picard *et al.*, 2004). However, the absence of Sirt1 in adipocytes promotes PPARγ hyperactivity and hence increases adipocytes differentiation (Picard *et al.*, 2004). On top of that, adipocyte-specific Sirt1 knock-out mice on HFD exhibited increased adipocyte hypertrophy and reduced sensitivity towards glucose and insulin (Mayoral *et al.*, 2015). It has been reported that overexpression of Sirt1 reduces the mRNA levels of C/EBPα, C/EBPδ and aP2 in 3T3-L1 preadipocytes further proving that Sirt1 negatively regulates adipocyte differentiation (Mayoral *et al.*, 2015).

Interestingly, numerous studies have reported Sirt1 inhibits adipogenesis through nucleocytoplasmic signaling modulation of FoxO1 (Lai *et al.*, 2012; Lee *et al.*, 2017; Tseng *et al.*, 2011). Sirt1 binds and deacetylates FoxO1 at three residues within the DNA binding domain to promote nuclear entry (Daitoku *et al.*, 2004; van der Heide & Smidt, 2005). Upon nuclear translocation, FoxO1 binds to peroxisome proliferator response elements (PPRE) then interferes with DNA occupancy of PPARγ and inhibits its transcriptional activity thereby impairs gene expression of adipogenic markers which is essential for the maturation of adipocytes (Armoni *et al.*, 2006; Wang & Tong, 2009). On the other hand, FoxO1 acetylation in the absence of Sirt1 promotes adipogenesis in 3T3-L1 preadipocytes by increasing the expression of adipogenic transcription factors (Ahn *et al.*, 2013). However, acetylation of FoxO1 promotes its phosphorylation by PI3K/Akt pathway that initiates nuclear export to the cytoplasm and eventually ubiquitination followed by proteasomal degradation (Matsuzaki *et al.*, 2005). Therefore, phosphorylation of FoxO1 transactivates PPARγ and induces adipogenesis in 3T3-L1 cells (Daitoku *et al.*, 2011).

2.5 Development of adipose tissues and obesity

Vertebrates store excess calories in the form of lipids in adipocytes and hence the building up of fat cells leads to the formation of adipose tissues in the body. Adipose tissues possess a crucial role in regulating the levels of free fatty acid and triglycerides in circulation, however, excessive accumulation results in promoting the progression of obesity. It has been shown that adipose tissue development is mediated either by hyperplasia or hypertrophy of adipocytes (Wolfram *et al.*, 2006). An increase in the adipocytes number by adipogenesis of preadipocytes is hyperplasia while an increase in the adipocyte size upon continuous lipid accumulation is termed as hypertrophy (Christodoulides *et al.*, 2009).

Adipocyte hypertrophy is associated with reduced insulin sensitivity which is a risk factor for developing type 2 diabetes even in lean individuals (Arner *et al.*, 2010). In addition, individuals with hypertrophic obesity are more susceptible to poorer metabolic profile as compared to those with hyperplastic obesity (Hoffstedt *et al.*, 2010). A short-term weight gain study in lean men showed that adipocytes size increased significantly following weight gain, yet adipocytes number remain unchanged (Salans *et al.*, 1971). Furthermore, several other reports also confirm that significant reduction in adipose tissue as a result of weight loss is mediated by the decrease in the adipocytes size and not due to a decrease in adipocytes number (Häger *et al.*, 1978; Spalding *et al.*, 2008). Generally, there are two types of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT) that play important regulatory roles in energy homeostasis (Figure 2.3).

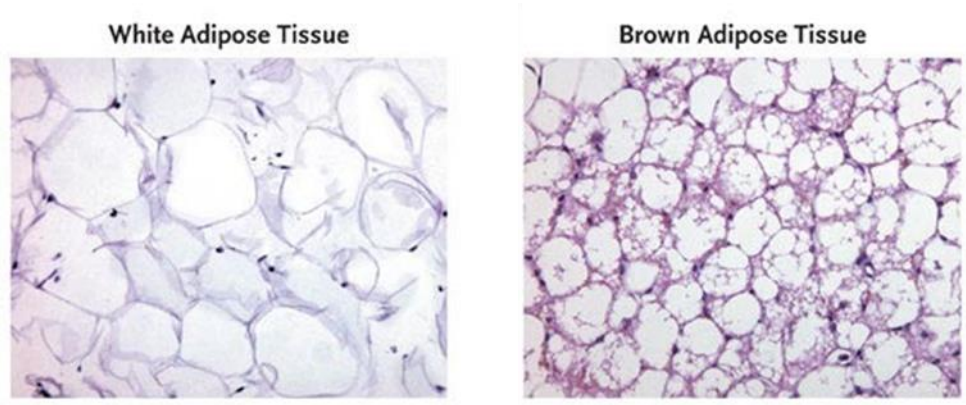

Figure 2.3: White and brown adipose tissue specimen stained with hematoxylin and eosin. WAT specimen shows a single large lipid vacuole in each white fat cell. BAT specimen shows granular cytoplasm containing mitochondria and multiple fat vacuoles. Adapted and modified from (van Marken Lichtenbelt *et al.*, 2009).

2.5.1 White Adipose Tissue

The white adipose tissue is composed of 25% of human body weight and its main role is to store energy in the form of lipids in the event of calorie abundant and act as an energy source during calorie deprivation. The fatty acids are stored in a large unilocular lipid droplet that occupies over 90% of the cell volume leaving very minimal space for other cellular organelles (Figure 2.3). It has been reported that approximately 60 to 80% of WAT cells are not homogenously mature adipocytes, but vascular stroma cells composed of preadipocytes, macrophages, stem cells, and endothelial cells (Tchoukalova *et al.*, 2004). In addition, WAT also serves as a thermal insulator and mechanical shock absorber for internal body organs (Trayhurn, 2007). Excessive accumulation of WAT has been involved in the development of obesity-related disorders. However, the distribution of fat is considered more important than its total amount in determining the risk of obesity-associated diseases. Adipose tissues accumulation in the upper body (abdominal fat) is mainly associated with the development of metabolic diseases (Goossens, 2017). Interestingly, WAT has metabolic (lipogenesis, fatty acid oxidation, and lipolysis) and

endocrine (production of adipokines) functions (Boudina & Graham, 2014). Moreover, WAT is also recognized as a crucial endocrine organ that secretes various adipokines (cytokines produced by adipocytes) such as leptin, adiponectin, tumor necrosis factor-α, interleukin-6, and resistin to modulate appetite, calorie expenditure, sensitivity towards insulin, inflammation and coagulation (Hauner, 2005). Therefore, the involvement of these secretory hormones in overall metabolic regulation is also directly linked to the development of obesity and its comorbidities.

2.5.2 Brown Adipose Tissue

The brown adipose tissue is an energy-expending and heat producing fat tissue found abundant in hibernating animals and infants that helps in maintaining body temperature. In contrast to WAT, brown adipocytes contain multilocular lipids in small-vesicles, central nucleus and numerous mitochondria which is attributed to the brown colour of BAT (Cinti, 2009) (Figure 2.3). Brown adipose tissue is distributed throughout the cervical, supraclavicular, axillary, paravertebral, mediastinal, and upper abdominal areas to warm the blood supply to important body organs (Wehrli *et al.*, 2007). The multiple small cytosolic lipids serve as fuel for oxidative phosphorylation and heat production that is predominantly controlled by high-density mitochondria in the BAT (Feldmann *et al.*, 2009). The ultimate role of BAT is to dissipate energy via heat production and this is mediated by the mitochondrial transmembrane protein, uncoupling protein-1 (UCP1) that consequently increases fatty acid oxidation as well as induces thermogenesis (Klingenberg, 1999; Mattson, 2010). It was previously shown that chronic cold exposure prevents diet-induced obesity in mice by boosting thermogenesis of BAT via UCP1 activation (Ma *et al.*, 2012).

2.6 Obesity

Obesity has become a global epidemic and hence considered as a major public health issue in both developed and developing countries. Earlier, obesity was prevalent among adults regardless of their genders, however, the worldwide incidence of childhood obesity has also increased dramatically in recent years (Sabin & Kiess, 2015). There are several factors that contribute to the development of obesity such as high intake of energy-dense foods, reduced physical activity, sedentary lifestyle, modern transportation and rapid urbanization (Jeffery & Utter, 2003). Obese individuals are highly susceptible to serious non-communicable diseases such as type 2 diabetes, cardiovascular disease, osteoarthritis, dyslipidemia and some cancers in which elevates the rate of obesity-mediated mortality and morbidity (Garg *et al.*, 2014; Martin-Rodriguez *et al.*, 2015).

Obesity is caused by impaired energy balance when calorie intake surpasses total calorie spent which is mediated by the basal level of metabolism, physical activities and thermogenesis (Hill *et al.*, 2012). Excess calorie intake with physical inactivity promotes energy storage in the form of lipids and leads to the expansion of adipose tissue. Over a period of time, abnormal or excessive fat accumulation in the body eventually end up with increased body weight and leads to the obese condition. Several methods to measure body fat have been identified which includes skinfold measurement, waist circumference and body mass index (BMI) (Pi-Sunyer, 2002). Among them, BMI is a widely used method to classify overweight and obesity in adults. By definition, BMI is the ratio of kilograms by the square to height in meters (kg/m^2) of a person. Asian individuals with $BMI \geq 23$ are overweight while those with $BMI \geq 27.5$ are classified as obese (Deurenberg *et al.*, 1998; Ko *et al.*, 2001). It has been reported that Asians are prone to type 2 diabetes, hypertension, and dyslipidemia at lower levels of BMI which has a greater degree of obesity compared with Caucasians (Zaki *et al.*, 2010). The WHO has projected that in 2030, Malaysia would have a 164% increase in total number of diabetics compared to the

patient population recorded three decades ago (Mafauzy, 2006). Hence, obesity has become a major clinical and public health issue that threatens to surpass already expended healthcare services in Malaysia. Table 2.1 shows the classification breakdown of BMI in details.

Table 2.1: BMI classification. Adapted from (Organization, 2017).

BMI (kg/m^2)	Classification
< 18.5	Underweight
18.5 - 22.9	Normal
23.0 – 27.5	Overweight
> 27.5	Obese

2.6.1 Insulin Resistance

Insulin resistance is a condition of reduced sensitivity of insulin-target tissues such as muscle, adipose and liver tissues as well as other body tissues towards insulin regardless of its normal or elevated level in the blood (Perseghin *et al.*, 2003). Insulin sensitivity is determined by body fat distribution. Studies on obese human subjects have shown that increased visceral but not subcutaneous adipose tissue mass contributes to the development of insulin resistance (Group & Group, 2003; McLaughlin *et al.*, 2011). Furthermore, abdominal fat is considered more lipolytic than subcutaneous fat, and it also hinders the antilipolytic action of insulin, which makes it highly important in causing insulin resistance, and increases the risk of developing diabetes (Roden *et al.*, 1996). Inflammation and hyperinsulinemia are important obesity-mediated risk factors for insulin abnormalities (Ye, 2013). The secretion of pro-inflammatory cytokines by adipose tissue-resident immune cells, mainly by macrophages facilitates the obesity-induced inflammation (Lee & Lee, 2014). As a result, the insulin signaling pathway in adipocytes

and hepatocytes is repressed through inhibition of IRS-1, reduction of PPARγ activity as well as stimulation of lipolysis (White, 2002; Ye, 2008).

Hyperinsulinemia, a condition of elevated fasting plasma insulin as a consequence of either increased insulin production or reduced insulin clearance may also contribute to insulin resistance in obese people (Ye, 2007). Overproduction of insulin in transgenic mice and an overdose of insulin in human has resulted in hyperinsulinemia and subsequently lead to insulin resistance (Shanik *et al.*, 2008). Insulin-mediated metabolic pathways such as glucose transport, glycogen synthesis and anti-lipolysis are severely impaired under the condition of insulin resistance which is also highly associated with the development of hypertension, type 2 diabetes, dyslipidemia and fatty liver diseases (Bray, 2004). In adipose tissue, reduced insulin sensitivity attenuates insulin-stimulated glucose uptake and anti-lipolytic effect of insulin thereby impairs the physiological functions of adipocytes (Hardy *et al.*, 2012). Dietary modification, calorie restriction and enhanced physical activity among obese people have been shown to reduce weight as well as improve insulin sensitivity (Murphy *et al.*, 2011). In addition, weight loss promoting drugs such as orlistat and sibutramine have also resulted in better glycemic control in parallel with body weight reduction in diabetic obese patients (McNulty *et al.*, 2003; Miles *et al.*, 2002). Furthermore, *in vivo* studies have demonstrated that boosted exercise in a high-fat-diet-induced obese rat resulted in inflammatory suppression in the liver, muscle and adipose tissues on par with enhanced insulin signaling (Oliveira *et al.*, 2011).

2.7 Managing Obesity and Related Complications

The main therapeutic approach in managing the prevalence of obesity and its comorbidities such as cardiovascular diseases is by weight management. Obese individuals can achieve significant weight reduction at individual-level through their diet and lifestyle modification as well as by switching to available pharmacological

intervention and surgery (Health, 1995; Wing *et al.*, 2001). A balance between calorie intake and expenditure is a must for weight management and hence, calorie restriction is one of the popular approaches for weight-loss in the obese subject (Hill *et al.*, 2012; Wadden *et al.*, 2012). Moreover, limiting calorie to ~ 1100 kcal/day promotes weight reduction, decreased liver lipid content, improved insulin sensitivity in skeletal muscle and liver as well as reduced basal glucose production rate in obese patients (Kirk *et al.*, 2009). A prior study has shown that dietary modification such as consuming more vegetables, fruits, whole grains, nuts, and yogurt negatively correlate with long-term weight gain, however, resulted otherwise in subjects consuming more junk foods, sugar-sweetened beverages, processed meats and fast food (Mozaffarian *et al.*, 2011).

In addition, implementing physically active lifestyle also contributes to the prevention of weight gain or induces weight loss. Active lifestyle in combination with diet management produces better results in obtaining and maintaining healthy body weight. A prior study in sports medicine has recommended that at least 150-250 minutes/week of moderate physical activities for overweight and obese individuals since it is associated with overall health improvement, long-term weight loss and prevention of weight regain when accompanied by dietary control (Donnelly *et al.*, 2009). In this regard, several other studies have shown that obese patients whom regularly engage in moderate to high-intensity physical exercise have reduced BMI, percentage body fat and hepatic steatosis, better glycemic control, as well as improvement in the cardiovascular health, insulin resistance and lipid profile (Bluher *et al.*, 2014; Ho *et al.*, 2012; Khoo *et al.*, 2015; Little *et al.*, 2014; Monteiro *et al.*, 2015; Oh *et al.*, 2014; Rock *et al.*, 2014).

On the other hand, obesity surgeries such as liposuction and bariatric surgery are also being practised as an alternative weight reduction method especially in a morbidly obese subject who are unable to withstand weight loss by nonsurgical means and suffers from

obesity-related comorbidities. Bariatric surgery has resulted in significant weight reduction, reduced the incidence of type-2 diabetic mellitus, reduced elevated blood pressure, cardiovascular diseases and improved serum lipid parameters in patients with severe obesity (Douglas *et al.*, 2015; Milone *et al.*, 2015). In addition, large-volume liposuction in extremely obese subjects reveals a positive improvement in plasma insulin, blood glucose level, insulin sensitivity, cardiovascular risk and blood pressure associated with weight loss (Boriani *et al.*, 2014; Hunstad, 1996; Narsete *et al.*, 2012). However, gastrointestinal ulceration, incisional and internal herniation and nutritional deficiency while thromboembolism, dehiscence, wound infections and seromas can be caused by bariatric surgery and liposuction in morbidly obese individuals (Eldar *et al.*, 2011; Narsete *et al.*, 2012). In addition to adopting a healthy diet, lifestyle modification and obesity surgery, the pharmacological intervention also has been proven successful in the management of obesity and its associated diseases, although some drugs are with limited efficacy or serious adverse effects.

2.8 Synthetic anti-obesity drugs

Weight-loss can be enhanced with medication, though several BMI criteria need to be met by an obese individual before receiving anti-obesity pharmacotherapies. Generally, obese patients with BMI >30kg/m^2 or BMI of 27 kg/m^2 accompanied by obesity-related co-morbidities are qualified to be prescribed with anti-obesity drugs (Apovian *et al.*, 2015). Nevertheless, obese subjects on weight-loss pharmacotherapies require long-term commitment towards the prescribed medications as many individuals are subjected to weight-regain upon withdrawal. Furthermore, patients consuming these daily medications also must be aware of the cost and possible side effects. Historically, the main class of synthetic drugs used for weight-reduction in obese population is appetite suppressor and gastrointestinal lipase inhibitor which respectively target to reduce food intake and fat absorption. However, there are several other potential weight-loss drugs that are currently

in the development phase. These drugs target central neuropeptide signaling, monoamine neurotransmission, intestinal peptide signaling, pancreatic hormone signaling, and adipose tissue hormone signaling (Colon-Gonzalez *et al.*, 2013). Table 2.2 shows commonly prescribed synthetic drugs for weight management in obese subjects and its associated adverse effects.

Table 2.2: Examples of common synthetic anti-obesity drugs.

Drug	Mode of action	Side effects	References
Phentermine* (Fastin)	Noradrenalin releaser Appetite suppressant	Headache, hypertension, insomnia, dizziness, palpitation, dry mouth and constipation	(Smith *et al.*, 2013) (Bain *et al.*, 2014)
Fluoxetine (Prozac)	Serotonin reuptake inhibitor Reduce food intake	Nervousness Mood depression	(Ward *et al.*, 1999) (Arnold *et al.*, 2002)
Sibutramine* (Meridia)	Norepinephrine and serotonin reuptake inhibitor Satiety inducer Increase thermogenesis	Headache, insomnia, dry mouth and constipation Risk of myocardial infarction and stroke in the long run	Li *et al.*, 2005) (Luque & Rey, 2002) (García-Morales *et al.*, 2006)
Rimonabant* (Acomplia)	Cannabinoid-1 receptor antagonist Induce lipolysis and fatty acid oxidation	Nausea, anxiety, joint pain, and diarrhoea Increase VLDL secretion	(Leite *et al.*, 2009) (Backhouse *et al.*, 2012)
Orlistat (Xenical)	Gastric and pancreatic lipase inhibitor Reduces dietary fat absorption Improves dyslipidemia	Diarrhoea, flatulence, bloating, abdominal pain and oily stool	(Sahebkar *et al.*, 2017) (Mannucci *et al.*, 2008) (Sahebkar *et al.*, 2017)

Note: *Withdrawn from the market due to life-threatening adverse effects

2.9 Naturally-derived Anti-adipogenic/Obesity Drugs

The pharmacological methods to address obesity are losing popularity since the presently available anti-obesity drugs have considerable serious adverse effects, prone to tolerance and only provide short-term weight loss. Therefore, there is still a need to find novel, safe and cost-effective therapeutic approaches although several possible targets to treat obesity have been recently discovered (Mayer *et al.*, 2009). Traditionally employed herbal based medicines have been very popular worldwide in recent years due to their natural origin, nutraceutical properties and acknowledgement by national health authorities (Who, 2013). In recent years, studies of natural products including crude extracts and isolated natural pure compounds have provided evidence for their preventive and therapeutic potentials in the management of obesity (Vermaak *et al.*, 2011). Several bioactive compounds abundantly found in medicinal plants, fruits and vegetables have been proven to inhibit adipogenesis, induce lipolysis and prevent the development of obesity *in vitro*, *in vivo* as well as in obese human subjects (Kim *et al.*, 2015; Song *et al.*, 2013; Wang *et al.*, 2014). The ability to modulate adipose tissue functions by phytochemicals underscores the potential of these molecules for the development of novel phytotherapeutic approaches for obesity treatment (Table 2.3).

Table 2.3: Potential phytochemicals for the treatment of obesity.

Phytochemical	Bioactive compound (Source)	Effects	References
Polyphenols	Ferulic acid (plant cell wall)	↓ high fat diet-induced obesity, serum triglyceride and cholesterol level in mice ↓ insulin resistance and serum leptin levels in obese mice	(Son *et al.*, 2010) (Naowaboot *et al.*, 2016)
	Resveratrol (skin of grapes, blueberries, raspberries, mulberries)	↓ adipogenesis ↓ the expression of adipogenic transcription factors and genes ↑ lipolysis and inhibit lipogenesis ↓ lipid accumulation in matured adipocytes ↑ insulin sensitivity	(Baile *et al.*, 2011) (Fischer-Posovszky *et al.*, 2010)
	Curcumin (Turmeric)	↓ lipid accumulation and plasma free fatty acid levels in high-fat diet induced obese rat Regulates lipid metabolism ↓ intracellular lipid accumulation	(Pongchaidecha *et al.*, 2009) (Alappat & Awad, 2010)
Flavonoid	Quercetin (plant pigment in onions, apples, Ginkgo biloba)	↓ adipogenic differentiation via AMPK pathway in 3T3-L1 cells ↓ hepatic triglyceride accumulation and dyslipidemia in obese mice	(Ahn *et al.*, 2008) (Jung *et al.*, 2013)

Table 2.3, continued

Alkaloids	Capsaicin (a spicy component of chilli pepper)	↓ body weight gain and adipose tissue size in high-fat diet fed the rat ↓ obesity-induced inflammation, metabolic disorders, and liver diseases	(Joo *et al.*, 2010) (Reyes-Escogido Mde *et al.*, 2011)
	Ephedrine (Ephedra plant)	Suppress appetite ↑ energy expenditure via thermogenesis ↓ total body fat, visceral fat Improves blood pressure in obese subjects	(Astrup *et al.*, 1995) (Carey *et al.*, 2015)
	Caffeine (Coffee beans, tea-leaves, cocoa beans)	↓ body weight and fat accumulation Improves blood lipid levels ↓ serum leptin levels in high-fat diet fed rats Induce lipolysis in matured adipocytes	(Xu *et al.*, 2015) (Liu *et al.*, 2015)
	Nicotine (tomato, potato, eggplant)	Promotes body weight reduction and appetite suppression ↑ thermogenesis in brown adipose tissue in obese rats Improves glucose homeostasis, insulin sensitivity ↓ adipose tissue inflammation	(Seoane-Collazo *et al.*, 2014) (Lakhan & Kirchgessner, 2011)

Table 2.3, continued

Terpenoids	Abscisic acid (plant hormone)	Improves glucose tolerance and obesity-induced inflammation in diabetic obese mice PPARγ agonist that improves sensitivity towards insulin ↓ infiltration of adipose tissue macrophages	(Guri *et al.*, 2007) (Guri *et al.*, 2008)
	Carotenoids (bright red, orange and yellow plant pigments)	↓ adipocyte differentiation and hypertrophy Improves insulin resistance ↓ blood glucose levels by regulating cytokine secretions from adipose tissue	(Lobo *et al.*, 2010) (Gammone & D'Orazio, 2015)
	Lycopene (red pigment found in fruits and vegetables)	↓ leptin, resistin and plasma IL-6 levels in diet-induced obese rats ↓ risk of cardiovascular diseases by inhibition of LDL oxidation and lipid peroxidation	(Luvizotto *et al.*, 2013) (Agarwal & Rao, 2000)
Organosulfur	Ajoene (garlic)	↓ cholesterol synthesis and improves blood pressure Regulate fat cell number through the apoptosis induction	(Cartea & Velasco, 2008) (Yang *et al.*, 2006)

Table 2.3, continued

	Sulforaphane (cruciferous vegetables like broccoli and cabbages)	Inhibit adipogenesis ↓ adipocyte hypertrophy, hepatic triglyceride accumulation *in vitro* and *in vivo* Induce adipocytes browning, lipolysis and fatty acid oxidation in 3T3-L1 cells	(Choi *et al.*, 2014) (Zhang *et al.*, 2016)
Phytosterols	Diosgenin (tubers of Dioscorea wild yam)	Improve dyslipidemia by decreasing the hepatic lipid content in diabetic mice ↓ obesity-induced endothelial dysfunction and improves blood pressure in obese rats	(Uemura *et al.*, 2011) (Szabo *et al.*, 2018)
	Protodioscin (puncture wine and fenugreek)	↓ serum triglyceride, cholesterol and LDL levels ↑ HDL levels	(Wang *et al.*, 2010)

Note: ↑= increase; ↓= decrease

2.10 *Eurycoma longifolia* Jack

Eurycoma longifolia Jack (EL) also known as Tongkat Ali (Figure 2.4) is a tropical medicinal plant classified under the order of Geranial's, genus of *Eurycoma* and family of Simaroubaceae. It is a flowering plant which grows up to 15-18 m in height and commonly found in lowland tropical forest floor up to 500 m above sea level. The leaves of this plant are about 20-40 cm long and it has 2–3 cm long ovoid-shaped green fruit which will turn dark brown after ripening (Bhat & Karim, 2010).

Figure 2.4: *Eurycoma longifolia* plant.
Retrieved from http://chengailimfruittrees.blogspot.my/2014/05/tongkat-ali.html

Moreover, this plant grows well in the presence of partially shady and well-drained soil with adequate water supply (Bhat & Karim, 2010). Typically, it may take up to 25 years for complete maturation of Tongkat Ali plant before it can be harvested (Keng, 1987). Tongkat Ali plant is indigenous to South East Asian countries and is found with various local names based on the place and country it belonging to. Other than Tongkat Ali, this evergreen plant is also locally known as Long Jack, Malaysian Ginseng, Natural Viagra, Setunjang Bumi, Pasak Bumi, Payung Ali, Penawar Pahit, Tongkat Ali Hitam, Cay ba Binh, Ian-don, and Jelaih especially in countries like Malaysia, Indonesia, Vietnam, Cambodia, Myanmar and Thailand (Rehman *et al.*, 2016).

2.10.1 Medicinal uses of *Eurycoma longifolia*

Eurycoma longifolia is an important medicinal plant and has always been a significant traditional remedy used mainly in South-East Asian countries. Almost all the parts of the plant are used traditionally for various medicinal purposes (Table 2.4).

Table 2.4: Traditional uses of *Eurycoma longifolia*.

Plant parts	Traditional Uses	References
Leaves	Washing itches	(Ang *et al.*, 2001)
Fruits	Curing dysentery	(Ang *et al.*, 2001)
Bark	As a vermifuge	(Ang *et al.*, 2001)
Taproots	Treat high blood pressure	(Kuo *et al.*, 2004)
Root bark	Treatment of diarrhoea and fever	(Kuo *et al.*, 2004)
Roots	Aches, ageing, aphrodisiac, antibiotic, appetite stimulant, cancer, diabetes, fever, high blood pressure, jaundice, cachexia, dropsy, malaria, osteoporosis, sexual dysfunction, androgen deficiency, glandular swelling, energy tonic and general health supplement	(Perry & Metzger, 1980) (Sobri *et al.*, 2007) (Chan *et al.*, 1989) (Darise *et al.*, 1982) (Mohd Effendy *et al.*, 2012) (Kuo *et al.*, 2004) (Ang *et al.*, 2001) (Jamal, 2006)

Nowadays, EL is primarily consumed in the form of raw crude powder, capsuled powder, additives in teas, coffees or energy drinks (Bhat & Karim, 2010). Due to the various traditional benefits of EL, many scientific studies have been performed to prove the above-mentioned healing potentials which also pave the way for the isolation of its many bioactive compounds.

2.10.2 Chemical Constituents

EL is rich in various classes of bioactive compound, such as quassinoids, alkaloids, triterpene-type tirucallane, squalene derivatives and phytosterols. Nevertheless, the bitter tasting quassinoids are the major chemical constituent abundantly found in the root of EL. Quassinoids are a group of highly oxygenated triterpenes which is mainly isolated from the plants of Simaroubaceae family and this group of bioactive compounds has gained much attention due to the dynamic pharmacological properties (Guo *et al.*, 2005b).

Eurycomanone, eurycomanol, laurycolactones, eurycomalactone, eurycomalides, longilactone, and eurycolactone are the major isolated quassinoids from EL that have shown various pharmacological effects including antimalarial, anticancer, antiplasmodial, anti-inflammatory and aphrodisiac activities in both *in vitro* and *in vivo* studies (Ang *et al.*, 1995; Chan *et al.*, 1989; Chan *et al.*, 1992; Low *et al.*, 2013; Tran *et al.*, 2014; Wong *et al.*, 2012).

2.10.2.1 Eurycomanone

Eurycomanone is one of the well-studied quassinoids that is predominantly found in the root of EL plant. It was found that the composition of eurycomanone in ethanolic, n-butanolic- and aqueous-based fractions of EL varies, hence Lethal Dose 50 and effective doses are different among the fractions. However, the aqueous-based fractions of EL are the safest among the others with the highest LD50 value of >3000 mg/kg while the n-butanol fraction is the most toxic due to the presence of eurycomanone. A pharmacokinetic study has reported eurycomanone to possess poor oral bioavailability, high volume of distribution and shorter biological half-life (Low *et al.*, 2005). Eurycomanone is a cytotoxic bioactive compound found in EL and it has shown cytotoxicity effects on a panel of human cancer cell lines including, breast, colon, fibrosarcoma, lung, melanoma, prostate, liver and leukemia by modulating cell cycle arrest and apoptotic pathways (Kardono *et al.*, 1991; Tong *et al.*, 2015; Zakaria *et al.*, 2009). In another *in vivo* study, eurycomanone is reported to enhance spermatogenesis through phosphodiesterase and aromatase inhibition in steroidogenesis suggesting the aphrodisiac property of EL root extract is due to the presence of eurycomanone (Low *et al.*, 2013). Furthermore, eurycomanone also showed a potent antimalarial effect against *P. falciparum* strains that have developed resistance towards chloroquine treatment (Chan *et al.*, 2004; Miller *et al.*, 1986). Besides, the derivative of eurycomanone has been

identified as a potent NF-κB inhibitor and this signifies the potential use of EL to treat or prevent inflammatory diseases (Tran *et al.*, 2014).

2.10.3 Standardized Quassinoids composition from *Eurycoma longifolia*

The standardized quassinoids composition from *Eurycoma longifolia* (SQEL) was prepared from the roots of EL by Low and colleagues (Low *et al.*, 2011). Previously, a toxicity study showed SQEL possess LD_{50} of >2000 mg/kg in rats suggesting that any dose administered below than this is safe in rodent experiments (Low *et al.*, 2014). The pharmacokinetic study showed eurycomanone has lower bioavailability and poor membrane permeability (Ahmad *et al.*, 2018; Low *et al.*, 2005). In a previous study, the author claimed that oral treatment was less effective than the intraperitoneal injection in investigating the anti-tumor effect of EL extract on tumor xenograft model (Al-Salahi *et al.*, 2014). Therefore, administration of SQEL using intraperitoneal injection is preferred as per used in other studies (Al-Salahi *et al*., 2013; Low *et al*., 2013; Tong *et al*., 2015). Interestingly, SQEL was shown to enhance spermatogenesis, improve endothelial dysfunction, possess antiangiogenic activity and anticancer effect on human prostate (LNCap) and leukemic (K-562) cancer cells in previous studies (Al-Salahi *et al.*, 2013; Al-Salahi *et al*., 2014; Low *et al.*, 2013; Tong *et al.*, 2015). Nevertheless, further studies are still warranted to identify other beneficial uses of SQEL, including its potential anti-adipogenic effects.

2.11 Rationale for the study

The involvement of EL in energy metabolism has been shown previously by the reduction of blood glucose in streptozotocin-induced hyperglycemic rats supplemented with aqueous extracts of EL for 10 days (Husen *et al.*, 2004). In addition, treatment with EL root extracts also improved insulin sensitivity by enhancing insulin-induced glucose uptake in 3T3-L1 cells (Lahrita *et al.*, 2015). Moreover, the effects of EL in the role of

adipogenesis were shown indirectly by the reduction of body weight and omentum fat mass in normal-diet-fed rats given aqueous EL extract for 2 weeks (Solomon *et al.*, 2014). On top of that, EL in combination studies with other bioactive compounds has shown a synergistic effect in body weight management (Talbott, 2007). Although EL is widely consumed as an energy drink to enhance overall well-being, its effect on lipid metabolism specifically on adipocyte differentiation is yet to be unveiled. Therefore, this study sought to elucidate the role of SQEL and eurycomanone on anti-adipogenic mechanisms in 3T3-L1 cells and high-fat-diet-induced obese mice. The specific objectives are as follow:

1. To investigate the effect of SQEL and eurycomanone on the differentiation of pre-adipocyte cells.

2. To determine the effect of SQEL and eurycomanone on adipogenic genes and proteins.

3. To study the anti-adipogenic effect of SQEL in HFD-induced obese mice.

CHAPTER 3: METHODOLOGY

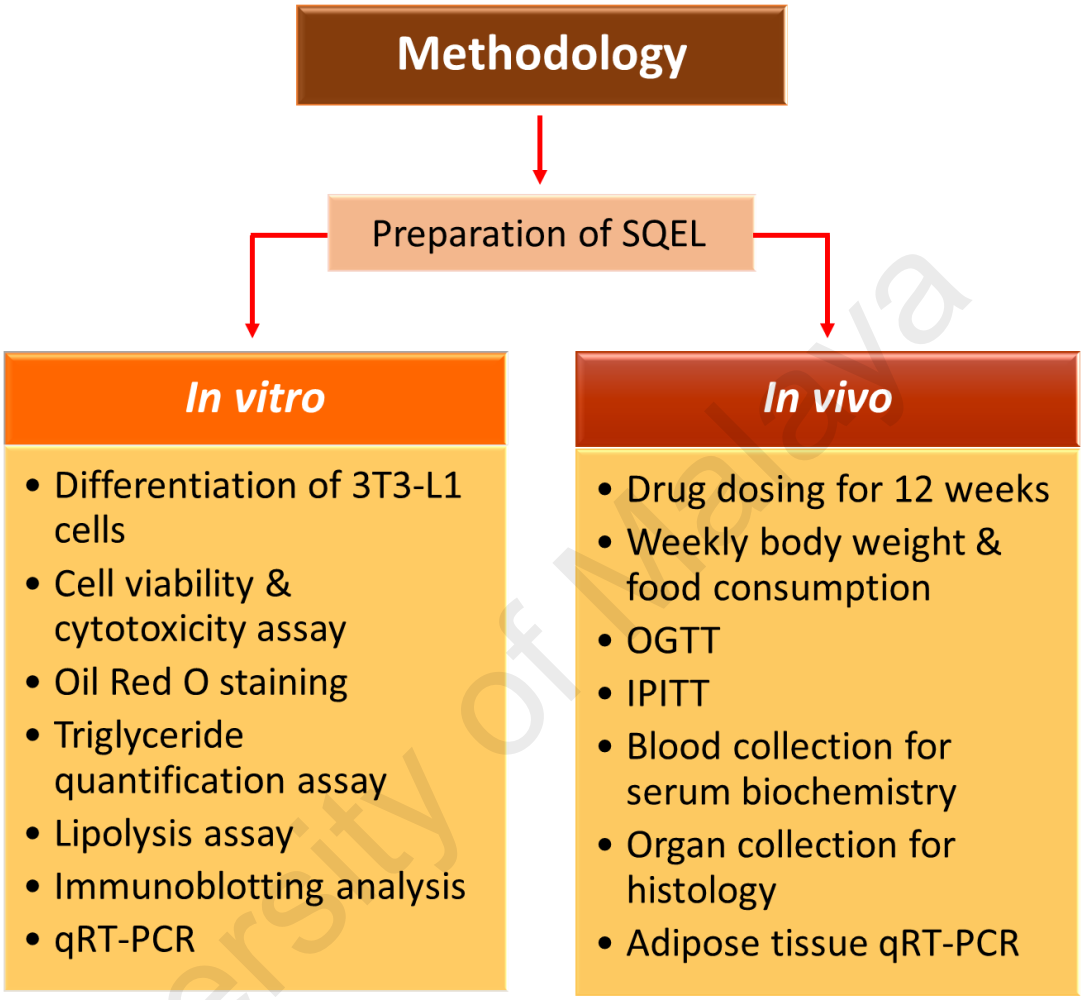

Figure 3.1: Summary of the methods.

3.1 Preparation of the Standardized Quassinoids composition from *Eurycoma longifolia* (SQEL)

The water-soluble standardized quassinoids composition from *Eurycoma longifolia* in powder form was extracted from the root of the plant according to the methods by (Low *et al.*, 2011) and provided by Professor Kit-Lam Chan, Universiti Sains Malaysia for this study. Briefly, the air-dried powdered roots (15 kg) of the EL were extracted with 6 × 4 L of 95% methanol for 6 days at 60°C. The combined methanol extract upon evaporation to dryness under partial vacuum produced a dark brown residue of 450 g (3% w/w). The residue was then chromatographed on a pre-packed Diaion HP 20 (Mitsubishi Chemical, Tokyo, Japan) resin column. The chosen quassinoid-rich fraction, SQEL was derived by elution with a gradient of H_2O-MeOH mixtures (1:0 to 0:1) at decreasing polarity (Low *et al.*, 2013), and subsequently dried under partial vacuum to 45 g (10% w/w of crude extract). The SQEL contains 14.49 ± 0.26% of eurycomanone, 7.39 ± 0.17% epoxyeurycomanone, 0.72 ± 0.06% 13,21-dihydroeurycomanone and 9.54 ± 0.22% w/w eurycomanol as quantified by high performance liquid chromatographic (HPLC) analysis (Al-Salahi *et al.*, 2013; Low *et al.*, 2013). These quassinoids were isolated and their purified structures (> 95%) were identified and confirmed following the protocol described previously (Teh *et al.*, 2011; Teh *et al.*, 2010; Teh-Murugaiyah, *et al.*, 2011). The purity of the compounds was determined with Empower 2 workstation software (Waters, Milford, MA, USA) operated in a Waters Delta Prep HPLC system equipped with a Waters 2996 photodiode array detector. The chemical structures of major quassinoids in SQEL are as illustrated in Figure 3.2.

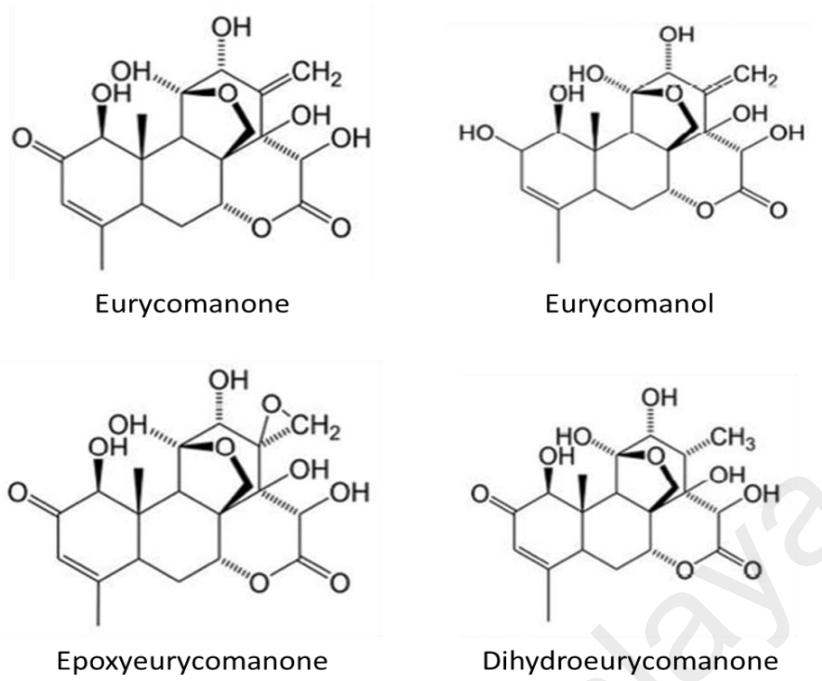

Figure 3.2: The bioactive ingredients of SQEL.

The pure compound, eurycomanone was purchased and its purity (> 96%) was verified using HPLC (Chengdu Biopurify Phytochemicals Ltd., Chengdu, China). A stock solution of 100 mg/mL SQEL and 25 mg/mL eurycomanone was prepared with dimethyl sulfoxide (DMSO) under the sterile condition and aliquoted in 1.5 mL sterile centrifuge tubes and kept at -20°C till further use.

3.2 Maintenance and Propagation of 3T3-L1 cells

Mouse 3T3-L1 preadipocyte cells (ATCC® CL-173™) were obtained from American Type Culture Collection (ATCC, USA) and cultured in 75 cm^2 sterile tissue culture flasks containing pre-warmed complete DMEM and incubated in a humidified atmosphere of 5% CO_2 at 37°C. The culture medium was replenished every 2 days and the cells were sub-cultured upon reaching 85-90% confluency. Briefly, the culture medium was removed from the flask completely and cells were washed with 10 mL of sterile 1X PBS. Then, 1 mL 1X 0.25% trypsin/EDTA (Biowest, USA) were added to the flask, shaken gently and incubated at 37°C for 5 minutes or until cells began to detach. Thereafter, the

flask was tapped gently and 9 mL of complete DMEM was added to inactivate the trypsin. Next, cells were re-suspended by aspiration and the cell suspension was transferred to a 15 mL centrifuge tube then centrifuged at 200 x g for 5 minutes. The supernatant was discarded and the cell pellet re-suspended in 1 mL of complete DMEM. Then, 100 µL of the cell suspension was transferred to a new culture flask with fresh pre-warmed complete DMEM. Cell passage number was recorded and monitored continuously. The cells were discarded at passage 12 and new cells were revived. For cell seeding, a total number of cells were counted. Ten microliter cell suspension was mixed with an equal volume of 0.4% trypan blue satin (Gibco, USA) and added to a haemocytometer counting chamber and viewed under the microscope. Cell number was estimated according to the following formula:

$$\text{Average cell number} \times 2 \times 10^4 = \text{number of cells/mL}$$

A prior dilution was made to get the final cell number needed for cell seeding according to the culture flask used.

3.3 Differentiation of 3T3-L1 cells

3T3-L1 pre-adipocyte cells were seeded at the optimal density (2000 cells/well) and maintained in pre-adipocyte culture medium (PCM) for 48 hours by incubating at 37°C and 5% CO_2. Then, the confluent pre-adipocytes were induced to differentiate in differentiation medium (DM) for two days. This medium was replaced with adipocyte maintenance medium (AMM) and used to maintain the culture for three days and then changed every three days (Tian *et al.*, 2017). The media formulations are as described in Table 3.1.

Table 3.1: Media formulations.

Medium	Formulation
Pre-adipocytes Culture Medium	Complete DMEM
Differentiation Medium	Complete DMEM, 0.5 mM 3-isobutyl-1-methylxanthine, 1 µM dexamethasone and 10 µg/mL insulin
Adipocyte Maintenance Medium	Complete DMEM and 10 µg/mL insulin

3.4 Cell viability and Cytotoxicity

Cell viability and cytotoxicity of SQEL and eurycomanone were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In MTT assay, the number of viable cells is directly proportional to the reduction amount of tetrazolium salt that forms purple colour formazan crystals (Mosmann, 1983). Cells were cultured in 96-well plate at an optimum density (2000 cells/well). After 24 hours, they were either treated with vehicle control or SQEL (0.4 – 100 µg/mL) or eurycomanone (0.4 – 100 µg/mL) in PCM for 72 hours. The viability of the differentiating pre-adipocytes was assessed by treating the 2 days post-confluent cells with SQEL (12.5 – 100 µg/mL) and eurycomanone (3.1 – 25 µg/mL) in DM for 48 hours.

To study the effects of SQEL and eurycomanone on the viability of matured adipocytes, 2 days post-confluent cells were induced to differentiate for 8 days as mentioned previously and treated with SQEL (12.5 – 100 µg/mL) and eurycomanone (3.1 – 25 µg/mL) in PCM for 48 hours. Then, 2 mg/mL of MTT in 1X PBS were added to each well and incubated for 3 hours at humidified atmosphere of 5% CO_2 at 37°C. The cell culture medium was removed, followed by the addition of 100 µL of DMSO and then

mixed at 300 rpm for 15 minutes. Absorbance reading was measured at 570 nm by using a multilabel microplate reader (Hidex Chameleon^{TM}, Finland). The effect of SQEL and eurycomanone on cell viability of 3T3-L1 pre-adipocytes, differentiating pre-adipocytes and matured adipocytes were determined as the percentage of cell viability by using the formula below: -

$$\% \text{ Cell viability} = \frac{\text{Abs (Treated - Blank)}}{\text{Abs (DMSO Control - Blank)}} \times 100\%$$

The half maximal inhibitory concentration (IC_{50}) was determined using GraphPad Prism software version 6.0 (GraphPad Software Inc., San Diego, CA).

3.5 Oil Red O Staining

Oil Red O staining was performed as described previously by (Chen *et al.*, 2015). Briefly, 3T3-L1 cells were seeded in 96-well plates at the optimal density (2000 cells/well) and allowed to achieve 100% confluence for 2 days. Then, cells were induced to differentiate as described previously and treated with vehicle control or SQEL (0.4 – 100 μg/mL) or eurycomanone (0.4 – 100 μg/mL) for 8 days. At the end of the treatment, the culture medium was removed and cells were fixed with 4% paraformaldehyde for 30 minutes. Cells were washed five times with 1X PBS and two times with distilled water and then stained for 1 hour with 0.36% Oil Red O solution. The dye solution was removed and cells were washed three times with distilled water for every 5 minutes interval. Then, stained lipid droplets were viewed under a microscope and the Oil Red O dye retained in cells was extracted with isopropanol and quantified by absorbance readout at 485 nm using the multilabel microplate reader (Hidex Chameleon^{TM}, Finland). The percentage inhibition of adipogenesis was calculated using the formula below: -

$$\% \text{ Adipogenesis Inhibition} = 100 - \frac{\text{Abs (Treated - Blank)} - \text{Abs (NDC - Blank)}}{\text{Abs (DMSO Control - Blank)}} \times 100\%$$

NDC, Non-differentiated control

3.6 Triglyceride Quantification Assay

3.6.1 Extraction of Intracellular Triglyceride from Matured Adipocytes

Cells were induced to differentiate as described above and treated with vehicle control or SQEL (12.5 – 100 µg/mL) or eurycomanone (3.1 - 25 µg/mL) or sulforaphane (0.9 µg/mL) for 8 days. Sulforaphane (0.9 µg/mL) was used as assay positive control (Choi *et al.*, 2012). At the end of treatment, the culture medium was removed and cells were washed two times with 1X PBS. Next, the cells were scraped gently and homogenized with 5% NP-40 solution (Tian *et al.*, 2017). Some cell homogenate was centrifuged at 15000 x g and 4°C for 10 minutes and the supernatant was collected and stored at -80°C for protein quantification while the remaining homogenate was used for triglyceride extraction. The remaining cell homogenate was heated to 100°C for 5 minutes or until it turns cloudy by dry block thermostat (Biosan, Latvia) and cooled to room temperature (25 ± 2°C) and this process was repeated twice. Lastly, the homogenate was centrifuged at 15 000 x g for 2 minutes and the triglyceride containing supernatant was collected and stored at -80°C to be used for triglyceride quantification assay.

3.6.2 Protein Quantification

Total protein content in cell homogenate was quantified using *DC*™ Protein Assay kit (Bio-Rad, USA) following the manufacturer's instruction. Briefly, the cell lysates were diluted 5-fold with distilled water and 5 µL were added to a flat bottom 96 well plate. Another set of samples was prepared for generating a standard curve by adding 5 µL of BSA standards whereby the 0 mg/mL well acts as assay blank. Subsequently, 25 µL of reagent A (1 mL reagent A + 20 µL reagent S) was added to each well. Then, 200 µL of reagent B was added to each well and the plate was agitated gently for 5 seconds. Bubbles were removed (if there is any) with a clean pipette tip and the plate was incubated in dark

at room temperature (25 ± 2°C) for 15 minutes. Lastly, the absorbance readout at 620 nm was measured using the multilabel microplate reader (Hidex Chameleon^{TM}, Finland). Standard and sample well readings were subtracted with blank reading. BSA Standard curve, Abs 620 nm versus BSA Standard concentration (mg/mL) was plotted and the linear equation y = mx + c was determined using GraphPad Prism software version 6.0 (GraphPad Software Inc., San Diego, CA) to calculate the sample protein concentration. Sample protein concentration was calculated using the formula below: -

Protein concentration (µg/µL) = Protein amount from standard curve (mg/mL) x Df

Df, dilution factor

3.6.3 Quantification of Triglyceride

Intracellular triglyceride contents were quantified using the Triglyceride Quantification Kit (BioVision, USA) following the manufacturer's protocol. Briefly, the cell lysates were diluted 10-fold with distilled water and 50 µL/well was added in triplicate into a flat bottom 96 well plate. Then, 40 µL of 1 mM triglyceride was mixed with 160 µl triglyceride assay buffer to prepare 0.2 mM triglyceride standard. Then, 0, 10, 20, 30, 40, 50 µl of the 0.2 mM triglyceride standard solution were added in duplicate into a series of wells and volume adjusted to 50 µl/well with triglyceride assay buffer to generate 0, 2, 4, 6, 8, 10 nM/well of triglyceride standard solution. The 0 nM well was used as assay blank. Then, 2 µl of lipase was added to blank and each standard and sample well, mixed and incubated for 20 minutes at room temperature (25 ± 2°C). After that, 50 µl/well triglyceride reaction mix were prepared as Table 3.2 and added to each well.

Table 3.2: Triglyceride Reaction Mix.

Triglyceride Reaction Mix	Volume (µl)
Triglyceride Assay Buffer	46
Triglyceride Probe	2
Triglyceride Enzyme Mix	2

The assay plate was incubated in dark at room temperature (25 ± 2°C) for 60 minutes. Lastly, the absorbance readout at 570 nm was measured using the multilabel microplate reader (Hidex Chameleon^{TM}, Finland). Standard and sample well readings were subtracted with blank reading. The triglyceride standard curve, abs 570 nm versus triglyceride (nM) was plotted and the linear equation y = mx + c was determined using GraphPad Prism software version 6.0 (GraphPad Software Inc., San Diego, CA) to calculate the sample triglyceride concentration. Normalized sample triglyceride concentration was calculated using the formula below: -

$$\text{Triglyceride concentration (nM/µg/µL)} = \frac{\text{Triglyceride amount from standard curve (nM) x Df}}{\text{Protein concentration (µg/µL)}}$$

Df, dilution factor

3.7 Lipolysis Assay

3T3-L1 cells were induced to differentiate as mentioned above. Then, the matured adipocytes were treated with vehicle control or SQEL (12.5 – 100 µg/mL) or eurycomanone (3.1 - 25 µg/mL) or isoproterenol (2.1 µg/mL) for 48 hours. Isoproterenol was used as a positive control for release of glycerol from triglycerides (Rondini *et al.*, 2017). The culture medium was collected at the end of the treatment. The collected medium was kept at -20°C until it was used for glycerol quantification. The extent of

lipolysis induced by the treatment was determined by quantifying the released glycerol into the medium using a Glycerol Quantification Kit (BioVision, USA) according to the manufacturer's instructions. Briefly, the collected media were diluted 10-fold with glycerol assay buffer and added 50 µL/well into a flat bottom 96 well plate. In addition, the 10-fold diluted culture medium was added as a blank for media. Ten microliters of 100 mM glycerol standard mixed with 990 µl glycerol assay buffer to prepare 1 mM glycerol standard solution. After that, 20 µL of 1 mM glycerol standard solution was further diluted with 180 µl glycerol assay buffer to get 100 µM working glycerol standard concentration. Then, 0, 2, 4, 6, 8 and 10 µl of the 100 µM glycerol standard were added in duplicate into a series of wells and volume adjusted to 50 µl/well with glycerol assay buffer to generate 0, 0.2, 0.4, 0.6, 0.8, 10 nM/well of glycerol standard solution. The 0 nM well was used as blank for glycerol standard. Then, 50 µl/well glycerol reaction mix was prepared as Table 3.3 and added to each well.

Table 3.3: Glycerol Reaction Mix.

Glycerol Reaction Mix	Volume (µl)
Glycerol Assay Buffer	46
Glycerol Probe	2
Glycerol Enzyme Mix	2

The assay plate was mixed gently for 5 seconds and incubated in dark at room temperature (25 ± 2°C) for 60 minutes. Finally, the absorbance readout at 570 nm was measured using the multilabel microplate reader (Hidex Chameleon^{TM}, Finland). Standard and sample well readings were subtracted with their respective blank readings. The glycerol standard curve, abs 570 nm versus glycerol (nM) was plotted and the linear equation $y = mx + c$ was determined using GraphPad Prism software version 6.0 (GraphPad Software Inc.,

San Diego, CA) to calculate the sample glycerol concentration. Sample glycerol concentration was calculated using the formula below: -

Glycerol concentration (nM) = Glycerol amount from standard curve (nM) x Df

Df, dilution factor

3.8 Immunoblotting Analysis

3.8.1 Preparation of Cell Lysates

3T3-L1 cells were washed twice with cold 1X PBS. Then, 100 µL/well of complete RIPA lysis buffer was added to 6 well plates. The plate was tilted several times to allow the RIPA lysis buffer to completely cover the cell surface and kept on ice for 10 minutes. Next, the lysed cells were scraped with a cell scraper and the cell lysates were harvested in pre-chilled 1.5 mL microcentrifuge tubes. Lastly, the tubes were centrifuged at 15 000 x g and 4°C for 30 minutes. Then, the fat cake (top layer) was removed and the clear cell lysates (middle layer) were collected in new pre-chilled 1.5 mL microcentrifuge tubes. The total protein concentration of the cell lysates was quantified with *DC*™ Protein Assay Kit (Biorad, USA) as described previously in section 3.6.2.

3.8.2 Preparation of Protein Sample

The 5X protein sample loading buffer solution containing 0.25 M Tris-HCl, pH 6.8, 10% SDS, 30% (v/v) glycerol, 10 mM DTT, 0.05% (w/v) bromophenol blue was prepared, aliquoted into 1.5 mL centrifuge tube and stored in -20°C until further use. The frozen 5X protein sample loading buffer solution was thawed at room temperature (25 ± 2°C) prior to use. An equal amount of protein was mixed with 5X protein sample loading buffer solution in a ratio of 4:1 then denatured at 100°C for 10 minutes by using a dry block thermostat (Biosan, Latvia). The protein loading samples were cooled to room

temperature (25 ± 2°C) before proceeding to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

3.8.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples (15-20 μg) were separated by SDS-PAGE for 2 hours at 110V and then transferred to a polyvinylidene difluoride membrane for 2 hours at 110V (Millipore, USA). The membranes were then blocked with 3% bovine serum albumin solution (0.3 g BSA + 1X TBS-T solution) (Santa Cruz, USA) at room temperature (25 ± 2°C) for 1 hour. Following washing with TBS-T, the membranes were incubated with the primary antibodies against PPARγ (1:1000), C/EBPα (1:1000), FAS (1:4000), Sirt1 (1:1000), FoxO3a (1:1000) and β-actin (1:20 000), overnight at 4°C. The next day, the membranes were washed five times with 1X TBS-T solution, five minutes each at room temperature (25 ± 2°C). The membranes were then incubated with respective horseradish peroxidase-conjugated secondary antibodies (DAKO, Denmark) for 2 hours at room temperature (25 ± 2°C). The bands were visualized with enhanced chemiluminescence (GE, UK) and exposed to X-ray film (Fujifilm, Japan). Densitometric analysis was performed using Image Studio Lite version 4.0.21 (LI-COR Biosciences) software. The protein levels were normalized to the housekeeping protein and expressed relative to the vehicle control.

3.9 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

3.9.1 RNA Extraction

Total RNA from 3T3-L1 cells was extracted using the RNAeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocols. The cells were washed with 1X PBS and harvested by trypsinization. Then, the cell pellet was washed again with 1X PBS and lysed by adding 350 μL of buffer RLT and vortexed for 1 min. Next, 350 μL of 70% ethanol (v/v) is added to the lysate and mixed well by pipetting up and down several times. Approximately 700 μL of the sample was transferred into a RNeasy spin column

with a 2 mL collection tube and centrifuged at 8000 x g and room temperature (25 ± 2°C) for 15 seconds. After that, the flow through was discarded and the sample was washed with 700 µL of buffer RW1 by resting for 1 min and then centrifuged at 8000 x g and room temperature (25 ± 2°C) for 15 seconds. The flow-through was discarded and the sample was washed twice with 500 µL of buffer RPE by centrifuging at 8000 x g and room temperature (25 ± 2°C) for 15 seconds and 2 minutes. Then, the spin column was centrifuged at top speed for 1 minute to flush away any remaining buffer and then transferred to a 1.5 mL collection tube. Lastly, 30 µL of RNase-free water was added to the column membrane and then centrifuged at 8000 x g and room temperature (25 ± 2°C) for 1 minute to elute the RNA. The collected RNA sample was stored at -80°C until further use.

3.9.2 cDNA Synthesis

Total RNA concentration and purity were determined using the NanoDrop™ 2000/2000c Spectrophotometer (Thermo Scientific, USA). RNase-free water was used as a blank. The RNA sample with an optical density 260/280 ratio greater than 1.8 (good RNA quality) was accepted and used for cDNA synthesis (Manchester, 1996). The cDNA was synthesized from 250 ng of total RNA by using the RT^2 First Strand Kit (Qiagen, Germany) according to the manufacturer's procedures. The reagents in the kit were thawed and briefly spun for 10-15 seconds prior to use. Then, the DNA elimination mix was prepared as Table 3.4, incubated at 42°C for 5 minutes and immediately placed on ice for 1 minute.

Table 3.4: Composition of DNA Elimination Mix.

Component	Amount (µL)
RNA (250 ng)	Variable
Buffer GE	2
RNase-free water	Variable
Total volume	10

After that, the 10 µL of DNA elimination mix was added to the reverse-transcription mix (Table 3.5) and mixed gently by pipetting. The mixture was incubated at 42°C for 15 minutes and then incubated at 95°C for 5 minutes to stop the reaction. Lastly, 91 µL of RNase-free water added to the reaction, mixed gently by pipetting and then placed on ice to proceed with the RT-PCR protocol.

Table 3.5: Composition of Reverse-transcription Mix.

Component	Amount (µL)
5X Buffer BC3	4
Control P2	1
RE3 Reverse Transcription Mix	2
RNase-free water	3
Total volume	10

3.9.3 mRNA Expression

The mRNA expressions of adipogenic genes were determined on a custom RT^2 Profiler PCR Array (APPENDIX C) using SYBR Green qPCR Mastermix according to

the manufacturer's instructions (Qiagen, Germany). The PCR components were prepared as summarized in Table 3.6.

Table 3.6: Composition of PCR mix.

Component	Amount (µL)
2X RT^2 SYBR Green Mastermix	12.5
cDNA synthesis reaction	1
RNase-free water	11.5
Total volume	25

The PCR mix (25 µL/well) was added into the array plate. Then, the plate was tightly sealed with an optical adhesive film, centrifuged at 1000 x g for 1 minutes and placed on a thermal cycler. PCR amplification was performed in a 96-well format Real-time PCR (Biosystem StepOne^{TM} Plus, Applied Biosystems, USA) thermal cycler inclusive of melt curve in PCR cycling program (Figure 3.3). The relative mRNA transcription levels were determined following the $2^{-\Delta\Delta}C_t$ method (Schmittgen & Livak, 2008).

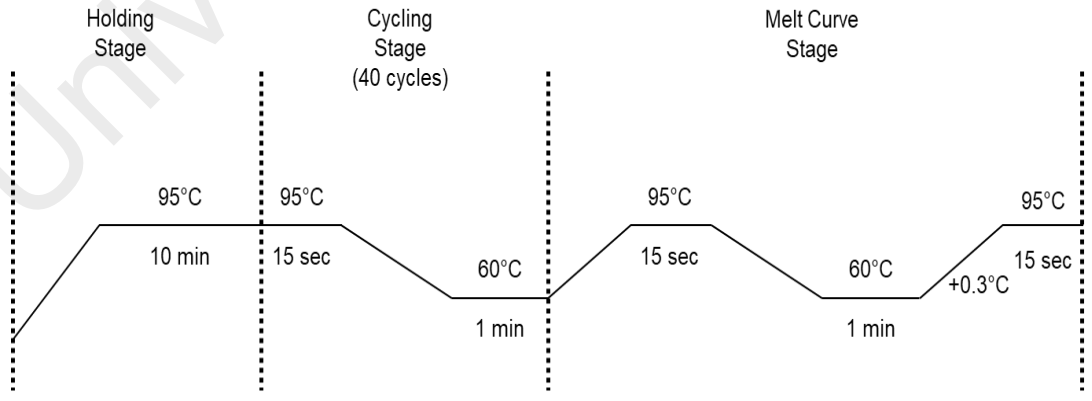

Figure 3.3: Biosystem StepOne^{TM} Plus PCR cycling program.

3.10 Animal Experiments

Six weeks old C57BL/6J male mice with uniform body weight were housed in individually ventilated cages under a controlled laboratory environment with 12 hours of the light-dark cycle in the AAALAC International accredited animal facility at the Department of Pharmacology, Faculty of Medicine, University of Malaya. Animal experimentation was performed according to the study protocol approved by the Institutional Animal Care and Use Committee, Faculty of Medicine, University of Malaya (Ethics Reference Number: 2016-170302/PHAR/R/DB). The animals were randomly divided into 4 experimental groups (n=8 for each group) respectively for normal diet and high-fat diet feeding groups as illustrated in Figure 3.4.

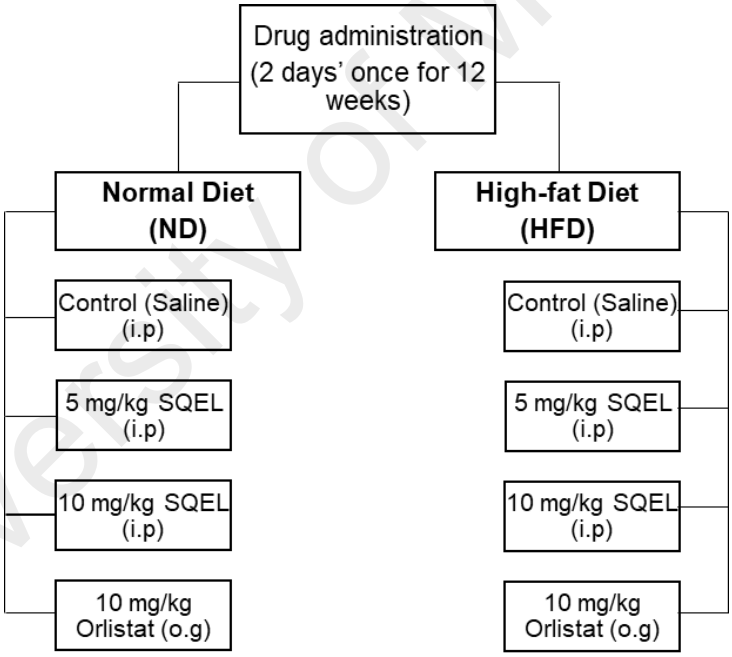

Figure 3.4: Animal study group segregation.

Normal diet (ND) group were fed with rodent maintenance diet (#1324, Altromin, Germany) and high-fat diet (HFD) group were fed with rodent high-fat diet (60% kcal fat) (C1090-60, Altromin, Germany) and water *ad libitum* for 12 weeks (Brandt *et al.*, 2018; Gao *et al.*, 2018). Both diet groups received 0.9% saline, 5 mg/kg and 10 mg/kg of SQEL via intraperitoneal route (i.p) while 10 mg/kg orlistat was given via oral route (o.g)

once every two days for 12 weeks (Al-Salahi *et al*., 2013; Al-Salahi *et al*., 2014; Low *et al*., 2013; Tong *et al*., 2015). Weekly food intake and mice body weight were measured. Prior to sacrifice at week 12, oral glucose tolerance test (OGTT) and intraperitoneal insulin tolerance test (IPITT) were performed. At the end of the treatment period, all mice were fasted for 6 hours and sacrificed by carbon dioxide inhalation. The liver, epididymal and perirenal white adipose tissues from all groups were excised, weighted and some were fixed in 10% formalin for histopathology. Blood samples from the animals were collected via cardiac puncture and serum was separated and stored at -20°C for biochemical analysis. Summary of the animal study is illustrated in Figure 3.5.

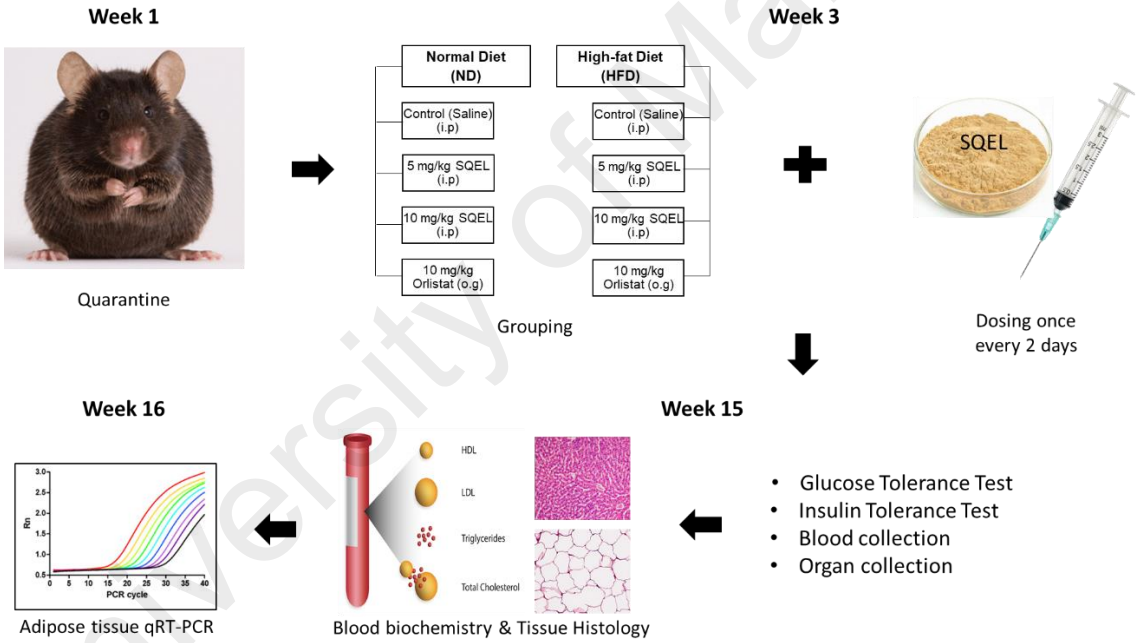

Figure 3.5: Summary of the animal study.

3.10.1 Oral Glucose Tolerance Test

Fasting blood glucose level was measured in all animals at 6 hours post-fasting via tail prick using an Accu-check® glucometer (Roche Diagnostics, Germany). Glucose (2 g/kg) was then administered via oral gavage and blood glucose was measured again at time points of 30, 60, 90 and 120 minutes post-gavage (Gao *et al.*, 2018). The area under

the curve of glucose level was calculated using GraphPad Prism software version 6.0 (GraphPad Software Inc., San Diego, CA).

3.10.2 Intraperitoneal Insulin Tolerance Test

Fasting blood glucose level was measured in all animals at 5 hours post-fasting via tail prick using an Accu-check® glucometer (Roche Diagnostics, Germany). Then, 0.75 IU/kg insulin was injected intraperitoneally and blood glucose was measured again at 15, 30, 60, 90 and 120 minutes post-injection (Gao *et al.*, 2018). The area under the curve of glucose level was calculated using GraphPad Prism software version 6.0 (GraphPad Software Inc., San Diego, CA).

3.10.3 Serum Biochemistry

Collected sera were sent to Haematology & Biochemistry Clinical Laboratory, Veterinary Laboratory Service Unit, Faculty of Veterinary Medicine, University Putra Malaysia to determine the total cholesterol and serum triglycerides levels using Dimension® Xpand® Plus clinical chemistry system (SIEMENS Healthcare Diagnostics Inc.).

3.10.4 Liver and Fat Tissues Histological Analysis

Liver and fat tissues were fixed in 10% buffered formalin, dehydrated and embedded in paraffin. Tissue sections of 4 μm thick were stained with hematoxylin and eosin to examine the morphology. Photomicrographs were taken using bright field LEICA DM 2000 microscope with LEICA ICC50 HD camera (Leica Microsystems, Germany). The mean diameter of the imaged adipocytes was measured using the ImageJ software with Adiposoft plugin at auto mode (Galarraga *et al.*, 2012). The Adiposoft application was calibrated to identify cells with diameter range (20-200 μm) and measuring the scale of 0.95 μm/pixel.

3.10.5 Fat Tissues qRT-PCR Analysis

Total RNA from fat tissues was extracted using the RNAeasy Mini Kit (Qiagen, Germany). The frozen fat tissue was thawed on ice for 10 minutes. Then, 5 µL of QIAzol™ Lysis Reagent (Qiagen, Germany) was added per mg of fat tissue and incubated for 10 minutes at room temperature (25 ± 2°C). Fat tissues were homogenised by using T10 basic ULTRA-TURRAX® (IKA Labortechnik, Malaysia) homogenizer and incubated at room temperature (25 ± 2°C) for 5 minutes. After that, 0.2 µL of chloroform was added per µL of tissue homogenate, vortexed for 15 seconds and incubated for 3 minutes at room temperature (25 ± 2°C). Then, the tissue homogenate was centrifuged at 12000 x g and 4°C for 15 minutes. Thereafter, the clear top layer of homogenate was collected in 1.5 mL microcentrifuge tubes then equal volume of 70% (v/v) of ethanol was added and vortexed for 15 seconds. Approximately 700 µL of the sample was transferred into a RNeasy spin column with a 2 mL collection tube and centrifuged at 8000 x g and room temperature (25 ± 2°C) for 15 seconds. After that, the flow through was discarded and the sample was washed with 700 µL of buffer RW1 by resting for 1 min and then centrifuged at 8000 x g and room temperature (25 ± 2°C) for 15 seconds. The flow-through was discarded and the sample was washed twice with 500 µL of buffer RPE by centrifuging at 8000 x g and room temperature (25 ± 2°C) for 15 seconds and 2 minutes. Then, the spin column was centrifuged at top speed for 1 minute to flush away any remaining buffer and then transferred to a 1.5 mL collection tube. Lastly, 30 µL of RNase-free water was added to the column membrane and then centrifuged at 8000 x g and room temperature (25 ± 2°C) for 1 minute to elute the RNA. The collected RNA sample was stored at -80°C until further use. cDNA was synthesized from 250 ng of total RNA as described previously at section 3.9.2. The mRNA expressions of AMPK and PGC-1α genes were determined according to the procedure described at section 3.9.3 by using the primers listed in Table 3.7.

Table 3.7 Forward and Reverse primers.

Gene	Forward	Reverse
AMPK	CTCAGTTCCTGGAGAAAGATGG	CTGCCGGTTGAGTATCTTCAC
PGC-1α	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG
B-actin	CTGTCCCTGTATGCCTCTG	ATGTCACGCACGATTTCC
GAPDH	GTGCAGTGCCAGCCTCGTCC	CAGGCGCCCAATACGGCCAA

The PCR components were prepared as summarized in Table 3.8 and 10 µL/well was added into the 0.2 mL low profile eight tube strips (ExtraGene, Taiwan). Then, the tube was centrifuged at 300 x rpm for 1 minutes and placed on a thermal cycler. PCR amplification was performed and the relative mRNA transcription levels were determined as defined previously at section 3.9.3.

Table 3.8 Composition of PCR mix.

Component	Amount (µL)
2X RT^2 SYBR Green Mastermix	5
cDNA synthesis reaction	1
Forward & Reverse primers	2
RNase-free water	2
Total volume	10

3.11 Statistical Analysis

All the data are expressed as a mean ± standard error of mean (SEM) from n number of experiments. Student's t-test was performed for comparison between two groups. Comparison for more than two groups was analysed by one-way ANOVA followed by Bonferroni's multiple comparison tests using Graph Pad Prism (Version 6.0, Graph Pad Software Inc., San Diego, CA). A p-value < 0.05 was considered to be statistically significant.

CHAPTER 4: RESULTS

4.1 Cytotoxicity effect of SQEL and eurycomanone

Potential cytotoxicity of both SQEL and eurycomanone on preadipocytes was evaluated using MTT assay before proceeding to study the adipogenesis effects of SQEL and eurycomanone. Eurycomanone exerted greater cytotoxicity to 3T3-L1 cells with an IC_{50} value of 70.8 ± 11.7 μg/mL compared to SQEL with an IC_{50} value of 160.3 ± 36.7 μg/mL (Figure 4.1). Hence, the concentration range of 12.5 – 100 μg/mL for SQEL and 3.1 – 25 μg/mL for eurycomanone were chosen for subsequent experiments. The chosen concentrations of SQEL and eurycomanone were further tested for potential cytotoxicity during the differentiation of pre-adipocytes and in the mature adipocytes, respectively. Both SQEL and eurycomanone were not cytotoxic to both the differentiating pre-adipocytes and matured adipocytes even at the highest tested concentration (Figure 4.2 A&B).

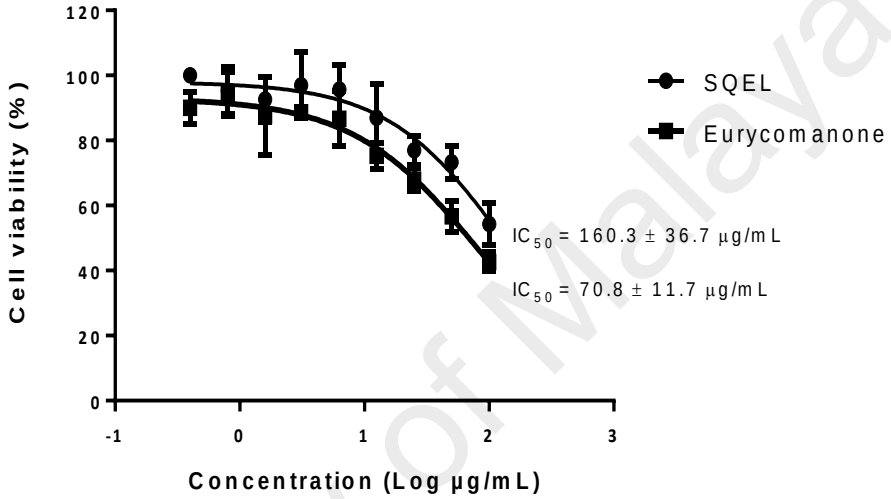

Figure 4.1: Effects of SQEL and eurycomanone on the viability of 3T3-L1 preadipocytes. The viability of 3T3-L1 preadipocytes treated with SQEL and eurycomanone (0.4 – 100 µg/mL) for 72 hours. The cell viability was determined by comparing with vehicle control (100%) and media blank (0%) by MTT reduction assay. Data are presented as the mean ± SEM of three independent experiments performed in triplicate.

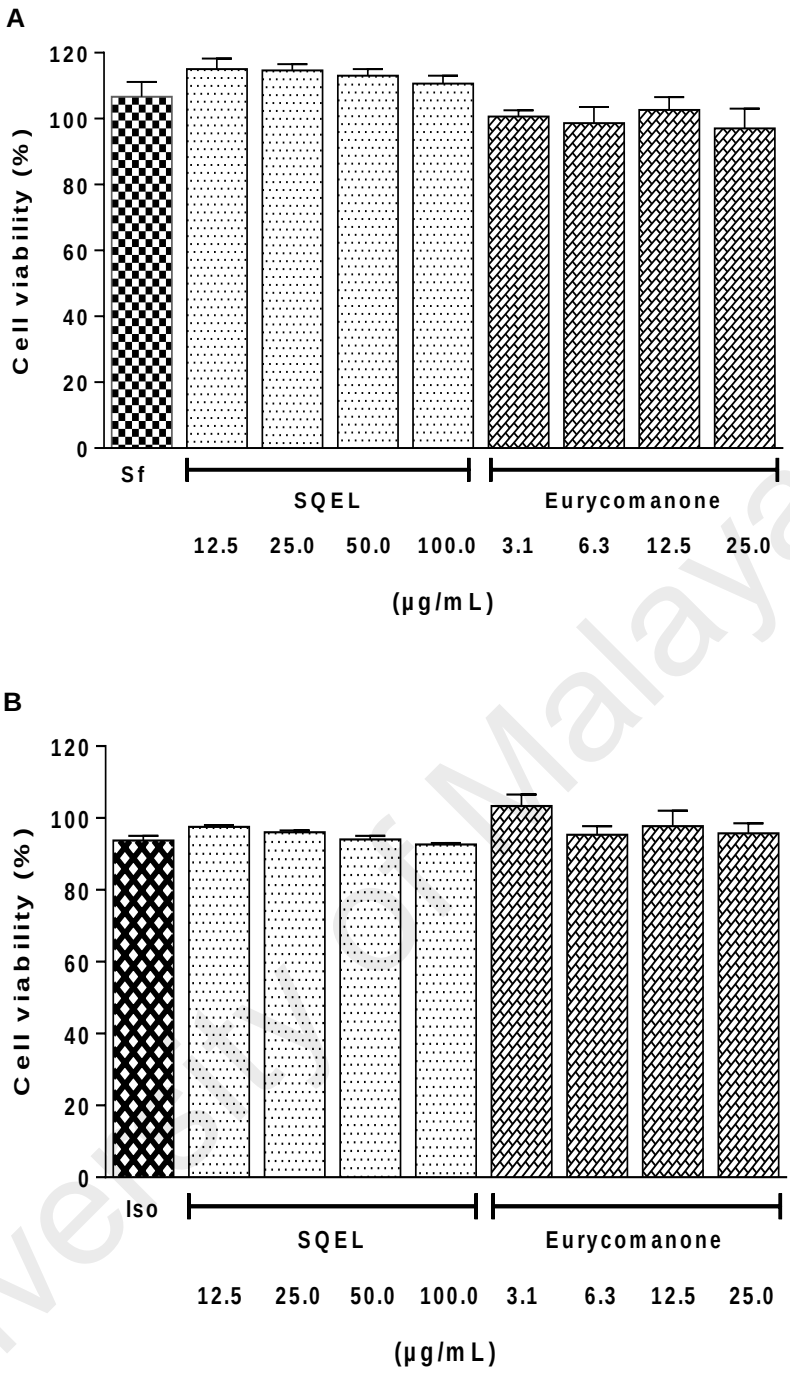

Figure 4.2: Effects of SQEL and eurycomanone on the viability of differentiating preadipocytes and matured adipocytes. (A) The viability of differentiating and (B) matured 3T3-L1 cells treated with SQEL (12.5 – 100 µg/mL) and eurycomanone (3.1 - 25 µg/mL) for 72 hours. The cell viability was determined by comparing with vehicle control (100%) and media blank (0%) by MTT reduction assay. Data are presented as the mean ± SEM of three independent experiments performed in triplicate. Sf, sulforaphane and Iso, isoproterenol.

4.2 SQEL and eurycomanone inhibit adipogenesis of 3T3-L1 cells

The potential anti-adipogenic effects of SQEL and eurycomanone in 3T3-L1 cells were assessed in the differentiating pre-adipocytes concurrently treated with SQEL (12.5 – 100 μg/mL) and eurycomanone (3.1 - 25 μg/mL) for 8 days. Microscopic inspection of the Oil Red O staining revealed a concentration-dependent reduction in the number of cells with stained lipid droplets following treatment with SQEL and eurycomanone compared to the control untreated cells (Figure 4.3A). Moreover, SQEL and eurycomanone treatment dose-dependently inhibited adipogenesis, thus validating the previous result (Figure 4.3B). Eurycomanone exerted stronger adipogenic inhibitory effect compared to SQEL as shown by 85% inhibition even with 25 μg/mL eurycomanone treatment while only 73% inhibition at 100 μg/mL SQEL concentration. The inhibition by SQEL and eurycomanone is comparable to the inhibition with sulforaphane, the positive control that attained 95% inhibition (Figure 4.3B). Since the anti-adipogenic effect of SQEL and eurycomanone was achieved at the concentrations that did not exert cytotoxicity, both have effectively inhibited adipogenic differentiation in 3T3-L1 cells (Figure 4.3B).

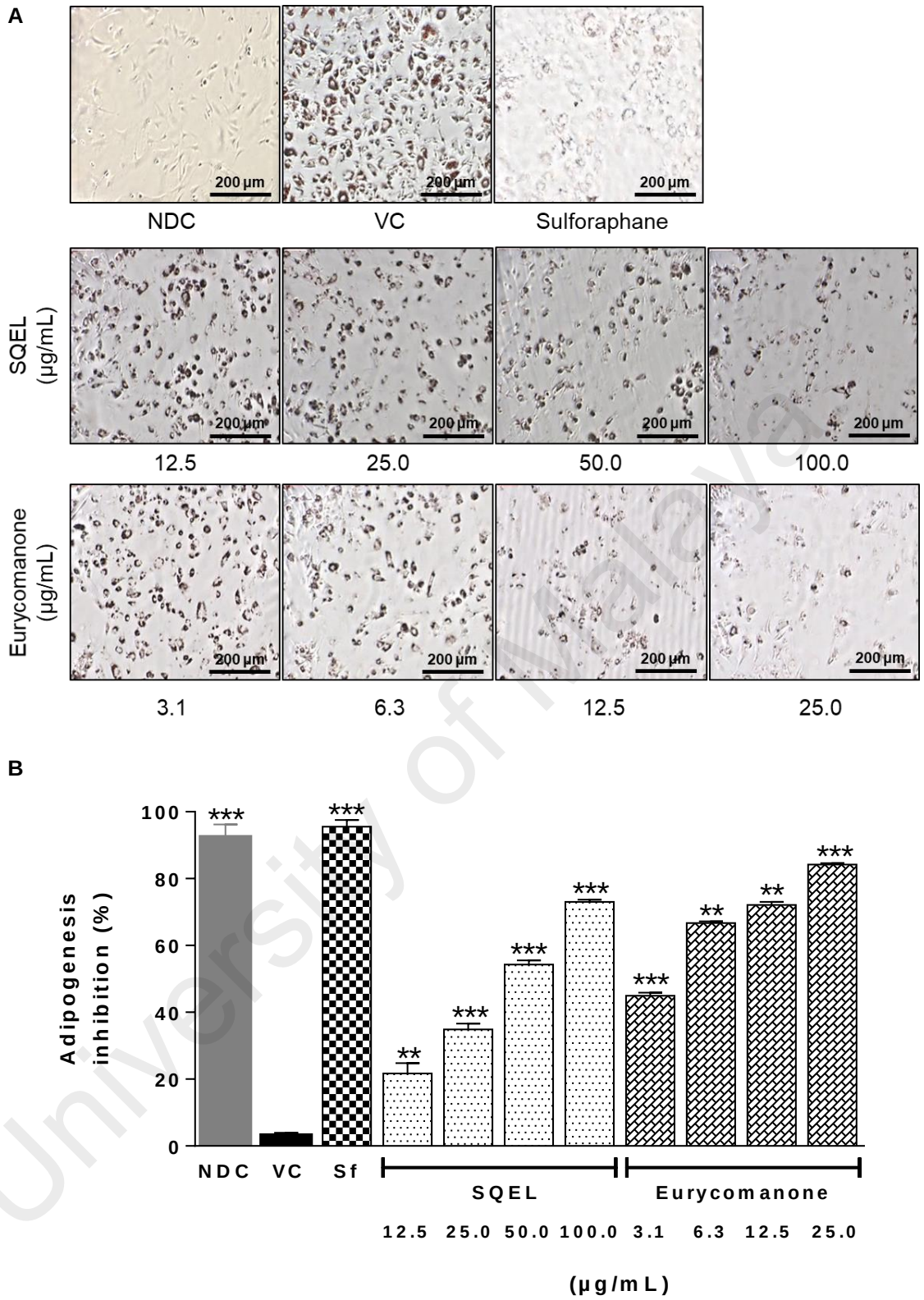

Figure 4.3: Adipogenic inhibitory effects of SQEL and eurycomanone in 3T3-L1 cells. (A) Oil Red O staining of differentiating 3T3-L1 cells treated with increasing concentrations of SQEL (12.5 – 100 µg/mL) and eurycomanone (3.1 - 25 µg/mL). Lipids accumulation in cells (dark brown stain) was photographed at 50x magnification after Oil Red O staining, scale bar: 200 µm. The accumulated dye was quantified as absorbance readout at 485 nm wavelength. (B) Adipogenesis inhibition by SQEL and eurycomanone.

Sulforaphane (0.9 μg/mL) was used as assay positive control. The percentage of adipogenesis inhibition was determined as the absorbance relative to that of vehicle control cells. Data are presented as the mean ± SEM of three independent experiments performed in triplicate, (*p< 0.05, **p< 0.01 and ***p< 0.001 versus vehicle control). NDC, non-differentiated control; VC, vehicle control, 0.5% DMSO and Sf, sulforaphane.

4.3 SQEL and eurycomanone reduce lipogenesis and induce lipolysis

Cytosolic lipid accumulation is the most prominent marker of adipogenesis and its quantification is used to assess the extent of *in vitro* adipocyte differentiation. Treatment of the differentiating 3T3-L1 cells with sulforaphane, the assay positive control for a period of 8 days reduced triglyceride accumulation (Figure 4.4A). Similarly, SQEL and eurycomanone treatment at various concentrations significantly and dose-dependently reduced triglyceride accumulation (Figure 4.4A). This further confirms that both the compounds inhibit adipogenesis. Triglycerides are hydrolyzed to form free fatty acids and glycerol in the event of energy demand, a metabolic process called lipolysis. Glycerol content hence indicates the degree of lipolysis in mature adipocytes. SQEL and eurycomanone significantly induced glycerol production in a dose-dependent manner after 48 hours of treatment in the matured adipocytes (Figure 4.4B). Isoproterenol, the assay positive control compound treatment for the same period has elevated the amount of glycerol released from matured adipocytes (Figure 4.4B). The increase in glycerol production was accompanied by a significant dose-dependent reduction in triglycerides contents at the concentrations that did not cause toxicity to matured adipocytes (Figure 4.4).

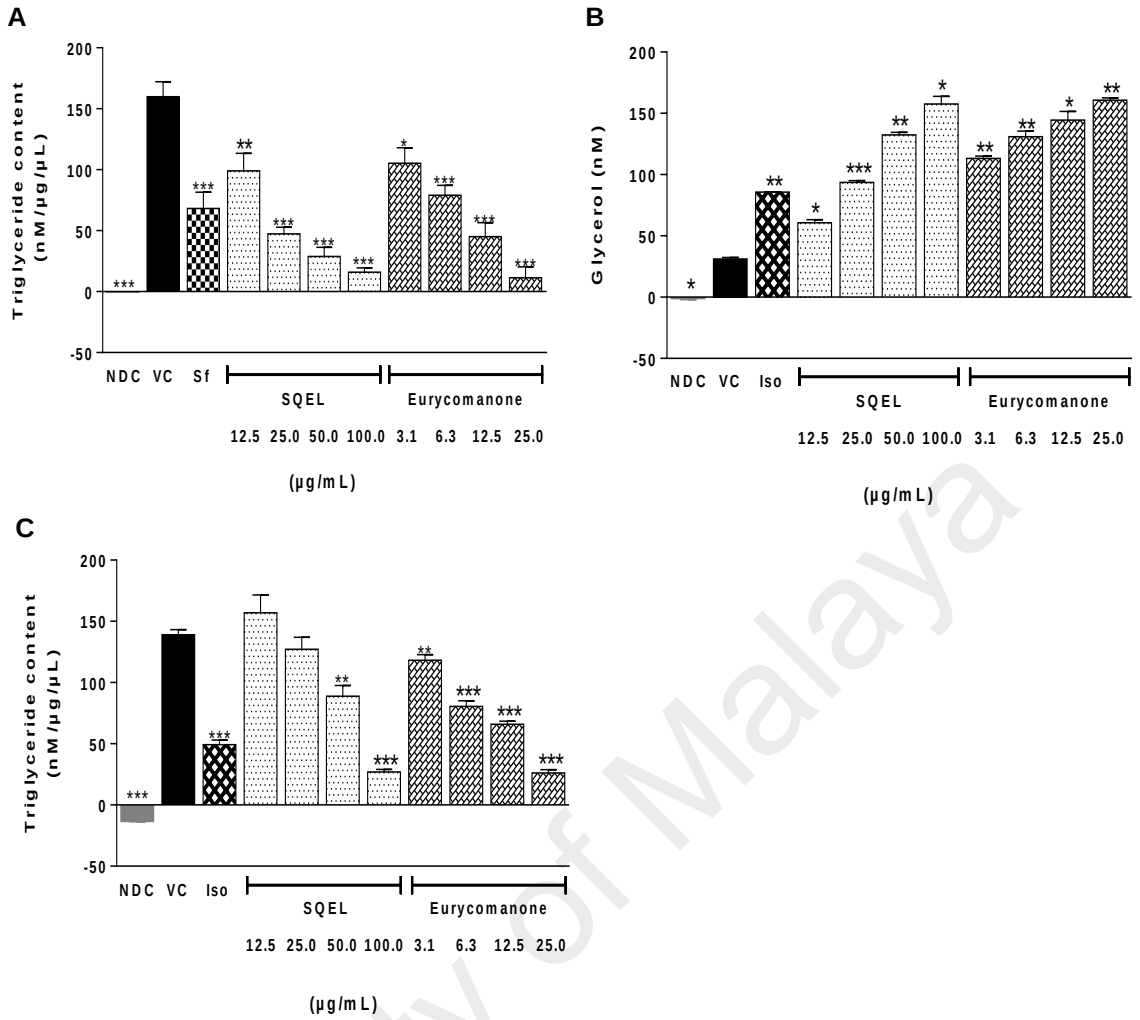

Figure 4.4: SQEL and eurycomanone inhibit the intracellular triglyceride accumulation and induce lipolysis in matured adipocytes. (A) Intracellular triglycerides content in the differentiating cells. Triglycerides levels were quantified with Triglyceride Quantification Kit and the amount (nM) was normalized to protein content (µg/µL) of the cells. Sulforaphane (0.9 µg/mL) was used as assay positive control. (B) Lipolysis in matured adipocytes. Matured adipocytes were treated with increasing concentrations of SQEL (12.5 – 100 µg/mL) and eurycomanone (3.1 – 25 µg/mL) in DMEM for 48 hours. Culture media collected were used to quantify the amount of glycerol produced with Glycerol Quantification Kit and (C) intracellular triglycerides upon lipolysis were quantified. Isoproterenol (2.1 µg/mL) was used as assay positive control. Data are presented as the mean ± SEM of three independent experiments performed in triplicate, (*p< 0.05, **p< 0.01 and ***p< 0.001 versus vehicle control). NDC, non-differentiated control; VC, vehicle control, 0.5% DMSO; Sf, sulforaphane and Iso, isoproterenol.

4.4 SQEL and eurycomanone reduce the expression of adipogenesis-associated proteins and genes in 3T3-L1 cells

Adipogenesis is tightly regulated by the chronological activation of numerous transcription factors including PPARγ and C/EBPα. These transcription factors are expressed immediately upon the induction of pre-adipocyte differentiation to activate the expression of a down-stream adipogenic-specific protein such as fatty acid synthase (FAS). Hence, the effect of SQEL and eurycomanone on the regulation of adipogenesis-associated proteins and genes in differentiating 3T3-L1 cells harvested on day 2 of differentiation was studied. The protein expressions of both PPARγ and C/EBPα were significantly reduced after SQEL and eurycomanone treatments (Figure 4.5 A&B). Among the PPARγ isomers (PPARγ1 and PPARγ2), the protein expression of PPARγ2 was reduced by two-fold by SQEL and eurycomanone treatments (Figure 4.5 A&B). Similarly, the protein expression of FAS was also reduced to half-fold after 2 days of SQEL and eurycomanone treatment (Figure 4.5 A&B). Nevertheless, sulforaphane treatment in differentiating cells for 48 hours has increased PPARγ1, PPARγ2, C/EBPα and FAS protein expression as compared to the vehicle control (Figure 4.5 A&B). Moreover, the mRNA expression of PPARγ decreased with eurycomanone treatment while both SQEL and eurycomanone significantly reduced the mRNA level of C/EBPα by two-fold in the differentiating cells (Figure 4.5C). In addition, the mRNA levels of lipoprotein lipase (LPL) and Kruppel-Like Factor 15 (KLF15) which are positively regulated by PPARγ were significantly reduced with SQEL and eurycomanone treatment as compared to the vehicle control (Figure 4.5C). Interestingly, significant upregulation by two-fold in the protein and mRNA expression of Sirtuin1 (Sirt1) was observed in the differentiating cells treated with SQEL and eurycomanone (Figure 4.5 A-C). Similarly, the protein expression of FoxO3 also two-fold increased by the same treatment in 3T3-L1 cells (Figure 4.5 A&B).

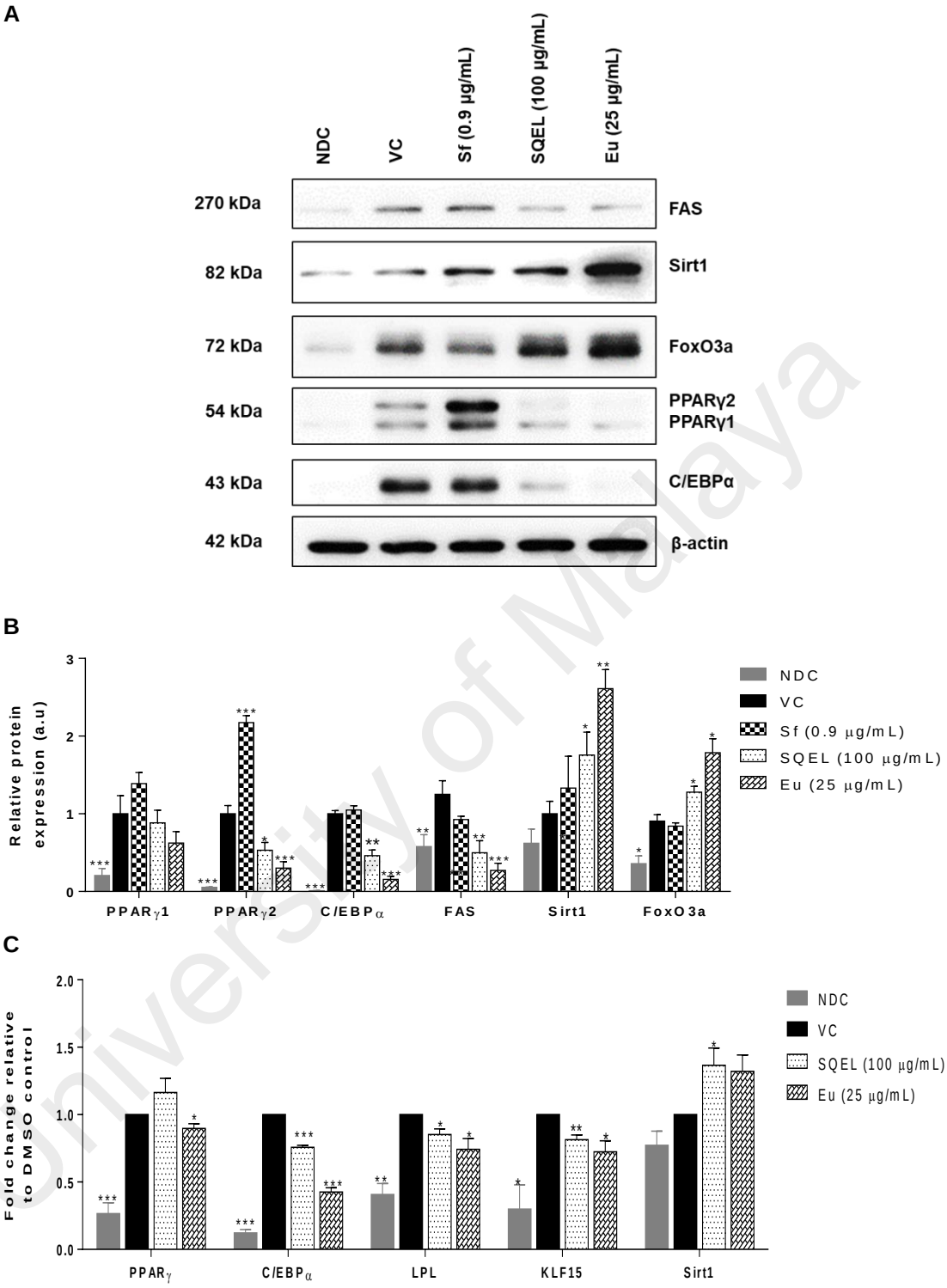

Figure 4.5: Effects of SQEL and eurycomanone on the expression of adipogenic markers in differentiating cells. (A&B) Protein and (C) mRNA expressions of adipogenic markers in differentiating pre-adipocytes. Intensities of the PPARγ1, PPARγ2, C/EBPα, Sirt1, FoxO3a and FAS protein bands were normalized to those of β-actin and relative protein expressions of the treated samples were obtained by comparing the normalized protein bands intensity to that of the vehicle control. Ct values of PPARγ,

C/EBPα, LPL, KLF15 and Sirt1 were normalized to those of actin and GAPDH and relative mRNA expressions of the treated samples were obtained by comparing to that of the vehicle control. Data are presented as the mean ± SEM, of three independent experiments performed in triplicate, (*p< 0.05, **p< 0.01 and ***p< 0.001) versus vehicle control. NDC, non-differentiated control; VC, vehicle control, 0.5% DMSO; Eu, eurycomanone; Sf, sulforaphane and a.u; arbitrary unit.

4.5 SQEL suppresses body weight gain in C57BL/6J mice

Anti-obesity effect of SQEL was evaluated in the high fat diet (HFD)-induced obese C57BL/6J mice. The body weight of mice fed with normal diet (ND) increased gradually with the highest weight of 30.94 ± 0.45 g observed at week 12 (Figure 4.6A). Whereas, the body weight of the mice fed with HFD increased drastically with the highest weight of 39.56 ± 1.23 g at week 12 (Figure 4.6B). Mice fed with HFD demonstrated 62% body weight gain compared to mice on ND which only gained about 28% of weight (Figure 4.6C), although there was no significant difference in their food intake (Figure 4.7 A&B). Interestingly, the mice groups that received 5 mg/kg and 10 mg/kg SQEL for the same study period showed significantly lower percentages of body weight gain with both types of diet, but higher weight reduction in mice fed with HFD than ND (Figure 4.6C). Mice on ND and received 5 mg/kg and 10 mg/kg SQEL have gained 11% and 16% of weight respectively (Figure 4.6C). However, HFD-fed mice received the same treatment respectively gained weight up to 31% and 26% which was similar to the percentage weight gain by saline control mice on ND (Figure 4.6C). The reduction in weight gain of both ND and HFD fed mice groups was comparable to mice given standard drug orlistat that respectively gained only 17% and 4% of weight compared to their initial body weight (Figure 4.6C).

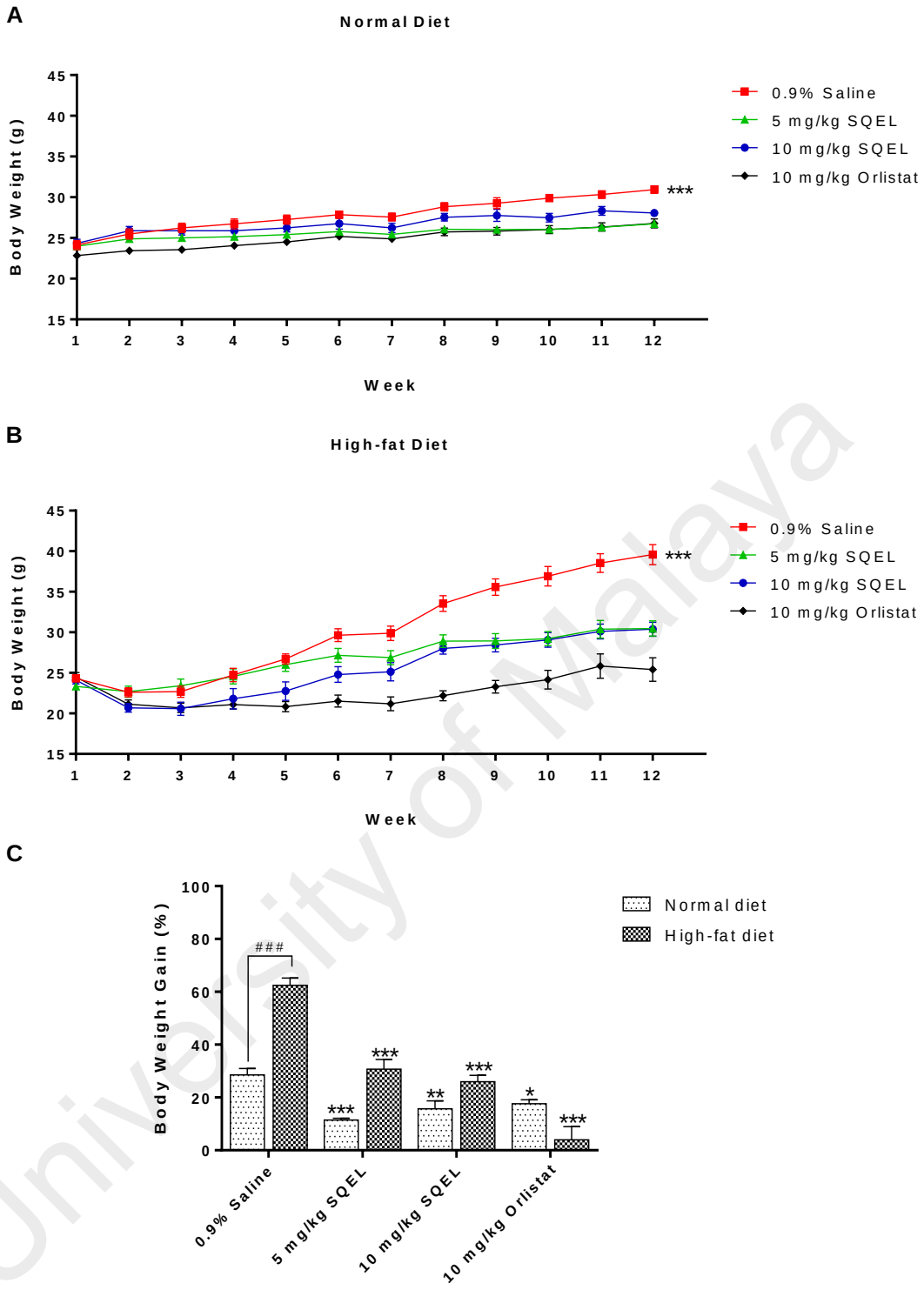

Figure 4.6: Effects of SQEL treatment on body weight of C57BL/6J mice. Weekly body weight (A&B) and (C) percentage body weight gain of mice group fed with normal diet and high-fat diet treated with 5 mg/kg SQEL and 10 mg/kg SQEL for 12 weeks. The percentage body weight gain was calculated by comparing the initial and final body weight. Data are presented as the mean ± SEM, n=7-8 (*p< 0.05, ** p<0.01 and ***p< 0.001 versus the respective control (0.9% saline) and $^{###}$p< 0.001 for high-fat diet versus normal diet 0.9% saline group.

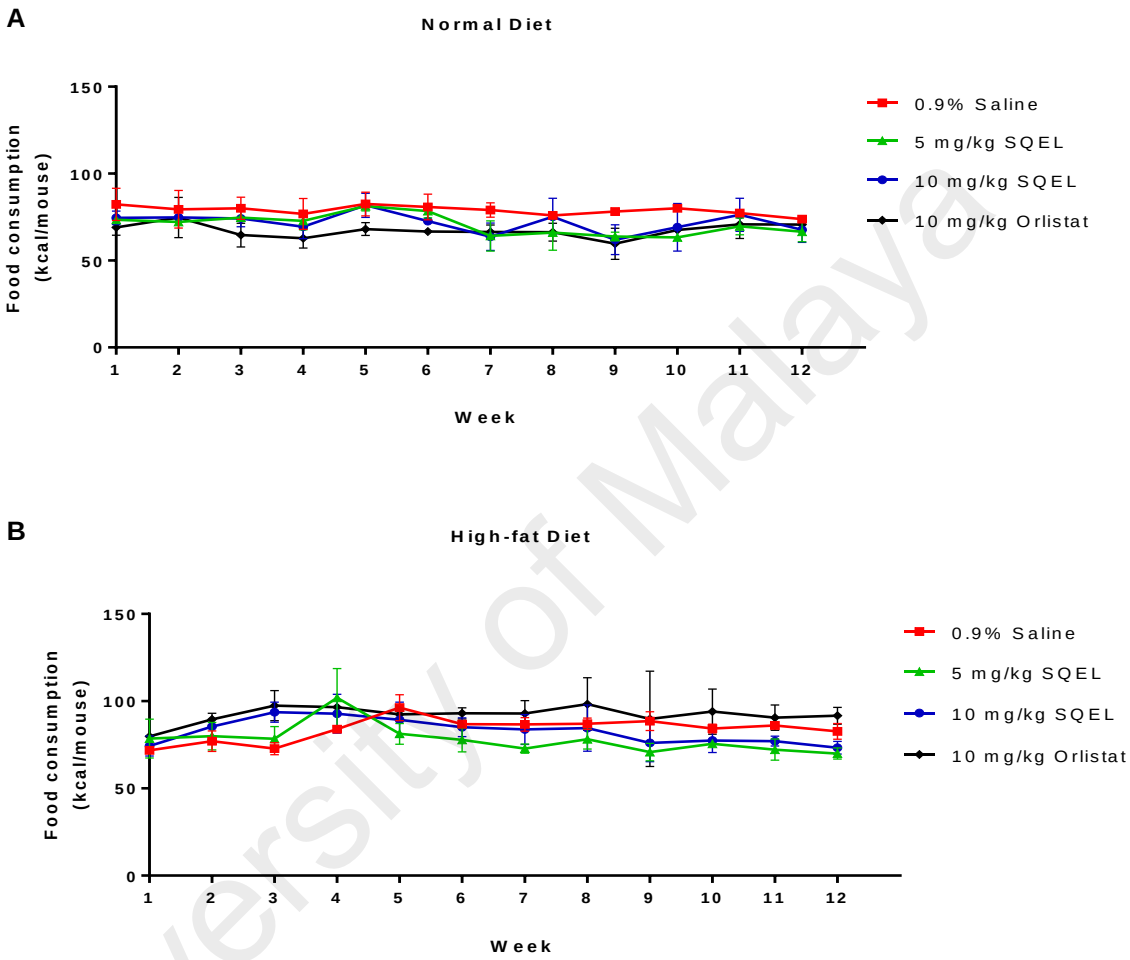

Figure 4.7: Effects of SQEL treatment on food consumption of C57BL/6J mice. Weekly food intake (A&B) of mice group fed with normal diet and high-fat diet treated with 5 mg/kg SQEL and 10 mg/kg SQEL for 12 weeks. Data are presented as the mean ± SEM, n=7-8.

4.6 SQEL improves impaired glucose tolerance but not insulin sensitivity in C57BL/6J mice

Prior to sacrifice on week 12, mice were subjected to OGTT and IPITT to study the effect of SQEL treatment on glucose and insulin sensitivity. The time course plasma glucose level curve and the area under the curve of glucose which indicates the total glucose amount within a specified period of time was plotted. Impaired glucose tolerance was observed in the HFD fed mice group compared to the ND fed group (Figure 4.8A). Significantly lower glucose intolerance observed in the ND fed mice group supplemented with 5 mg/kg and 10 mg/kg SQEL as well as in the HFD fed mice group treated with 10 mg/kg SQEL compared to its respective saline control mice group (Figure 4.8B). Orlistat treatment has significantly reduced glucose intolerance in both the ND and HFD fed mice groups (Figure 4.8B). On the other hand, insulin sensitivity was not affected in mice on both ND and HFD with and without SQEL treatment (Figure 4.8C). However, orlistat treatment significantly improved insulin sensitivity in mice group given HFD (Figure 4.8D).

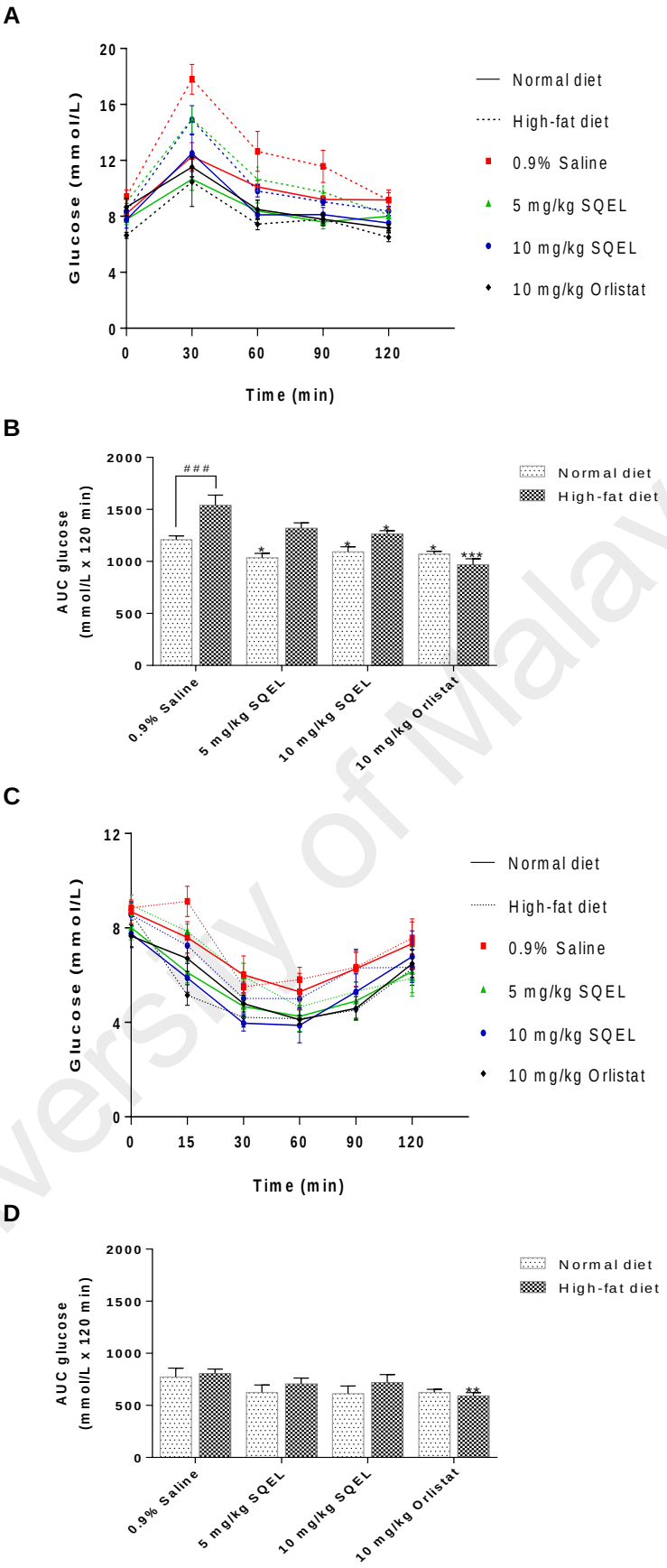

Figure 4.8: Effects of SQEL treatment on glucose and insulin sensitivity of C57BL/6J mice. (A) Oral glucose tolerance curve and its (B) area under the curve and (C) insulin tolerance test curve and its (D) area under the curve of mice group fed with

normal diet and high-fat diet treated with 5 mg/kg SQEL and 10 mg/kg SQEL for 12 weeks. Data are presented as the mean ± SEM, n=7-8 (*p< 0.05, ** p<0.01 and ***p< 0.001 versus the respective control (0.9% saline) and $^{###}$p< 0.001 for high-fat diet versus normal diet 0.9% saline group.

4.7 SQEL reduces adipose tissue mass in C57BL/6J mice

At the end of 12th week, mice were fasted for 6 hours and euthanized by carbon dioxide asphyxiation. The epididymal and perirenal fat pads and livers were harvested and weighed. Saline or SQEL-treated ND fed mice possessed smaller epididymal fat pad compared to those fed with HFD (Figure 4.9). SQEL treatment has impaired the development of epididymal fat in mice on HFD (Figure 4.9). Moreover, in the HFD fed mice group treated with SQEL, epididymal and perirenal fat pad mass was reduced significantly compared to its saline control (Figure 4.10 A&B). The decrease in fat mass was comparable with the reduction seen with standard anti-obesity drug orlistat (Figure 4.10 A&B). An unanticipated finding was noted, whereby, the ND fed mice possessed 5.0 ± 0.11 g/100 g BW of liver weight, which is significantly higher as compared to mice fed with HFD that only have 3.89 ± 0.05 g/100 g BW of liver weight in 12 weeks (Figure 4.10C). However, there was an insignificant reduction in the liver weight of mice fed with ND and treated with SQEL for the same period (Figure 4.10C). On the other hand, SQEL treatment in mice fed with HFD showed a significant increase in liver weight to 4.72 ± 0.05 and 5.28 ± 0.33 g/100 g BW at respective dose when compared to its saline control (Figure 4.10C). However, SQEL-mediated increase in liver weight of mice on HFD was not significantly higher than the liver weight of ND fed saline control mice (Figure 4.10C). The mice group on orlistat treatment and consumed HFD only exhibited a very mild reduction in their liver weight (Figure 4.10C).

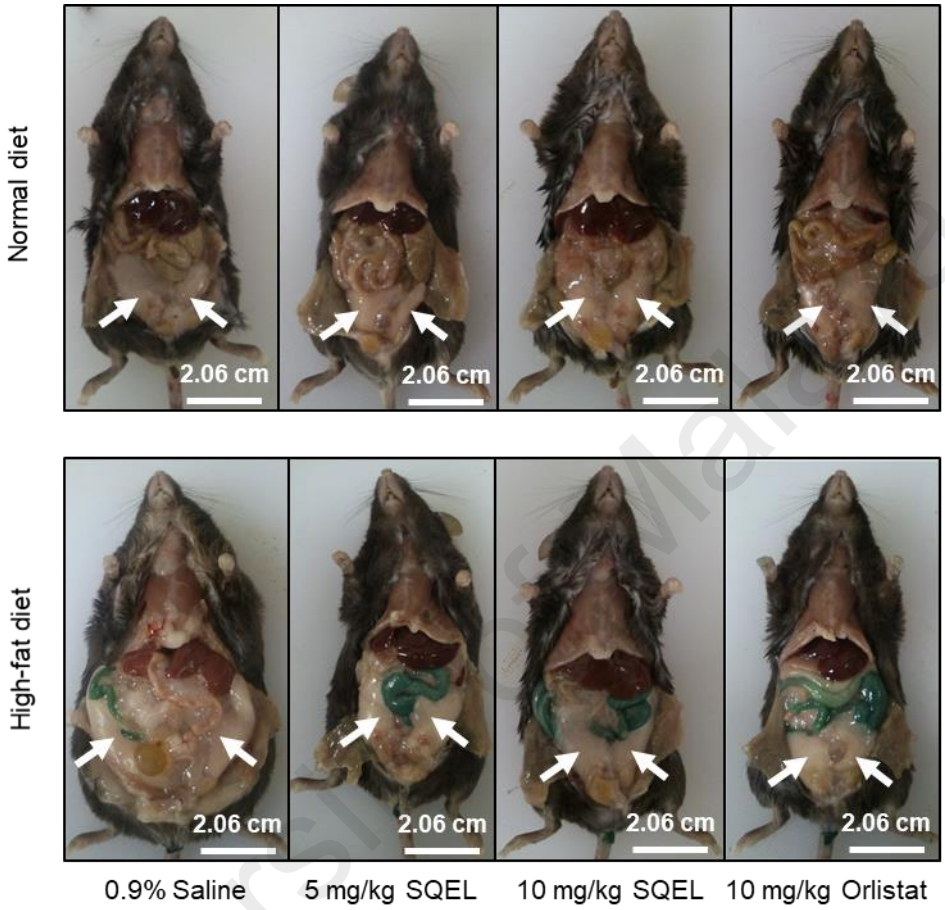

Figure 4.9: Effects of SQEL treatment on the adipose tissue development in C57BL/6J mice. Epididymal fat pads of mice on normal and high-fat diet treated with 0.9% saline, 5 mg/kg SQEL, 10 mg/kg SQEL and 10 mg/kg orlistat for 12 weeks. White arrows indicate epididymal fat pads. Scale bar: 2.06 cm.

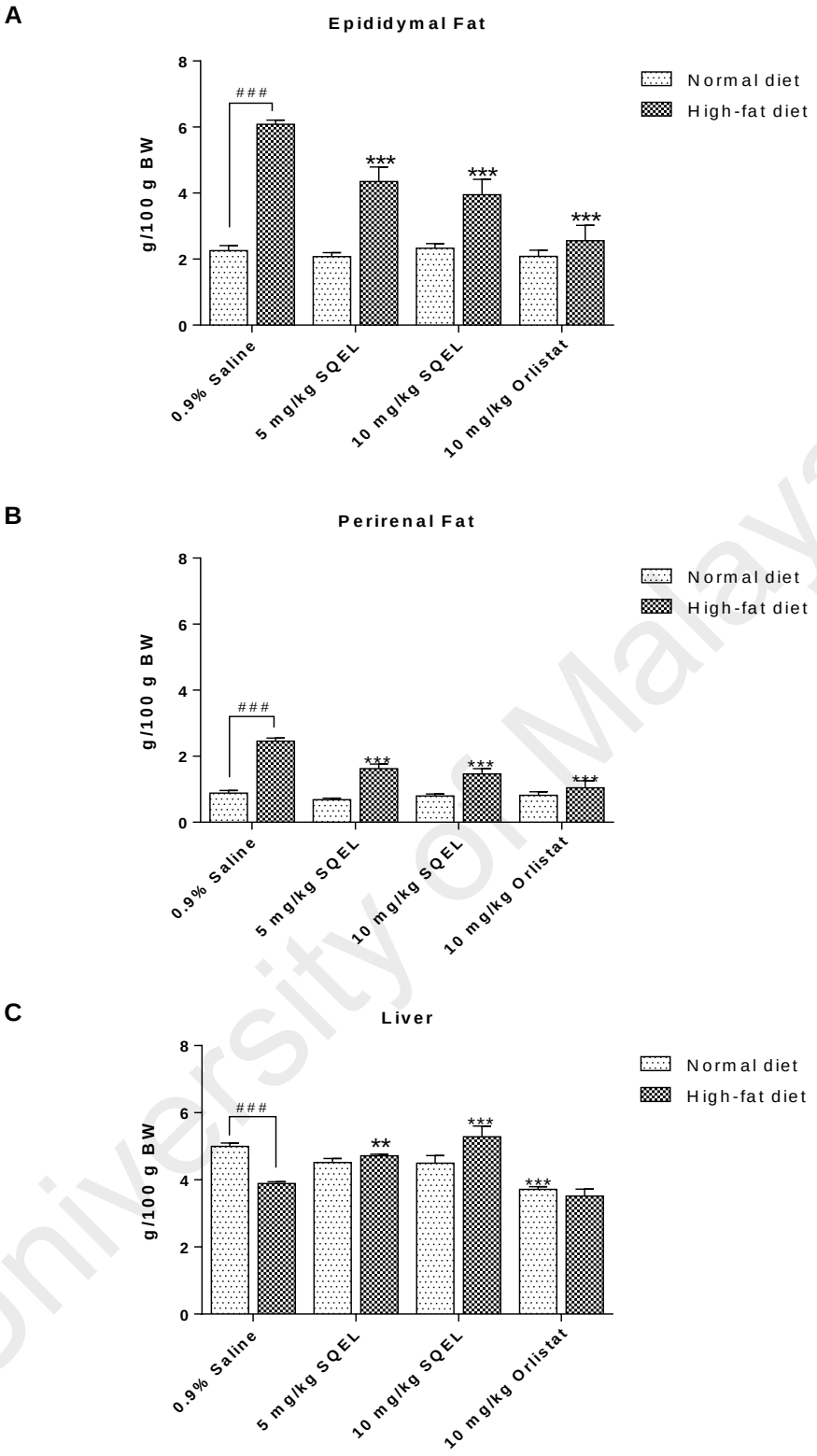

Figure 4.10: Effects of SQEL treatment on the adipose and liver tissue mass in C57BL/6J mice. Normalized weights of (A) epididymal fat, (B) perirenal fat and (C) liver of mice on normal and high-fat diet treated with 0.9% saline, 5 mg/kg SQEL, 10 mg/kg SQEL and 10 mg/kg orlistat for 12 weeks. Data are presented as the mean ± SEM, n=7-8. (**p< 0.01 and ***p< 0.001) versus respective control (0.9% saline) and $^{###}$p< 0.001 versus normal diet 0.9% saline.

4.8 Effects of SQEL treatment on fat accumulation in the liver and adipose tissue hypertrophy in C57BL/6J mice

Hepatic steatosis as indicated by the presence of numerous fat globules was observed in the liver histological section of the saline group mice consumed HFD compared to the ND fed group (Figure 4.11A). Moreover, fat globules were absent in the SQEL-treated mice fed with ND (Figure 4.11A). Interestingly, a significant reduction in the number of fat globules was observed in the SQEL treated HFD fed mice when compared to the saline control group of the HFD mice (Figure 4.11A). Similarly, a significant increase in the size of adipocyte was observed in the saline group of the HFD fed mice compared to the saline group of the ND fed mice (Figure 4.11B). The average adipocyte diameter of the saline control mice group on HFD and ND was 82.22 ± 5.4 μm and 64.98 ± 3.75 μm respectively. Nevertheless, the adipose tissue diameter of 10 mg/kg SQEL-treated mice group fed with HFD was reduced to 71.88 ± 3.84 μm in which significantly lower when compared to the saline control mice group (Figure 4.11C) and appeared phenotypically similar to those of mice on ND and orlistat-treated mice fed HFD (Figure 4.11B). These findings demonstrated that SQEL significantly reduced the accumulation of lipid in the liver and adipose tissue hypertrophy in C57BL/6J mice.

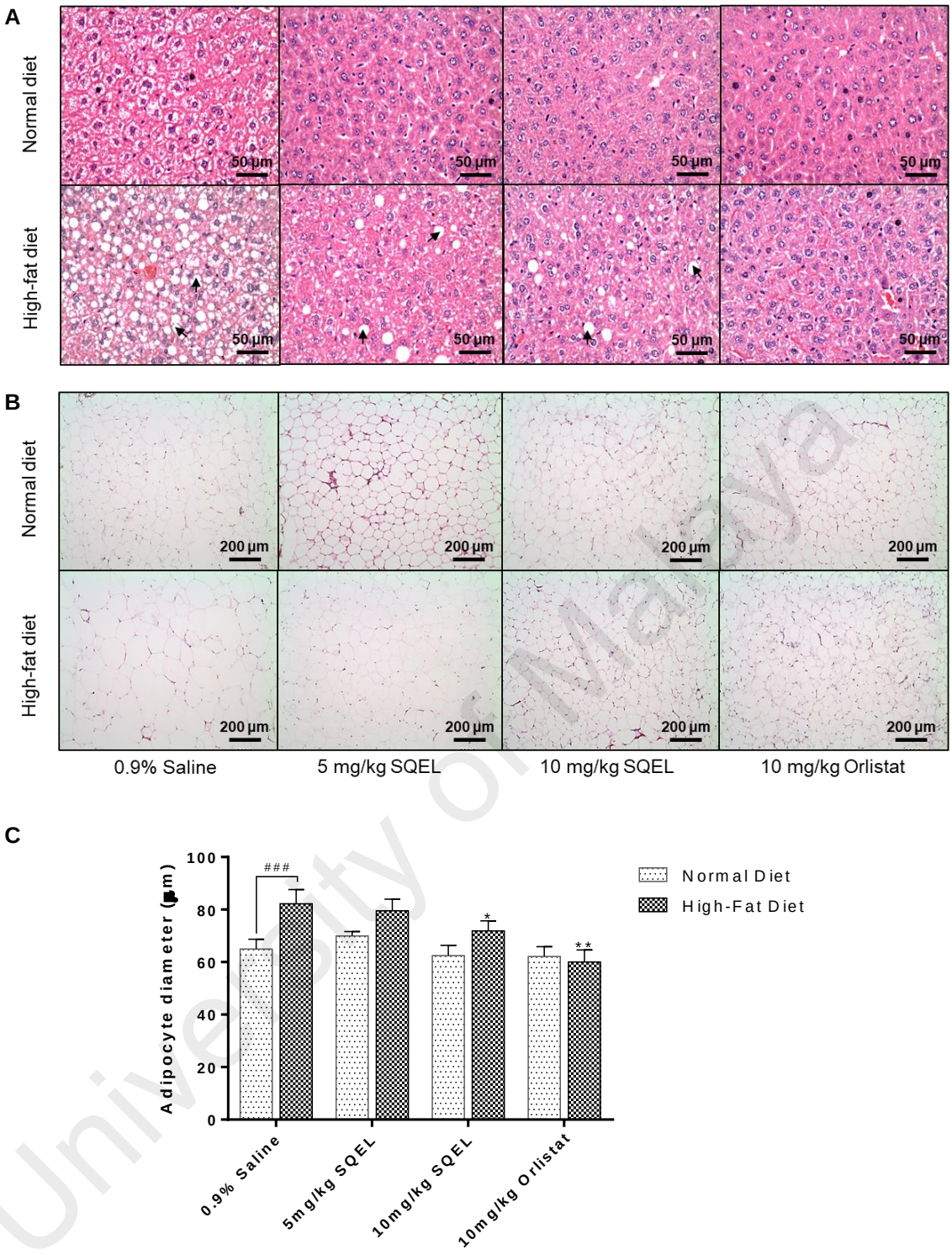

Figure 4.11: Effects of SQEL treatment on adipose and liver tissues in C57BL/6J mice. H&E-stained histological images of (A) liver and (B) adipose tissues and (C) adipose tissue size of mice on normal and high-fat diet treated with 0.9% saline, 5 mg/kg SQEL, 10 mg/kg SQEL and 10 mg/kg orlistat for 12 weeks. Data are presented as the mean ± SEM, n=7-8. (*p< 0.05 and ** p<0.01) versus respective control (0.9% saline) and $^{###}$p< 0.001 versus normal diet 0.9% saline. The presence of lipid globules in the mice livers is indicated by black arrows. Magnification: 400x (A) & 100x (B). Scale bar: 50 µm (A) & 200 µm (B).

4.9 Effects of SQEL treatment on lipid profile and fasting blood glucose in C57BL/6J mice

After 12 weeks of SQEL treatment, mice fasted for 6 hours, euthanized by carbon dioxide asphyxiation and blood was withdrawn through cardiac puncture. Serum was separated, stored at -20°C and sent for lipid profiling to study the effects of SQEL treatment on lipid metabolism. Serum total cholesterol level was elevated in mice fed with HFD compared to those on ND (Table 4.1). A slight increase in triglyceride level was observed in mice fed with HFD compared to those on ND but it was not significant (Table 4.1). Treatment with SQEL significantly decreased the total cholesterol and triglyceride levels in mice fed with ND and HFD as observed similar to the orlistat-treated group compared to their respective diet saline control (Table 4.1). Saline control mice fed HFD have higher fasting glucose compared to mice consumed ND. However, SQEL treatment has reduced the fasting glucose level in both ND and HFD fed mice group although the reduction was not significant (Table 4.1).

Table 4.1: Effect of SQEL treatment on blood serum parameters in C57BL/6 mice.

Parameter	Normal diet (Mean ± SEM)			
	0.9 % Saline (n=8)	5 mg/kg SQEL (n=8)	10 mg/kg SQEL (n=8)	10 mg/kg Orlistat (n=7)
T. Cholesterol (mMol/L)	3.51 ± 0.07	3.09 ± 0.12[a]	2.84 ± 0.19[b]	2.67 ± 0.19[b]
Triglyceride (mMol/L)	2.53 ± 0.16	1.92 ± 0.12[a]	1.66 ± 0.11[c]	1.37 ± 0.08[c]
Fasting blood glucose (mMol/L)	8.2 ± 0.44	7.78 ± 0.37	7.76 ± 0.61	8.66 ± 0.63

Parameter	High-fat diet (Mean ± SEM)			
	0.9 % Saline (n=8)	5 mg/kg SQEL (n=8)	10 mg/kg SQEL (n=8)	10 mg/kg Orlistat (n=7)
T. Cholesterol (mMol/L)	7.13 ± 0.37†	4.89 ± 0.40[c]	5.11 ± 0.23[c]	4.83 ± 0.28[c]
Triglyceride (mMol/L)	2.69 ± 0.20	2.26 ± 0.17	2.45 ± 0.19	1.68 ± 0.15[b]
Fasting blood glucose (mMol/L)	9.41 ± 0.48	9.03 ± 0.36	8.38 ± 0.36	9.35 ± 0.29

Lipid profile and blood glucose of mice given normal diet and high-fat diet and treated with 0.9% saline, 5 mg/kg SQEL, 10 mg/kg SQEL and 10 mg/kg orlistat for 12 weeks. Data are presented as the mean ± SEM, n=7-8, [a]$p < 0.05$, [b]$p < 0.01$ and [c]$p < 0.001$ versus control (0.9% saline) and [†]$p < 0.0001$ versus 0.9% saline of normal diet.

4.10 SQEL treatment increases the mRNA levels of AMPK and PGC-1α in adipose tissues of C57BL/6J mice

PGC-1α possess important role in regulating the mitochondrial biogenesis that stimulates energy expenditure. AMPK is an energy sensor that modulate the expression of PGC-1α by extracellular signaling in controlling metabolism (Cantó & Auwerx, 2009). Since SQEL treatment has reduced the mice body weight independent of total calorie intake, the possible involvement of mitochondrial biogenic gene expression was studied in adipose tissues of C57BL/6J mice. The mRNA levels of both AMPK and PGC-1α were increased in mice fed ND compared to mice on HFD (Figure 4.12 A&B). Treatment with SQEL at 10 mg/kg dose significantly elevated the mRNA levels of AMPK and PGC-1α by half-fold in mice group fed with HFD as well as in the orlistat-treated group (Figure 4.12 A&B). The AMPK mRNA expression increased in SQEL and orlistat treated mice group on ND, but it was not significant when compared to its saline control group (Figure 4.12A). SQEL treatment reduced the mRNA level of PGC-1α in mice fed ND when compared to its saline control group (Figure 4.12B). However, the PGC-1α mRNA level was unaltered in mice group fed with ND and treated with orlistat (Figure 4.12B).

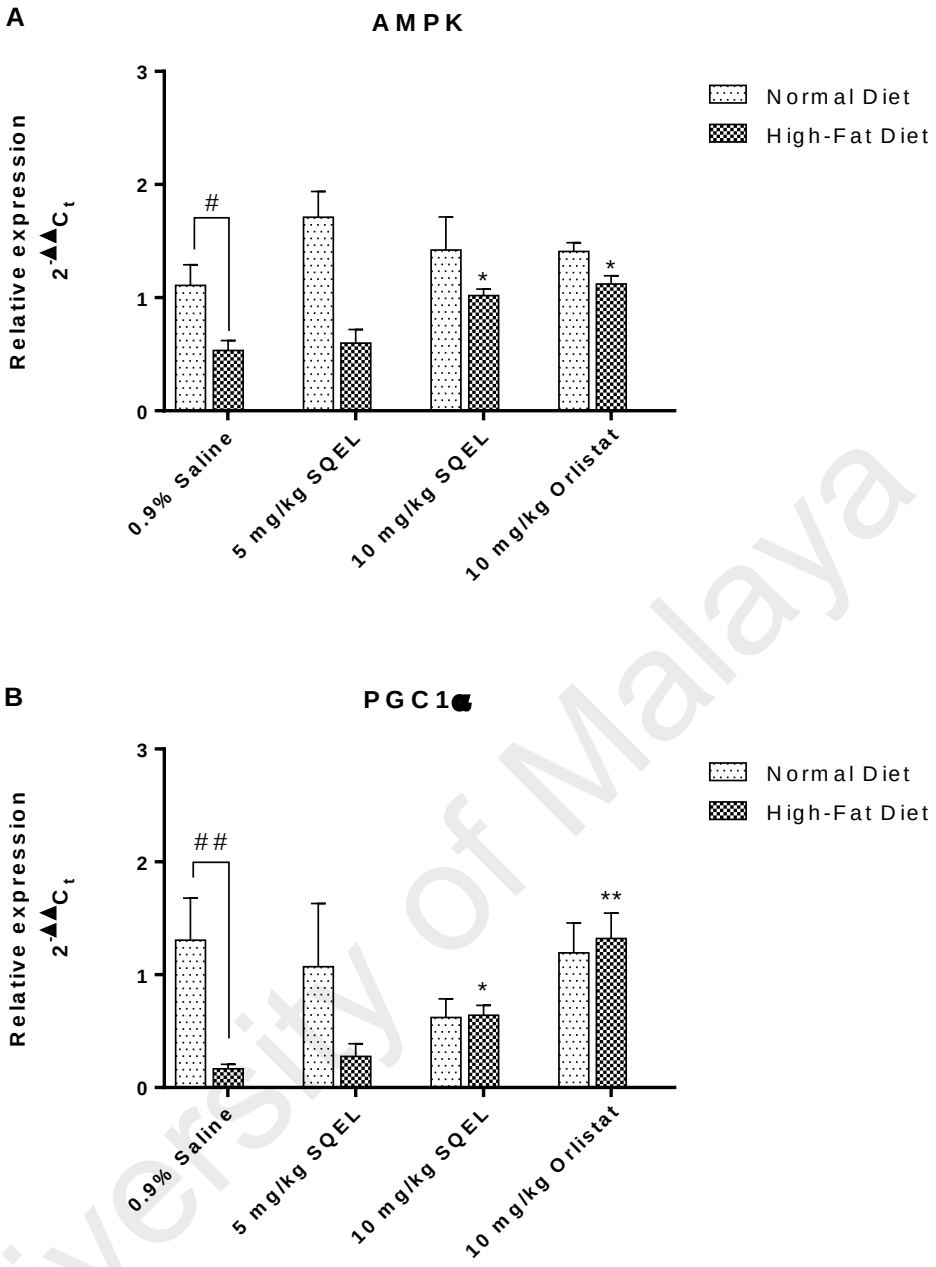

Figure 4.12: Effects of SQEL and eurycomanone on the mRNA expression of AMPK and PGC-1α in adipose tissues of C57BL/6J mice. mRNA expressions of (A) AMPK and (B) PGC-1α in adipose tissue of mice on normal and high-fat diet treated with 0.9% saline, 5 mg/kg SQEL, 10 mg/kg SQEL and 10 mg/kg orlistat for 12 weeks. Ct values of AMPK and PGC-1α were normalized to those of beta-actin and GAPDH and relative mRNA expressions of the treated samples were obtained by comparing to that of the vehicle control. Data are presented as the mean ± SEM, n=7-8. (*$p< 0.05$ and ** $p<0.01$) versus respective control (0.9% saline) and $^{\#}p< 0.05$ and $^{\#\#}p< 0.01$ versus normal diet 0.9% saline.

CHAPTER 5: DISCUSSION

Quassinoids are bioactive constituents of bitter principles from the plants belonging to the Simaroubaceae family. Their broad biological properties have attracted much attention from the scientific community. Various quassinoids isolated from Simaroubaceous plants possess antimalarial, anti-inflammatory, anti-tumor, anti-cancer and anti-parasitic effects (Guo *et al.*, 2005a; Jiwajinda *et al.*, 2002). *Eurycoma longifolia* Jack is a plant classified under the same family and is well-known as a traditional herb used for the treatment of various ailments. Six major quassinoids can be found in the root of this plant which includes, eurycomanone, 13,21-dihydroeurycomanone, 13α(21)-epoxyeurycomanone, 14,15β-dihydroxyklaineanone, eurycomalactone, and longilactone (Han *et al.*, 2015). In recent years, natural products have been receiving notable attention as alternative sources to develop safe and effective drugs for weight management. To date, there are various dietary phytochemicals including polyphenols, alkaloids, terpenoids, organosulfur and phytosterols that have been reported to possess anti-obesity effects (Mohamed *et al.*, 2014). However, the effects of quassinoids in adipogenesis and the underlying mechanisms involved remain to be investigated.

In the present study, the anti-adipogenic effect of a quassinoids composition (SQEL) and the pure compound eurycomanone from *Eurycoma longifolia* Jack was investigated. Therefore, SQEL that contains enriched quassinoids (Low *et al.*, 2013) and the commercially available purified eurycomanone were used in this study. In addition, sulforaphane, an isothiocyanate compound found in cruciferous plants that were previously shown the anti-adipogenic effect was used as a positive control in this study (Choi *et al.*, 2012). Fibroblast-like preadipocytes including 3T3-L1, 3T3-F422A, 1246, Ob1771, TA1, and 30A5 cells are able to differentiate into adipocytes and derived from transgenic mice (Sarjeant & Stephens, 2012). The 3T3-L1 cell is one of the best cellular

models widely used to study *in vitro* adipocyte differentiation due to their capacity to develop a homogeneous mature adipocytes population, minimal drop in differentiation potential and better sensitivity towards lipogenic and lipolytic drug treatment (Ali *et al.*, 2013; MacDougald & Lane, 1995; Sarjeant & Stephens, 2012). Pre-adipocytic 3T3-L1 cells are committed to adipocyte differentiation via intracellular lipid accumulation upon hormonal induction (Rosen & MacDougald, 2006). Interestingly, adipogenesis and accumulation of lipid droplets are directly associated with the development of obese condition (Gregoire *et al.*, 1998). A flavonoid treatment has impaired cellular lipid accumulation in 3T3-L1 adipocytes as well as significantly decreased subcutaneous white adipose tissue weight in mice (Inafuku *et al.*, 2013). In another study, adipogenesis and body weight gain were prevented respectively in 3T3-L1 cells and high-fat diet induced obese mice with blueberry extract treatment (Song *et al.*, 2013). Thus, inhibiting adipogenic differentiation may prevent adipocyte hypertrophy and reduce its lipid content as shown previously by several findings (Inafuku *et al.*, 2013; Park *et al.*, 2012; Song *et al.*, 2013).

Prior to the study on the adipogenic inhibitory effects of SQEL and eurycomanone in 3T3-L1 cells, potential cytotoxicity effects of both the compounds were first ascertained. The chosen concentrations of SQEL and eurycomanone were not cytotoxic to the differentiating adipocytes, however, significantly inhibited adipogenesis by reducing the accumulation of cytosolic lipid in differentiating 3T3-L1 cells. Hence, the anti-adipogenic effect shown by these compounds was independent of non-specific cell toxicity. In this study, the pure compound, eurycomanone was found to have greater *in vitro* anti-adipogenic effect compared to the purified aqueous SQEL that contains about 32% quassinoids including 14% of eurycomanone.

Lipolysis is the catabolic process of triglyceride breakdown into free fatty acids and glycerol in adipocytes (Lass *et al.*, 2011). This is an important energy homeostatic process whereby; the free fatty acids are taken up by other body tissues to be utilized for β-oxidation and subsequent ATP generation in the event of energy demand (Kolditz & Langin, 2010). As lipolysis is a critical factor to reduce the amount of stored lipid in adipocytes, it can be one of the effective therapeutic targets to combat obesity. Interestingly, both SQEL and eurycomanone induced lipolysis in mature adipocytes and resulted in an increase in glycerol content but reduced intracellular triglycerides at the concentration that did not exert cytotoxicity. This finding is consistent with a previous study whereby, eurycomanone isolated from the powdered root of EL enhanced glycerol release and reduced lipid accumulation without cytotoxic effects in matured 3T3-L1 adipocytes (Lahrita *et al.*, 2017). The same study also elucidated that the lipolytic effects of eurycomanone are mediated by the direct activation of PKA, a potent activator of lipid hydrolyzing enzyme, hormone-sensitive lipase (Lahrita *et al.*, 2017).

Adipogenesis is a highly controlled process governed by a complex network of transcription factors. Cultured pre-adipocytes will proliferate to achieve growth arrest phase and begin to express the transcription initiator proteins PPARγ and C/EBPα upon adipogenic induction by a cocktail of hormones (Farmer, 2006; Gregoire *et al.*, 1998). PPARγ and C/EBPα are key adipogenic transcription factors that initiate the expression of pro-adipogenic markers for the phenotypical establishment of matured adipocyte (Moseti *et al.*, 2016; Rosen, 2005). Nonetheless, several earlier reports have shown that down-regulation of PPARγ and C/EBPα expression during the initial phase of differentiation have successfully prevented adipocyte maturation and triglyceride accumulation in 3T3-L1 cells (Inafuku *et al.*, 2013; Ono & Fujimori, 2011). Comparably, SQEL and eurycomanone treatment have also significantly reduced the protein expression of PPARγ and C/EBPα during the early stage of differentiation in 3T3-L1

cells. In the current study, SQEL and eurycomanone treatment for 48 hours reduced the expression of both PPARγ isomers but significant changes were observed only in PPARγ2 protein expression. Zhu and colleagues have reported that PPARγ is expressed in two isoforms: PPARγ1 and PPARγ2 as a result of splicing and using alternate promoter (Zhu *et al.*, 1995). However, PPARγ2 is the isoform that plays the regulatory role in adipogenesis while PPARγ1 has no effect in adipogenic differentiation (Ren *et al.*, 2002). This suggests that the down-regulation of PPARγ2 has a significant implication on adipocyte differentiation. Surprisingly, sulforaphane treatment resulted in the up-regulation of PPARγ expression during the early stage of adipogenesis but significantly reduced terminal differentiation and triglyceride accumulation in 3T3-L1 cells suggesting that the compound may reduce the expression of PPARγ in the later stage of adipogenic differentiation. These results are in agreement with a previous report whereby, significant suppression of PPARγ expression was observed only after 6 days of sulforaphane treatment in differentiation medium (Choi *et al.*, 2012).

On the other hand, PPARγ and C/EBPα are also responsible for the expression of adipocyte-specific genes including FAS, LPL and KLF15 which are involved in the formation of mature adipocytes (Farmer, 2006). FAS regulates *de novo* lipogenesis from acetyl-CoA, malonyl-CoA, and NADPH and is abundantly expressed in adipose tissue, liver, and lung (Ranganathan *et al.*, 2006). An earlier study showed that the rate of FAS synthesis is increased during the early phase of adipogenic differentiation which is attributed to the deposition of cytoplasmic lipid in 3T3-L1 cells (Student *et al.*, 1980). In addition, LPL, an early marker for adipogenesis was found to be elevated in adipocytes (Vu *et al.*, 1996) and its overexpression in mice has been shown to promote obesity (Delezie *et al.*, 2012). Furthermore, the DNA-binding transcriptional regulator, KLF15 is also induced during adipogenesis to increase the PPARγ2 gene promoter activity and its inhibition blocked differentiation in 3T3-L1 cells (Mori *et al.*, 2005). Findings from the

present study showed that the expression of FAS protein, mRNA expression levels of LPL and KLF15 were significantly downregulated in 3T3-L1 cells treated with SQEL and eurycomanone compared to vehicle control cells. Therefore, the reduction of adipogenic-specific genes and protein expression is directly contributed by the suppression of PPARγ and C/EBPα transcription factors. This finding suggests that SQEL and eurycomanone inhibit differentiation of pre-adipocytes and lipid accumulation in matured adipocytes by inhibiting the expression of adipogenic transcriptional factors and their downstream target proteins.

In addition to PPARγ and C/EBPα that play a pivotal role in the transcriptional control of adipocyte differentiation *in vitro,* earlier reports have identified the involvement of metabolic regulatory factors such as sirtuin (Sirt) proteins in adipogenesis (Cho *et al.*, 2009). The activation of Sirt1 has been shown to impair adipocyte formation while its inhibition leads to adipocyte hyperplasia and eventually promotes the expression of adipogenic markers (Backesjo *et al.*, 2006). The upregulation of Sirt1 protein during the early stage of adipogenesis, could reduce the expression of PPARγ and inhibit adipocyte maturation (Kim *et al.*, 2015). In addition, Mayoral and colleagues have demonstrated that Sirt1 knock-out mice on high-fat diet displayed PPARγ hyperactivity and eventually leads to adipocyte hypertrophy (Mayoral *et al.*, 2015). Moreover, the overexpression of Sirt1 was also previously shown to block triglyceride accumulation in 3T3-L1 cells (Imamura *et al.*, 2017), suggesting that Sirt1 could be the upstream regulator of adipogenic transcription factors PPARγ and C/EBPα in modulating adipogenesis. In an earlier study, the nuclear entry of Sirt1 was reported to reduce the expression of PPARγ and its downstream adipogenic proteins which then inhibits 3T3-L1 adipocyte differentiation (Wang *et al.*, 2016). Picard and colleagues have shown that Sirt1 blocks adipogenesis by repressing the transactivation of PPARγ upon binding to the PPARγ cofactors (repression domain 1 and CBF/Su(H) interaction domain) in 3T3-L1 cells

(Picard *et al.*, 2004). In addition, an increased Sirt1 expression during adipogenesis also promotes deacetylation of PPARγ which then impairs PPARγ transcriptional activity and obstructs adipogenesis in 3T3-L1 cells (Kim *et al.*, 2015). In the present study, the mRNA and protein expression of Sirt1 during the early phase of adipogenesis was significantly upregulated together with down-regulation of PPARγ, C/EBPα and other adipogenic-associated markers by SQEL and eurycomanone treatment. FoxOs are key regulators of metabolism in insulin-sensitive tissues such as liver, adipose, and muscle cells (Gross *et al.*, 2008). Overexpression of FoxO1 and FoxO3a suppress adipogenesis in 3T3-L1 cells by down-regulating PPARγ and C/EBPα (Lai *et al.*, 2012; Nakae *et al.*, 2003). Comparably, the protein expression of FoxO3a was dramatically elevated along with suppression of key adipogenic transcription factors by SQEL and eurycomanone treatment in differentiating 3T3-L1 cells. It is known that Sirt1 is the upstream regulator of FoxOs in adipogenic inhibition, the finding from the present study is suggestive of the possible involvement of Sirt1/FoxO3a/PPARγ signaling in SQEL and eurycomanone-mediated adipogenesis inhibition in 3T3-L1 cells. It is shown that several phytochemicals including agrimol B, fisetin, xanthigen and resveratrol, were also shown to inhibit adipogenesis via Sirt1/PPARγ or Sirt1/FoxO/PPARγ signaling pathway (Costa *et al.*, 2011; Kim *et al.*, 2015; Lai *et al.*, 2012; Wang *et al.*, 2016).

Several reports have shown the correlation between adipogenesis and the development of obese condition (Nishimura *et al.*, 2007; Spiegelman & Flier, 1996). This is because the number of adipocytes in a given fat mass increases in obesity due to the uncontrolled adipogenesis that contributes to the formation of new adipocytes from precursor cells (Hausman *et al.*, 2001; Tchoukalova *et al.*, 2004). Studies on human have shown that higher fat intake is accompanied by body weight gain and eventually can lead to obesity and other related comorbidities. Rodent is suitable models to study both the mechanistic and therapeutic aspects of obesity since they are susceptible to weight gain upon feeding

with HFDs in a relatively short duration (Buettner *et al.*, 2007). Most rodents on obesogenic diet readily become obese, however, inbred mouse strain such as C57BL/6J mice are more prone to obesity when given HFDs (Rossmeisl *et al.*, 2003). Moreover, the HFD-induced obese C57BL/6J mice also develop features of obesity-associated diseases such as hypertriglyceridemia, hyperglycemia, insulin resistance and hepatic steatosis supporting the suitability of this mice strain as an experimental rodent model of metabolic syndrome (Fernandes-Santos *et al.*, 2009; Fraulob *et al.*, 2010). Although dietary intake of 60 kcal% fat in a human would be physiologically irrelevant, the said diets are generally used to enhance weight gain in rodents (Jiang *et al.*, 2009; Johnston *et al.*, 2007). Thus, obesity-related disease models can be established quickly for pre-clinical interventional studies in a shorter period of time.

There has been a growing number of phytochemicals including curcumin, rutin and clerodane diterpene that has been shown to inhibit the progression of adipogenesis in 3T3-L1 cells as well as obesity in C57BL/6J mouse model (Beg *et al.*, 2015; Choi *et al.*, 2006; Ejaz *et al.*, 2009). In the present study, SQEL treatment not only decreased the weight of epididymal and perirenal adipose tissues but has also reduced the adipose tissue size of mice on HFD. This shows that the development of epididymal and perirenal adipose tissues was reduced in the presence of SQEL, possibly due to the inhibitory effect of triglyceride accumulation in fat cells as demonstrated by the inhibition of adipogenesis and cytosolic lipid accumulation *in vitro*. These findings hence, suggest that SQEL-mediated suppression of body weight gain in mice consumed high-fat diet was due to the reduction in adipose tissue size and weight.

Interestingly, body weight gain was prevented in SQEL treated-mice without caloric restriction which is on par with other previous studies that showed weight gain in animal models could also be prevented without the suppression of food intake (Beg *et al.*, 2015;

Inafuku *et al.*, 2013; Sharma *et al.*, 2017; Song *et al.*, 2013). This then suggests that SQEL may regulate body weight gain by enhancing calorie expenditure. It is shown that increased calorie expenditure could be mediated through the action of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (Gao *et al.*, 2009). PGC-1α is a transcriptional coactivator and an inducer of transcription factors involved in cellular energy metabolism and also acts as a master regulator of mitochondrial biogenesis (Cantó & Auwerx, 2009). Overexpression of PGC-1α in white adipocytes leads to upregulation in the expression of mitochondrial respiratory chain proteins and metabolic enzymes involved in fatty acid oxidation (Tiraby *et al*. 2003). Moreover, a previous study has demonstrated the reduction of body weight gain in mice which was mediated by an increase in energy expenditure and fatty acid oxidation was through the actions of PGC-1α on butyrate supplementation (Gao *et al.*, 2009). Furthermore, adipose tissue-specific deletion of PGC-1α in mice has resulted in severe weight gain, increase in circulating lipids, insulin resistance, impaired glucose homeostasis along with reduced expression of genes modulating lipid metabolism and fatty acid uptake in adipose tissues (Kleiner *et al.*, 2012). Nevertheless, the mRNA expression of PGC-1α was also diminished in adipose tissue of mice on HFD compared to those on low-fat diet for 6 weeks (Cummins *et al*. 2014). Similarly, in the present study, mice fed with HFD had significantly lower mRNA level of PGC-1α when compared to ND fed mice group. Nonetheless, other studies have demonstrated the involvement of the adenosine monophosphate-activated protein kinase (AMPK) in increased calorie expenditure in mice models (Choi *et al.*, 2017; Kudo *et al.*, 2015). AMPK has been shown to regulate PGC-1α and mitochondrial biogenesis in several tissues, including adipose tissues (Gaidhu *et al.*, 2009). AMPK regulates energy metabolism by stimulating the interaction between PGC1α and PRDM16 resulting in increased mitochondrial biogenesis, thermogenesis, and energy expenditure (Zhang *et al.*, 2016). Another study further

demonstrated that AMPK could increase the expression of Sirt1 and enhance mitochondrial biogenesis through PGC1α (Fernandez-Marcos & Auwerx, 2011). Interestingly, SQEL treatment has elevated the mRNA expression of AMPK and PGC1α in adipose tissue of mice fed HFD. This finding suggests that SQEL could also alter the energy balance through an increased calorie expenditure via AMPK- PGC1α signaling in white adipose tissue in preventing weight gain among mice on HFD.

Hyperglycemia and insulin resistance are strongly associated with obesity. Prior studies have shown that C57BL/6J mice fed with high-fat diet have elevated blood glucose level (Winzell & Ahren, 2004) and an anti-obesity compound treatment has improved glucose tolerance (Chen *et al.*, 2017). Results from this study show that the development of glucose intolerance in HFD mice was significantly inhibited by 12 weeks of SQEL treatment. Even though the time-course of glucose clearance in HFD fed saline control mice was delayed compared to that of the ND fed mice, SQEL also improved glucose clearance significantly in mice on a normal diet. Insulin is the key regulator of adipocyte morphology whereby it promotes the differentiation of pre-adipocytes into adipocytes and stimulates lipogenesis as well as inhibiting lipolysis in mature adipocytes (Kahn & Flier, 2000). Adipocytes are one of the cell types that are more sensitive to insulin and hence impaired response towards insulin signaling on metabolism and glucose transport in adipose tissue will lead to insulin resistance in obese individual (Kahn & Flier, 2000). However, the attempt to elucidate the effect of SQEL treatment on insulin sensitivity could not be accomplished since insulin resistance was not observed in both ND and HFD fed mice groups, likely due to a shorter study period. Previously, significant insulin resistance was observed in mice fed with HFD for 16 weeks (Fraulob *et al.*, 2010) and targeted mutagenesis or transgenesis of genes required for insulin action and insulin secretion in mice (Nandi *et al.*, 2004) would yield better animal model to be used for studies on insulin resistant. As PPARγ activation has been shown to induce the expression

of a number of genes involved in the insulin signaling cascade (Leonardini *et al.,* 2010) inhibition of PPARγ by SQEL treatment could have led to the lack of effect on insulin sensitivity in normal although glucose intolerance was reduced.

Histological analysis of liver sections of HFD fed mice revealed the accumulation of abundant lipid droplets compared to mice on ND, a prominent sign of hepatic steatosis strongly associated with obesity (Fabbrini *et al.*, 2010). Liver sections of SQEL treated mice fed with ND exhibited normal histology similar to orlistat treatment. Moreover, mice on HFD supplemented with SQEL significantly attenuated the progression of fatty liver disease evidenced by a drastic reduction in the number of lipid droplets in the liver. On the other hand, liver histology of mice received SQEL did not show any sign of inflammation or cytotoxicity, suggesting that increased liver weight was not attributed to liver damage. Interestingly, these findings are comparable to the effects of orlistat treatment in preventing the development of steatohepatitis in obese individuals (Harrison *et al.,* 2003) and indicate that SQEL can intensely suppress the pathological sign in the liver.

Hypertriglyceridemia and hypercholesterolemia are associated with increased risk for obesity as there is a significant correlation between impaired lipid metabolism and obesity (Eisinger *et al.*, 2014; Subramanian & Chait, 2012). Therefore, the effects of SQEL treatment in the regulation of lipid metabolism were studied in both normal and high-fat diet consumed mice. Previously, it was shown that C57BL/6J mice on the obesogenic diet had elevated levels of serum triglycerides and cholesterol compared to those on ND (Beg *et al.*, 2015; Song *et al.*, 2013). However, in the present study, serum total cholesterol but not triglyceride levels were significantly higher in mice fed with HFD compared to those on ND. Nevertheless, decreased level of circulating triglycerides and insignificant change in serum triglycerides have also been found in C57BL/6J mice fed with HFD compared

to mice fed with standard diet previously (Eisinger *et al.*, 2014; Ji *et al.*, 2014). Moreover, high-fat consumption or reduced carbohydrate intake could lessen triglyceride production and/or induce triglyceride clearance that eventually lowers circulating level of triglycerides in obese mouse model (Guo *et al.*, 2009). Results from the present study demonstrated that SQEL treatment reduces both total cholesterol and triglycerides levels in mice regardless of the types of diet given. These outcomes indicate that SQEL modulates triglyceride and cholesterol metabolism both in normal and obese mice.

CHAPTER 6: CONCLUSION

6.1 Conclusion

Adipogenesis dysregulation has been associated with the development of fat tissue mass that leads to obesity, a public health issue owing to its high prevalence, morbidity and mortality. Despite the advancement in pharmacotherapy and other non-pharmacological methods advocated to combat this serious health concern, unsatisfactory outcome and serious side effects warrant the search for a novel and/or alternative phytomedicine for the treatment of obesity. The current study is an effort to investigate the potentials of naturally derived bioactive compounds, SQEL as an anti-adipogenic agent to halt adipogenesis and eventually the progression to obese condition. In the present study, the cytotoxicity, anti-adipogenic and anti-obesity effect of SQEL were investigated in 3T3-L1 cells and diet-induced obese C57BL/6J mice.

Results from the present study revealed that the tested SQEL and eurycomanone concentrations are not cytotoxic to both differentiating pre-adipocytes and matured adipocytes. Concurrent treatment of SQEL and eurycomanone at non-cytotoxic concentrations to differentiating 3T3-L1 cells blocked adipogenesis. This finding was further strengthened by the observation of a concentration-dependent reduction in the number of cells with stained lipid droplets treated with SQEL and eurycomanone through microscopic inspection of the Oil Red O staining. A stronger adipogenic inhibitory effect showed by eurycomanone suggests that the anti-adipogenic property of SQEL could be mediated by the presence of eurycomanone which comprises 14% of quassinoid composition of SQEL. SQEL and eurycomanone also impaired lipogenesis as revealed by a dose-dependent reduction in the level of cytosolic triglyceride. Furthermore, SQEL and eurycomanone treatment also enhanced lipolysis that eventually reduced the

intracellular triglyceride content in mature adipocytes. These findings suggest that SQEL and eurycomanone block adipogenesis and regulate lipid metabolism in adipocytes.

The inhibitory effect of adipogenesis induced by SQEL and eurycomanone on the molecular regulation of transcription factors together with its downstream adipogenic-specific markers in differentiating 3T3-L1 cells was also studied. In the present study, the transcription factors PPARγ and C/EBPα that expressed immediately upon the induction of pre-adipocyte differentiation were significantly downregulated by both SQEL and eurycomanone treatment. Subsequently, the expression of adipogenic markers such as FAS, LPL and KLF15 which are necessary for the phenotypical establishment of matured adipocyte were also impaired by the same treatment. On the other hand, Sirt1 and FoxO3a expression was enhanced by SQEL and eurycomanone during the early phase of adipogenesis in 3T3-L1 cells. These outcomes propose that the blockade of adipogenesis in differentiating 3T3-L1 cells following treatment with SQEL and eurycomanone is due to the repressive effect of the adipogenic-specific transcription factors and markers in which probably modulated by the Sirt1/FoxO3a/PPARγ signalling.

As the involvement of adipogenesis is crucial in obesity progression, the anti-obesity effect of SQEL was assessed in the ND and HFD fed C57BL/6J mice. The administration of SQEL via intraperitoneal route for 12 consecutive weeks prevented both ND and HFD fed mice from significant body weight gain independent of their total calories consumed. Moreover, SQEL treatment has induced energy expenditure in adipose tissue of mice on HFD by elevating the expression of thermogenic genes, AMPK and PGC-1α. Similarly, SQEL treatment also attenuated adipose tissue hypertrophy as evidenced by a significant reduction in the epididymal and perirenal fat pad mass in mice consumed HFD. Conclusively, these findings suggest SQEL consumption could prevent body weight gain by impairing the enlargement of adipose tissue that in turn reduces fatty tissue mass in

HFD-induced obese mice. Supplementation of SQEL improved glucose clearance, decreased serum triglycerides and cholesterol levels in both ND and HFD fed mice. Additionally, the development of hepatic steatosis was also significantly prevented in the obese mouse supplemented with SQEL. These findings suggest that SQEL treatment not only possesses anti-obesity property but also prevents the development of obesity-associated diseases in C57BL/6J mice.

Collectively, the outcomes of the present study are strongly suggestive of the novel effect of SQEL in inhibiting adipogenesis and has potential as a natural therapeutic agent in preventing the development of obesity.

6.2 Limitations of the study and future direction

Adipogenesis is a closely regulated process that involves the transition of cell proliferation to cell differentiation where both events leading to the commitment of the pre-adipocytes maturation into adipocyte (Fajas, 2003). The clonal expansion, a cell cycle re-entry is necessary to induce metabolic gene expression for the accumulation of cytosolic lipid droplets in adipocytes (Reichert & Eick, 1999). Therefore, *in vitro* adipogenic inhibitors could also act as the cell cycle blocking agents that impair the 3T3-L1 cell differentiation. Several bioactive compounds such as vitisin A and sulforaphane have been shown to induce cell cycle arrest at various stages of a clonal expansion that eventually blocked adipogenesis in 3T3-L1 cells (Choi *et al.*, 2012; Kim *et al.*, 2008). Hence, further study can be conducted to identify the possible modulation of the cell cycle by SQEL and eurycomanone-mediated adipogenesis inhibition in differentiating pre-adipocytes.

Lipolysis is the mobilization of triglycerides in adipose tissue that is hydrolyzed into free fatty acids and glycerol to provide energy to the body when needed. This catabolic process plays a crucial role in regulating triglycerides turnover and it is dysregulated in

an obese individual thus contributing to several pathological conditions (Arner *et al.*, 2011). Previous studies have demonstrated that drugs that induce lipolysis prevent adipocyte hypertrophy, promote energy expenditure, reduced hyperlipidemia, hyperglycemia and attenuates obesity in mice (Chen *et al.*, 2017; Xiong *et al.*, 2015). Similarly, SQEL induced lipolysis as indicated by significant increase in glycerol production and a decrease in accumulated triglycerides in matured adipocytes. However, the mechanism of SQEL-induced lipolysis *in vitro* and its lipolytic effect in obese rodent has yet to be unveiled and warrants further studies. In the present study, the *in vivo* triglycerides and LDL lowering effects of SQEL was only moderately achieved. Hence, improvement to the current preparation of SQEL to yield greater efficacy is recommended. Furthermore, as EL is commonly taken orally, preparation given via oral route rather than intraperitoneal route can be tested.

Hypertension is one of the major contributory factors in the cardiovascular morbidity and mortality that strongly associated with obesity. Obesity is well known as a causative or a coexisting factor in the pathogenesis of blood pressure that leads to myocardial infraction, stroke and kidney failure (Susic & Varagic, 2017). In reality, dietary factors, metabolic, endothelial, vascular and inflammatory dysfunction are the potential mechanism linking obesity to hypertension (DeMarco *et al.*, 2014). A previous study found a purified fraction from *Eurycoma longifolia* plant has hypotensive properties and suggested it could be used as a conventional treatment for the control of hypertension (Tee *et al.*, 2016). However, the study was limited to the investigation of the purified fraction only in normal but not in the obese rodent. Since the results from current study strongly suggest the obesity preventive effects of SQEL in a diet-induced obese mouse model, the potential anti-hypertensive properties of SQEL in the obese animal model is worth exploring further.

REFERENCES

Agarwal, A. K., & Garg, A. (2002). A novel heterozygous mutation in peroxisome proliferator-activated receptor-γ gene in a patient with familial partial lipodystrophy. *The Journal of Clinical Endocrinology & Metabolism, 87*(1), 408-408.

Agarwal, S., & Rao, A. V. (2000). Tomato lycopene and its role in human health and chronic diseases. *Canadian Medical Association Journal, 163*(6), 739-744.

Ahmad, N., Samiulla, D., Teh, B., Zainol, M., Zolkifli, N., Muhammad, A., . . . Ismail, Z. (2018). Bioavailability of Eurycomanone in Its Pure Form and in a Standardised Eurycoma longifolia Water Extract. *Pharmaceutics, 10*(3), 90.

Ahn, J., Lee, H., Jung, C. H., Jeon, T. I., & Ha, T. Y. (2013). MicroRNA-146b promotes adipogenesis by suppressing the SIRT1-FOXO1 cascade. *EMBO Molecular Medicine, 5*(10), 1602-1612.

Ahn, J., Lee, H., Kim, S., Park, J., & Ha, T. (2008). The anti-obesity effect of quercetin is mediated by the AMPK and MAPK signaling pathways. *Biochemical and Biophysical Research Communications, 373*(4), 545-549.

Ailhaud, G. (1982). Adipose cell differentiation in culture. *Molecular and Cellular Biochemistry, 49*(1), 17-31.

Al-Salahi, O. S., Kit-Lam, C., Majid, A. M., Al-Suede, F. S., Mohammed Saghir, S. A., Abdullah, W. Z., . . . Yusoff, N. M. (2013). Anti-angiogenic quassinoid-rich fraction from Eurycoma longifolia modulates endothelial cell function. *Microvascular Research, 90*, 30-39.

Al-Salahi, O. S. A., Ji, D., Majid, A. M. S. A., Kit-Lam, C., Abdullah, W. Z., Zaki, A., . . . Majid, A. S. A. (2014). Anti-tumor activity of Eurycoma longifolia root extracts against K-562 cell line: in vitro and in vivo study. *PloS One, 9*(1), e83818.

Alappat, L., & Awad, A. B. (2010). Curcumin and obesity: evidence and mechanisms. *Nutrition Reviews, 68*(12), 729-738.

Ali, A. T., Hochfeld, W. E., Myburgh, R., & Pepper, M. S. (2013). Adipocyte and adipogenesis. *European Journal of Cell Biology, 92*(6-7), 229-236.

Ang, H. H., Chan, K. L., & Mak, J. W. (1995). In vitro antimalarial activity of quassinoids from Eurycoma longifolia against Malaysian chloroquine-resistant Plasmodium falciparum isolates. *Planta Medica, 61*(2), 177-178.

Ang, H. H., Ikeda, S., & Gan, E. K. (2001). Evaluation of the potency activity of aphrodisiac in Eurycoma longifolia Jack. *Phytotherapy Research, 15*(5), 435-436.

Apovian, C. M., Aronne, L. J., Bessesen, D. H., McDonnell, M. E., Murad, M. H., Pagotto, U., . . . Endocrine, S. (2015). Pharmacological management of obesity: an endocrine Society clinical practice guideline. *Journal of Clinical Endocrinology and Metabolism, 100*(2), 342-362.

Armoni, M., Harel, C., Karni, S., Chen, H., Bar-Yoseph, F., Ver, M. R., . . . Karnieli, E. (2006). FOXO1 represses peroxisome proliferator-activated receptor-γ1 and-γ2 gene promoters in primary adipocytes a novel paradigm to increase insulin sensitivity. *Journal of Biological Chemistry, 281*(29), 19881-19891.

Arner, E., Westermark, P. O., Spalding, K. L., Britton, T., Rydén, M., Frisén, J., . . . Arner, P. (2010). Adipocyte turnover: relevance to human adipose tissue morphology. *Diabetes, 59*(1), 105-109.

Arner, P., Bernard, S., Salehpour, M., Possnert, G., Liebl, J., Steier, P., . . . Spalding, K. L. (2011). Dynamics of human adipose lipid turnover in health and metabolic disease. *Nature, 478*(7367), 110-113.

Arnold, L. M., McElroy, S. L., Hudson, J. I., Welge, J. A., Bennett, A. J., & Keck Jr, P. E. (2002). A placebo-controlled, randomized trial of fluoxetine in the treatment of binge-eating disorder. *The Journal of Clinical Psychiatry*.

Astrup, A., Breum, L., & Toubro, S. (1995). Pharmacological and clinical studies of ephedrine and other thermogenic agonists. *Obesity, 3*(S4).

Backesjo, C. M., Li, Y., Lindgren, U., & Haldosen, L. A. (2006). Activation of Sirt1 decreases adipocyte formation during osteoblast differentiation of mesenchymal stem cells. *Journal of Bone and Mineral Research, 21*(7), 993-1002.

Backhouse, K., Sarac, I., Shojaee-Moradie, F., Stolinski, M., Robertson, M. D., Frost, G. S., . . . Umpleby, A. M. (2012). Fatty acid flux and oxidation are increased by rimonabant in obese women. *Metabolism: Clinical and Experimental, 61*(9), 1220-1223.

Baile, C. A., Yang, J. Y., Rayalam, S., Hartzell, D. L., Lai, C. Y., Andersen, C., & Della‐Fera, M. A. (2011). Effect of resveratrol on fat mobilization. *Annals of the New York Academy of Sciences, 1215*(1), 40-47.

Bain, J. A., Dority, J. S., & Cook, A. M. (2014). Subarachnoid hemorrhage in a patient taking phentermine for weight loss. *Journal of the American Pharmacists Association, 54*(5), 548-551.

Barak, Y., Nelson, M. C., Ong, E. S., Jones, Y. Z., Ruiz-Lozano, P., Chien, K. R., . . . Evans, R. M. (1999). PPARγ is required for placental, cardiac, and adipose tissue development. *Molecular Cell, 4*(4), 585-595.

Beg, M., Shankar, K., Varshney, S., Rajan, S., Singh, S. P., Jagdale, P., . . . Gaikwad, A. N. (2015). A clerodane diterpene inhibit adipogenesis by cell cycle arrest and ameliorate obesity in C57BL/6 mice. *Molecular and Cellular Endocrinology, 399*, 373-385.

Benito, M., Porras, A., Nebreda, A. R., & Santos, E. (1991). Differentiation of 3T3-L1 fibroblasts to adipocytes induced by transfection of ras oncogenes. *Science, 253*(5019), 565-568.

Bennett, C. N., Ross, S. E., Longo, K. A., Bajnok, L., Hemati, N., Johnson, K. W., . . . MacDougald, O. A. (2002). Regulation of Wnt signaling during adipogenesis. *Journal of Biological Chemistry, 277*(34), 30998-31004.

Benvenuti, S., Cellai, I., Luciani, P., Deledda, C., Baglioni, S., Giuliani, C., . . . Mannucci, E. (2007). Rosiglitazone stimulates adipogenesis and decreases osteoblastogenesis in human mesenchymal stem cells. *Journal of Endocrinological Investigation, 30*(9), RC26-RC30.

Berndt, J., Kovacs, P., Ruschke, K., Klöting, N., Fasshauer, M., Schön, M., . . . Blüher, M. (2007). Fatty acid synthase gene expression in human adipose tissue: association with obesity and type 2 diabetes. *Diabetologia, 50*(7), 1472-1480.

Bhat, R., & Karim, A. A. (2010). Tongkat Ali (Eurycoma longifolia Jack): a review on its ethnobotany and pharmacological importance. *Fitoterapia, 81*(7), 669-679.

Bieker, J. J. (2001). Krüppel-like factors: three fingers in many pies. *Journal of Biological Chemistry, 276*(37), 34355-34358.

Birkenmeier, E. H., Gwynn, B., Howard, S., Jerry, J., Gordon, J. I., Landschulz, W. H., & McKnight, S. L. (1989). Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. *Genes and Development, 3*(8), 1146-1156.

Birsoy, K., Chen, Z., & Friedman, J. (2008). Transcriptional regulation of adipogenesis by KLF4. *Cell Metabolism, 7*(4), 339-347.

Blüher, M., Michael, M. D., Peroni, O. D., Ueki, K., Carter, N., Kahn, B. B., & Kahn, C. R. (2002). Adipose tissue selective insulin receptor knockout protects against obesity and obesity-related glucose intolerance. *Developmental Cell, 3*(1), 25-38.

Bluher, S., Petroff, D., Wagner, A., Warich, K., Gausche, R., Klemm, T., . . . Keller, A. (2014). The one year exercise and lifestyle intervention program KLAKS: Effects on anthropometric parameters, cardiometabolic risk factors and glycemic control in childhood obesity. *Metabolism: Clinical and Experimental, 63*(3), 422-430.

Boriani, F., Villani, R., & Morselli, P. G. (2014). Metabolic effects of large-volume liposuction for obese healthy women: a meta-analysis of fasting insulin levels. *Aesthetic Plastic Surgery, 38*(5), 1050-1056.

Bost, F., Aouadi, M., Caron, L., & Binetruy, B. (2005). The role of MAPKs in adipocyte differentiation and obesity. *Biochimie, 87*(1), 51-56.

Bost, F., Aouadi, M., Caron, L., Even, P., Belmonte, N., Prot, M., . . . Pouysségur, J. (2005). The extracellular signal–regulated kinase isoform ERK1 is specifically required for in vitro and in vivo adipogenesis. *Diabetes, 54*(2), 402-411.

Boudina, S., & Graham, T. E. (2014). Mitochondrial function/dysfunction in white adipose tissue. *Experimental Physiology, 99*(9), 1168-1178.

Bouvy-Liivrand, M., Heinaniemi, M., John, E., Schneider, J. G., Sauter, T., & Sinkkonen, L. (2014). Combinatorial regulation of lipoprotein lipase by microRNAs during mouse adipogenesis. *RNA Biology, 11*(1), 76-91.

Bray, G. A. (2004). Medical consequences of obesity. *Journal of Clinical Endocrinology and Metabolism, 89*(6), 2583-2589.

Brandt, N., Kotowska, D., Kristensen, C. M., Olesen, J., Lützhøft, D. O., Halling, J. F., . . . Kiilerich, P. (2018). The impact of exercise training and resveratrol supplementation on gut microbiota composition in high-fat diet fed mice. *Physiological reports, 6*(20), e13881.

Buettner, R., Schölmerich, J., & Bollheimer, L. C. (2007). High-fat diets: modeling the metabolic disorders of human obesity in rodents. *Obesity, 15*(4), 798-808.

Camp, H. S., & Tafuri, S. R. (1997). Regulation of peroxisome proliferator-activated receptor γ activity by mitogen-activated protein kinase. *Journal of Biological Chemistry, 272*(16), 10811-10816.

Cao, Z., Umek, R. M., & McKnight, S. L. (1991). Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes and Development, 5*(9), 1538-1552.

Cantó, C., & Auwerx, J. (2009). PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure. *Current Opinion in Lipidology*, 20(2), 98.

Caprio, M., Feve, B., Claës, A., Viengchareun, S., Lombès, M., & Zennaro, M.-C. (2007). Pivotal role of the mineralocorticoid receptor in corticosteroid-induced adipogenesis. *The Federation of American Societies for Experimental Biology Journal, 21*(9), 2185-2194.

Carey, A. L., Pajtak, R., Formosa, M. F., Van Every, B., Bertovic, D. A., Anderson, M. J., . . . Kingwell, B. A. (2015). Chronic ephedrine administration decreases brown adipose tissue activity in a randomised controlled human trial: implications for obesity. *Diabetologia, 58*(5), 1045-1054.

Cartea, M. E., & Velasco, P. (2008). Glucosinolates in Brassica foods: bioavailability in food and significance for human health. *Phytochemistry Reviews, 7*(2), 213-229.

Chan, K.-L., Choo, C.-Y., Abdullah, N. R., & Ismail, Z. (2004). Antiplasmodial studies of Eurycoma longifolia Jack using the lactate dehydrogenase assay of Plasmodium falciparum. *Journal of Ethnopharmacology, 92*(2-3), 223-227.

Chan, K., Lee, S., Sam, T., & Han, B. (1989). A quassinoid glycoside from the roots of Eurycoma longifolia. *Phytochemistry, 28*(10), 2857-2859.

Chan, K. L., Iitaka, Y., Noguchi, H., Sugiyama, H., Saito, I., & Sankawa, U. (1992). 6α-Hydroxyeurycomalactone, a quassinoid from Eurycoma longifolia. *Phytochemistry, 31*(12), 4295-4298.

Chawla, A., Schwarz, E. J., Dimaculangan, D. D., & Lazar, M. A. (1994). Peroxisome proliferator-activated receptor (PPAR) gamma: adipose-predominant expression and induction early in adipocyte differentiation. *Endocrinology, 135*(2), 798-800.

Chen, G., Li, H., Zhao, Y., Zhu, H., Cai, E., Gao, Y., . . . Zhang, L. (2017). Saponins from stems and leaves of Panax ginseng prevent obesity via regulating thermogenesis, lipogenesis and lipolysis in high-fat diet-induced obese C57BL/6 mice. *Food and Chemical Toxicology, 106*(Pt A), 393-403.

Chen, J., Sang, Z., Li, L., He, L., & Ma, L. (2017). Discovery of 5-methyl-2-(4-((4-(methylsulfonyl) benzyl) oxy) phenyl)-4-(piperazin-1-yl) pyrimidine derivatives as novel GRP119 agonists for the treatment of diabetes and obesity. *Molecular Diversity, 21*(3), 637-654.

Chen, Q., Shou, P., Zheng, C., Jiang, M., Cao, G., Yang, Q., . . . Zhang, X. (2016). Fate decision of mesenchymal stem cells: adipocytes or osteoblasts? *Cell Death and Differentiation, 23*(7), 1128.

Chen, Z.-L., Shao, W.-J., Xu, F., Liu, L., Lin, B.-S., Wei, X.-H., . . . Weng, J.-P. (2015). Acute Wnt pathway activation positively regulates leptin gene expression in mature adipocytes. *Cellular Signalling, 27*(3), 587-597.

Cho, Y.-W., Hong, S., Jin, Q., Wang, L., Lee, J.-E., Gavrilova, O., & Ge, K. (2009). Histone methylation regulator PTIP is required for PPARγ and C/EBPα expression and adipogenesis. *Cell Metabolism, 10*(1), 27-39.

Choi, H. S., Jeon, H. J., Lee, O. H., & Lee, B. Y. (2014). Indole-3-carbinol, a vegetable phytochemical, inhibits adipogenesis by regulating cell cycle and AMPKalpha signaling. *Biochimie, 104*, 127-136.

Choi, I., Park, Y., Choi, H., & Lee, E. H. (2006). Anti-adipogenic activity of rutin in 3T3-L1 cells and mice fed with high-fat diet. *Biofactors, 26*(4), 273-281.

Choi, J., Kim, K.-J., Koh, E.-J., & Lee, B.-Y. (2017). Gelidium elegans Regulates the AMPK-PRDM16-UCP-1 Pathway and Has a Synergistic Effect with Orlistat on Obesity-Associated Features in Mice Fed a High-Fat Diet. *Nutrients, 9*(4), 342.

Choi, J. S., Kim, J. H., Ali, M. Y., Jung, H. J., Min, B. S., Choi, R. J., . . . Jung, H. A. (2015). Anti-adipogenic effect of epiberberine is mediated by regulation of the Raf/MEK1/2/ERK1/2 and AMPKalpha/Akt pathways. *Archives of Pharmacal Research, 38*(12), 2153-2162.

Choi, K. M., Lee, Y. S., Kim, W., Kim, S. J., Shin, K. O., Yu, J. Y., . . . Yoo, H. S. (2014). Sulforaphane attenuates obesity by inhibiting adipogenesis and activating the AMPK pathway in obese mice. *The Journal of Nutritional Biochemistry, 25*(2), 201-207.

Choi, K. M., Lee, Y. S., Sin, D. M., Lee, S., Lee, M. K., Lee, Y. M., . . . Yoo, H. S. (2012). Sulforaphane Inhibits Mitotic Clonal Expansion During Adipogenesis Through Cell Cycle Arrest. *Obesity, 20*(7), 1365-1371.

Christodoulides, C., Lagathu, C., Sethi, J. K., & Vidal-Puig, A. (2009). Adipogenesis and WNT signalling. *Trends in Endocrinology and Metabolism, 20*(1), 16-24.

Chung, S., Yao, H., Caito, S., Hwang, J.-w., Arunachalam, G., & Rahman, I. (2010). Regulation of SIRT1 in cellular functions: role of polyphenols. *Archives of Biochemistry and Biophysics, 501*(1), 79-90.

Cinti, S. (2009). Transdifferentiation properties of adipocytes in the adipose organ. *American Journal of Physiology-Endocrinology and Metabolism, 297*(5), E977-E986.

Coe, N. R., & Bernlohr, D. A. (1998). Physiological properties and functions of intracellular fatty acid-binding proteins. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 1391*(3), 287-306.

Colon-Gonzalez, F., Kim, G. W., Lin, J. E., Valentino, M. A., & Waldman, S. A. (2013). Obesity pharmacotherapy: What is next? *Molecular Aspects of Medicine, 34*(1), 71-83.

Costa, C. d. S., Rohden, F., Hammes, T. O., Margis, R., Bortolotto, J. W., Padoin, A. V., . . . Guaragna, R. M. (2011). Resveratrol upregulated SIRT1, FOXO1, and adiponectin and downregulated PPARγ1–3 mRNA expression in human visceral adipocytes. *Obesity Surgery, 21*(3), 356-361.

Covas, D. T., Panepucci, R. A., Fontes, A. M., Silva, W. A., Orellana, M. D., Freitas, M. C., . . . Jamur, M. C. (2008). Multipotent mesenchymal stromal cells obtained from diverse human tissues share functional properties and gene-expression profile with CD146+ perivascular cells and fibroblasts. *Experimental Hematology, 36*(5), 642-654.

Cristancho, A. G., & Lazar, M. A. (2011). Forming functional fat: a growing understanding of adipocyte differentiation. *Nature Reviews: Molecular Cell Biology, 12*(11), 722-734.

Cummins, T. D., Holden, C. R., Sansbury, B. E., Gibb, A. A., Shah, J., Zafar, N., ... & Bhatnagar, A. (2014). Metabolic remodeling of white adipose tissue in obesity. *American Journal of Physiology-Endocrinology and Metabolism*, 307(3), E262-E277.

Dagon, Y., Avraham, Y., & Berry, E. M. (2006). AMPK activation regulates apoptosis, adipogenesis, and lipolysis by eIF2α in adipocytes. *Biochemical and Biophysical Research Communications, 340*(1), 43-47.

Daitoku, H., Hatta, M., Matsuzaki, H., Aratani, S., Ohshima, T., Miyagishi, M., . . . Fukamizu, A. (2004). Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity. *Proceedings of the National Academy of Sciences of the United States of America, 101*(27), 10042-10047.

Daitoku, H., Sakamaki, J.-i., & Fukamizu, A. (2011). Regulation of FoxO transcription factors by acetylation and protein–protein interactions. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1813*(11), 1954-1960.

Darise, M., Kohda, H., Mizutani, K., & Tanaka, O. (1982). Eurycomanone and eurycomanol, quassinoids from the roots of Eurycoma longifolia. *Phytochemistry, 21*(8), 2091-2093.

Dave, S., Kaur, N. J., Nanduri, R., Dkhar, H. K., Kumar, A., & Gupta, P. (2012). Inhibition of adipogenesis and induction of apoptosis and lipolysis by stem bromelain in 3T3-L1 adipocytes. *PloS One, 7*(1), e30831.

Delezie, J., Dumont, S., Dardente, H., Oudart, H., Gréchez-Cassiau, A., Klosen, P., . . . Challet, E. (2012). The nuclear receptor REV-ERBα is required for the daily balance of carbohydrate and lipid metabolism. *The Federation of American Societies for Experimental Biology Journal, 26*(8), 3321-3335.

DeMarco, V. G., Aroor, A. R., & Sowers, J. R. (2014). The pathophysiology of hypertension in patients with obesity. *Nature Reviews: Endocrinology, 10*(6), 364-376.

Deurenberg, P., Yap, M., & van Staveren, W. A. (1998). Body mass index and percent body fat: a meta analysis among different ethnic groups. *International Journal of Obesity and Related Metabolic Disorders, 22*(12), 1164-1171.

Donnelly, J. E., Blair, S. N., Jakicic, J. M., Manore, M. M., Rankin, J. W., & Smith, B. K. (2009). American College of Sports Medicine Position Stand. Appropriate physical activity intervention strategies for weight loss and prevention of weight regain for adults. *Medicine and Science in Sports and Exercise, 41*(2), 459-471.

Douglas, I. J., Bhaskaran, K., Batterham, R. L., & Smeeth, L. (2015). Bariatric surgery in the United Kingdom: a cohort study of weight loss and clinical outcomes in routine clinical care. *PLoS Medicine, 12*(12), e1001925.

Eisinger, K., Liebisch, G., Schmitz, G., Aslanidis, C., Krautbauer, S., & Buechler, C. (2014). Lipidomic analysis of serum from high fat diet induced obese mice. *International Journal of Molecular Sciences, 15*(2), 2991-3002.

Ejaz, A., Wu, D., Kwan, P., & Meydani, M. (2009). Curcumin inhibits adipogenesis in 3T3-L1 adipocytes and angiogenesis and obesity in C57/BL mice. *Journal of Nutrition, 139*(5), 919-925.

Elberg, G., Gimble, J. M., & Tsai, S. Y. (2000). Modulation of the murine peroxisome proliferator-activated receptor γ2 promoter activity by CCAAT/enhancer-binding proteins. *Journal of Biological Chemistry, 275*(36), 27815-27822.

Eldar, S., Heneghan, H., Brethauer, S., & Schauer, P. (2011). Bariatric surgery for treatment of obesity. *International Journal of Obesity, 35*(S3), S16.

Essayan, D. M. (2001). Cyclic nucleotide phosphodiesterases. *Journal of Allergy and Clinical Immunology, 108*(5), 671-680.

Esteve Ràfols, M. (2014). Adipose tissue: Cell heterogeneity and functional diversity. *Endocrinología y Nutrición (English Edition), 61*(2), 100-112.

Evans, R. M., Barish, G. D., & Wang, Y.-X. (2004). PPARs and the complex journey to obesity. *Nature Medicine, 10*(4), 355.

Fabbrini, E., Sullivan, S., & Klein, S. (2010). Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. *Hepatology, 51*(2), 679-689.

Fajas, L. (2003). Adipogenesis: a cross-talk between cell proliferation and cell differentiation. *Annals of Medicine, 35*(2), 79-85.

Fan, H., Wu, D., Tian, W., & Ma, X. (2013). Inhibitory effects of tannic acid on fatty acid synthase and 3T3-L1 preadipocyte. *Biochimica et Biophysica Acta, 1831*(7), 1260-1266.

Farmer, S. R. (2006). Transcriptional control of adipocyte formation. *Cell Metabolism, 4*(4), 263-273.

Feldmann, H. M., Golozoubova, V., Cannon, B., & Nedergaard, J. (2009). UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. *Cell Metabolism, 9*(2), 203-209.

Fernandes-Santos, C., Carneiro, R. E., Mendonca, L. D., Aguila, R. B., & Mandarim-de-Lacerda, C. A. (2009). Rosiglitazone aggravates nonalcoholic fatty pancreatic disease in C57BL/6 mice fed high-fat and high-sucrose diet. *Pancreas, 38*(3), E80-E86.

Fernandez-Marcos, P. J., & Auwerx, J. (2011). Regulation of PGC-1α, a nodal regulator of mitochondrial biogenesis. *American Journal of Clinical Nutrition, 93*(4), 884S-890S.

Fève, B. (2005). Adipogenesis: cellular and molecular aspects. *Best Practice & Research Clinical Endocrinology & Metabolism, 19*(4), 483-499.

Fischer-Posovszky, P., Kukulus, V., Tews, D., Unterkircher, T., Debatin, K. M., Fulda, S., & Wabitsch, M. (2010). Resveratrol regulates human adipocyte number and function in a Sirt1-dependent manner. *American Journal of Clinical Nutrition, 92*(1), 5-15.

Fraulob, J. C., Ogg-Diamantino, R., Fernandes-Santos, C., Aguila, M. B., & Mandarim-de-Lacerda, C. A. (2010). A mouse model of metabolic syndrome: insulin resistance, fatty liver and non-alcoholic fatty pancreas disease (NAFPD) in C57BL/6 Mice fed a high fat diet. *Journal of Clinical Biochemistry and Nutrition, 46*(3), 212-223.

Fritsche, L., Weigert, C., Haring, H. U., & Lehmann, R. (2008). How insulin receptor substrate proteins regulate the metabolic capacity of the liver-implications for health and disease. *Current Medicinal Chemistry, 15*(13), 1316-1329.

Fruhbeck, G., Gomez-Ambrosi, J., Muruzabal, F. J., & Burrell, M. A. (2001). The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. *American Journal of Physiology: Endocrinology and Metabolism, 280*(6), E827-847.

Gaidhu, M. P., Fediuc, S., Anthony, N. M., So, M., Mirpourian, M., Perry, R. L., & Ceddia, R. B. (2009). Prolonged AICAR-induced AMP-kinase activation promotes energy dissipation in white adipocytes: novel mechanisms integrating HSL and ATGL. *Journal of Lipid Research, 50*(4), 704-715.

Galarraga, M., Campión, J., Muñoz-Barrutia, A., Boqué, N., Moreno, H., Martínez, J. A., . . . Ortiz-de-Solórzano, C. (2012). Adiposoft: automated software for the analysis of white adipose tissue cellularity in histological sections. *Journal of Lipid Research, 53*(12), 2791-2796.

Gammone, M. A., & D'Orazio, N. (2015). Anti-obesity activity of the marine carotenoid fucoxanthin. *Marine Drugs, 13*(4), 2196-2214.

Gao, J., Song, J., Du, M., & Mao, X. (2018). Bovine α-Lactalbumin Hydrolysates (α-LAH) Ameliorate Adipose Insulin Resistance and Inflammation in High-Fat Diet-Fed C57BL/6J Mice. *Nutrients, 10*(2), 242.

Gao, Z., Yin, J., Zhang, J., Ward, R. E., Martin, R. J., Lefevre, M., . . . Ye, J. (2009). Butyrate improves insulin sensitivity and increases energy expenditure in mice. *Diabetes, 58*(7), 1509-1517.

García-Morales, L. M., Berber, A., Macias-Lara, C. C., Lucio-Ortiz, C., Del-Rio-Navarro, B. E., & Dorantes-Alvárez, L. M. (2006). Use of sibutramine in obese mexican adolescents: A 6-month, randomized, double-blind, placebo-controlled, parallel-group trial. *Clinical Therapeutics, 28*(5), 770-782.

Garg, S. K., Maurer, H., Reed, K., & Selagamsetty, R. (2014). Diabetes and cancer: two diseases with obesity as a common risk factor. *Diabetes, Obesity & Metabolism, 16*(2), 97-110.

Garin-Shkolnik, T., Rudich, A., Hotamisligil, G. S., & Rubinstein, M. (2014). FABP4 attenuates PPARgamma and adipogenesis and is inversely correlated with PPARgamma in adipose tissues. *Diabetes, 63*(3), 900-911.

Goossens, G. H. (2017). The metabolic phenotype in obesity: fat mass, body fat distribution, and adipose tissue function. *Obesity Facts, 10*(3), 207-215.

Gray, S., Feinberg, M. W., Hull, S., Kuo, C. T., Watanabe, M., Sen, S., . . . Jain, M. K. (2002). The Krüppel-like factor KLF15 regulates the insulin-sensitive glucose transporter GLUT4. *Journal of Biological Chemistry, 277*(37), 34322-34328.

Gray, S., Wang, B., Orihuela, Y., Hong, E.-G., Fisch, S., Haldar, S., . . . Kahn, B. B. (2007). Regulation of gluconeogenesis by Krüppel-like factor 15. *Cell Metabolism, 5*(4), 305-312.

Gray, S. L., Dalla Nora, E., Grosse, J., Manieri, M., Stoeger, T., Medina-Gomez, G., . . . Yeo, G. S. (2006). Leptin deficiency unmasks the deleterious effects of impaired peroxisome proliferator–activated receptor γ function (P465L PPARγ) in mice. *Diabetes, 55*(10), 2669-2677.

Green, H., & Kehinde, O. (1975). An established preadipose cell line and its differentiation in culture II. Factors affecting the adipose conversion. *Cell, 5*(1), 19-27.

Green, H., & Kehinde, O. (1979). Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line. *Journal of Cellular Physiology, 101*(1), 169-171.

Green, H., & Meuth, M. (1974). An established pre-adipose cell line and its differentiation in culture. *Cell, 3*(2), 127-133.

Greenwood, M. (1985). The relationship of enzyme activity to feeding behavior in rats: lipoprotein lipase as the metabolic gatekeeper. *International Journal of Obesity, 9*, 67-70.

Gregoire, F. M., Smas, C. M., & Sul, H. S. (1998). Understanding adipocyte differentiation. *Physiological Reviews, 78*(3), 783-809.

Gross, D., Van Den Heuvel, A., & Birnbaum, M. (2008). The role of FoxO in the regulation of metabolism. *Oncogene, 27*(16), 2320.

Group, D.-D. S., & Group, E. D. E. (2003). Age, body mass index and type 2 diabetes—associations modified by ethnicity. *Diabetologia, 46*(8), 1063-1070.

Guo, J., Jou, W., Gavrilova, O., & Hall, K. D. (2009). Persistent diet-induced obesity in male C57BL/6 mice resulting from temporary obesigenic diets. *PloS One, 4*(4), e5370.

Guo, Z., Vangapandu, S., Sindelar, R. W., Walker, L. A., & Sindelar, R. D. (2005a). Biologically active quassinoids and their chemistry: Potential leads for drug design. *Current Medicinal Chemistry, 12*(2), 173-190.

Guri, A. J., Hontecillas, R., Ferrer, G., Casagran, O., Wankhade, U., Noble, A. M., . . . Bassaganya-Riera, J. (2008). Loss of PPAR gamma in immune cells impairs the ability of abscisic acid to improve insulin sensitivity by suppressing monocyte chemoattractant protein-1 expression and macrophage infiltration into white adipose tissue. *The Journal of Nutritional Biochemistry, 19*(4), 216-228.

Guri, A. J., Hontecillas, R., Si, H., Liu, D., & Bassaganya-Riera, J. (2007). Dietary abscisic acid ameliorates glucose tolerance and obesity-related inflammation in db/db mice fed high-fat diets. *Clinical Nutrition, 26*(1), 107-116.

Hadie, D., & Carling, D. (1997). The AMP-activated protein kinase-fuel gauge of the mammalial cell. *European Journal of Biochemistry, 246*, 259-273.

Häger, A., Sjörström, L., Arvidsson, B., Björntorp, P., & Smith, U. (1978). Adipose tissue cellularity in obese school girls before and after dietary treatment. *The American Journal of Clinical Nutrition, 31*(1), 68-75.

Han, L., Zhou, R., Niu, J., McNutt, M. A., Wang, P., & Tong, T. (2010). SIRT1 is regulated by a PPARγ–SIRT1 negative feedback loop associated with senescence. *Nucleic Acids Research, 38*(21), 7458-7471.

Han, Y. M., Jang, M., Kim, I. S., Kim, S. H., & Yoo, H. H. (2015). Simultaneous quantitation of six major quassinoids in Tongkat Ali dietary supplements by liquid chromatography with tandem mass spectrometry. *Journal of Separation Science, 38*(13), 2260-2266.

Hardy, O. T., Czech, M. P., & Corvera, S. (2012). What causes the insulin resistance underlying obesity? *Current Opinion in Endocrinology, Diabetes, and Obesity, 19*(2), 81.

Harrison, S. A., Ramrakhiani, S., Brunt, E. M., Anbari, M. A., Cortese, C., & Bacon, B. R. (2003). Orlistat in the treatment of NASH: a case series. *American Journal of Gastroenterology, 98*(4), 926-930.

Hauner, H. (2005). Secretory factors from human adipose tissue and their functional role. *Proceedings of the Nutrition Society, 64*(2), 163-169.

Haunerland, N. H., & Spener, F. (2004). Fatty acid-binding proteins–insights from genetic manipulations. *Progress in Lipid Research, 43*(4), 328-349.

Hausman, D., DiGirolamo, M., Bartness, T., Hausman, G., & Martin, R. (2001). The biology of white adipocyte proliferation. *Obesity Reviews, 2*(4), 239-254.

He, W., Barak, Y., Hevener, A., Olson, P., Liao, D., Le, J., . . . Evans, R. M. (2003). Adipose-specific peroxisome proliferator-activated receptor γ knockout causes insulin resistance in fat and liver but not in muscle. *Proceedings of the National Academy of Sciences, 100*(26), 15712-15717.

He, Y., Li, Y., Zhao, T., Wang, Y., & Sun, C. (2013). Ursolic acid inhibits adipogenesis in 3T3-L1 adipocytes through LKB1/AMPK pathway. *PloS One, 8*(7), e70135.

Health, N. I. o. (1995). *National Heart, Lung, and Blood Institute.* Paper presented at the Global Initiative for Asthma Management and Prevention. NHLBI/WHO Workshop Report.

Hevener, A. L., He, W., Barak, Y., Le, J., Bandyopadhyay, G., Olson, P., . . . Olefsky, J. (2003). Muscle-specific Pparg deletion causes insulin resistance. *Nature Medicine, 9*(12), 1491.

Hill, J. O., Wyatt, H. R., & Peters, J. C. (2012). Energy balance and obesity. *Circulation, 126*(1), 126-132.

Hirosumi, J., Tuncman, G., Chang, L., Görgün, C. Z., Uysal, K. T., Maeda, K., . . . Hotamisligil, G. S. (2002). A central role for JNK in obesity and insulin resistance. *Nature, 420*(6913), 333.

Ho, S. S., Dhaliwal, S. S., Hills, A. P., & Pal, S. (2012). The effect of 12 weeks of aerobic, resistance or combination exercise training on cardiovascular risk factors in the overweight and obese in a randomized trial. *BMC Public Health, 12*, 704.

Hoffstedt, J., Arner, E., Wahrenberg, H., Andersson, D., Qvisth, V., Löfgren, P., . . . Palmer, M. (2010). Regional impact of adipose tissue morphology on the metabolic profile in morbid obesity. *Diabetologia, 53*(12), 2496-2503.

Huang, B., Yuan, H. D., Kim, D. Y., Quan, H. Y., & Chung, S. H. (2011). Cinnamaldehyde prevents adipocyte differentiation and adipogenesis via regulation of peroxisome proliferator-activated receptor-γ (PPARγ) and AMP-activated protein kinase (AMPK) pathways. *Journal of Agricultural and Food Chemistry*, *59*(8), 3666-3673.

Hunstad, J. P. (1996). Liposuction for obesity. *Operative Techniques in Plastic and Reconstructive Surgery*, *3*(2), 124-131.

Husen, R., Pihie, A. H. L., & Nallappan, M. (2004). Screening for antihyperglycaemic activity in several local herbs of Malaysia. *Journal of Ethnopharmacology*, *95*(2), 205-208.

Hwang, C.-S., Loftus, T. M., Mandrup, S., & Lane, M. D. (1997). Adipocyte differentiation and leptin expression. *Annual Review of Cell and Developmental Biology*, *13*(1), 231-259.

Imamura, H., Nagayama, D., Ishihara, N., Tanaka, S., Watanabe, R., Watanabe, Y., . . . Tatsuno, I. (2017). Resveratrol attenuates triglyceride accumulation associated with upregulation of Sirt1 and lipoprotein lipase in 3T3-L1 adipocytes. *Molecular Genetics and Metabolism Reports*, *12*, 44-50.

Inafuku, M., Nugara, R. N., Kamiyama, Y., Futenma, I., Inafuku, A., & Oku, H. (2013). Cirsium brevicaule A. GRAY leaf inhibits adipogenesis in 3T3-L1 cells and C57BL/6 mice. *Lipids in Health and Disease*, *12*(1), 124.

Jamal, J. A. (2006). Malay traditional medicine. *Tech Monitor (Special Feature: traditional Medicine: S & T Advancement)*, 37-49.

Jeffery, R. W., & Utter, J. (2003). The changing environment and population obesity in the United States. *Obesity*, *11*(S10).

Jeffrey, K. L., Camps, M., Rommel, C., & Mackay, C. R. (2007). Targeting dual-specificity phosphatases: manipulating MAP kinase signalling and immune responses. *Nature Reviews Drug Discovery*, *6*(5), 391.

Ji, E., Jung, M. Y., Park, J. H., Kim, S., Seo, C. R., Park, K. W., . . . Lee, S. (2014). Inhibition of adipogenesis in 3T3-L1 cells and suppression of abdominal fat accumulation in high-fat diet-feeding C57BL/6J mice after downregulation of hyaluronic acid. *International Journal of Obesity*, *38*(8), 1035-1043.

Jiang, L., Wang, Q., Yu, Y., Zhao, F., Huang, P., Zeng, R., . . . Liu, Y. (2009). Leptin contributes to the adaptive responses of mice to high-fat diet intake through suppressing the lipogenic pathway. *PloS One*, *4*(9), e6884.

Jin, Q., Zhang, F., Yan, T., Liu, Z., Wang, C., Ge, X., & Zhai, Q. (2010). C/EBPalpha regulates SIRT1 expression during adipogenesis. *Cell Research*, *20*(4), 470-479.

Jiwajinda, S., Santisopasri, V., Murakami, A., Sugiyama, H., Gasquet, M., Riad, E., . . . Ohigashi, H. (2002). In vitro anti-tumor promoting and anti-parasitic activities of the quassinoids from Eurycoma longifolia, a medicinal plant in Southeast Asia. *Journal of Ethnopharmacology*, *82*(1), 55-58.

Johnston, S. L., Souter, D. M., Tolkamp, B. J., Gordon, I. J., Illius, A. W., Kyriazakis, I., & Speakman, J. R. (2007). Intake compensates for resting metabolic rate variation in female C57BL/6J mice fed high-fat diets. *Obesity, 15*(3), 600-606.

Joo, J. I., Kim, D. H., Choi, J.-W., & Yun, J. W. (2010). Proteomic analysis for antiobesity potential of capsaicin on white adipose tissue in rats fed with a high fat diet. *Journal of Proteome Research, 9*(6), 2977-2987.

Jung, C. H., Cho, I., Ahn, J., Jeon, T. I., & Ha, T. Y. (2013). Quercetin reduces high-fat diet-induced fat accumulation in the liver by regulating lipid metabolism genes. *Phytotherapy Research, 27*(1), 139-143.

Kahn, B. B., Alquier, T., Carling, D., & Hardie, D. G. (2005). AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metabolism, 1*(1), 15-25.

Kahn, B. B., & Flier, J. S. (2000). Obesity and insulin resistance. *Journal of Clinical Investigation, 106*(4), 473-481.

Kardono, L. B., Angerhofer, C. K., Tsauri, S., Padmawinata, K., Pezzuto, J. M., & Kinghorn, A. D. (1991). Cytotoxic and antimalarial constituents of the roots of Eurycoma longifolia. *Journal of Natural Products, 54*(5), 1360-1367.

Keng, H. (1987). *Orders and families of Malayan seed plants*: NUS Press.

Kern, P. A. (1997). Potential role of TNFα and lipoprotein lipase as candidate genes for obesity. *The Journal of Nutrition, 127*(9), 1917S-1922S.

Kersten, S. (2002). Peroxisome proliferator activated receptors and obesity. *European Journal of Pharmacology, 440*(2-3), 223-234.

Khoo, J., Dhamodaran, S., Chen, D. D., Yap, S. Y., Chen, R. Y., & Tian, R. H. (2015). Exercise-induced weight loss is more effective than dieting for improving adipokine profile, insulin resistance, and inflammation in obese men. *International Journal of Sport Nutrition and Exercise Metabolism, 25*(6), 566-575.

Kim, E. K., Lim, S., Park, J. M., Seo, J. K., Kim, J. H., Kim, K. T., . . . Suh, P. G. (2012). Human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by AMP-activated protein kinase. *Journal of Cellular Physiology, 227*(4), 1680-1687.

Kim, H. L., Sim, J. E., Choi, H. M., Choi, I. Y., Jeong, M. Y., Park, J., . . . Um, J. Y. (2014). The AMPK pathway mediates an anti-adipogenic effect of fruits of Hovenia dulcis Thunb. *Food & Function, 5*(11), 2961-2968.

Kim, S.-h., Park, H.-S., Lee, M.-s., Cho, Y.-J., Kim, Y.-S., Hwang, J.-T., . . . Kwon, D. Y. (2008). Vitisin A inhibits adipocyte differentiation through cell cycle arrest in 3T3-L1 cells. *Biochemical and Biophysical Research Communications, 372*(1), 108-113.

Kim, S. C., Kim, Y. H., Son, S. W., Moon, E. Y., Pyo, S., & Um, S. H. (2015). Fisetin induces Sirt1 expression while inhibiting early adipogenesis in 3T3-L1 cells. *Biochemical and Biophysical Research Communications, 467*(4), 638-644.

Kim, S. Y., Oh, M. R., Kim, M. G., Chae, H. J., & Chae, S. W. (2015). Anti-obesity effects of Yerba Mate (Ilex Paraguariensis): a randomized, double-blind, placebo-controlled clinical trial. *BMC Complementary and Alternative Medicine, 15*, 338.

Kinnunen, P. K., Jackson, R. L., Smith, L. C., Gotto, A. M., Jr., & Sparrow, J. T. (1977). Activation of lipoprotein lipase by native and synthetic fragments of human plasma apolipoprotein C-II. *Proceedings of the National Academy of Sciences of the United States of America, 74*(11), 4848-4851.

Kirk, E., Reeds, D. N., Finck, B. N., Mayurranjan, S. M., Patterson, B. W., & Klein, S. (2009). Dietary fat and carbohydrates differentially alter insulin sensitivity during caloric restriction. *Gastroenterology, 136*(5), 1552-1560.

Kleiner, S., Mepani, R. J., Laznik, D., Ye, L., Jurczak, M. J., Jornayvaz, F. R., ... & Spiegelman, B. M. (2012). Development of insulin resistance in mice lacking PGC-1α in adipose tissues. *Proceedings of the National Academy of Sciences,* 109(24), 9635-9640.

Klingenberg, M. (1999). Uncoupling Protein—A Useful Energy Dissepator. *Journal of Bioenergetics and Biomembranes, 31*(5), 419-430.

Ko, G. T., Tang, J., Chan, J. C., Sung, R., Wu, M. M., Wai, H. P., & Chen, R. (2001). Lower BMI cut-off value to define obesity in Hong Kong Chinese: an analysis based on body fat assessment by bioelectrical impedance. *British Journal of Nutrition, 85*(2), 239-242.

Kolditz, C.-I., & Langin, D. (2010). Adipose tissue lipolysis. *Current Opinion in Clinical Nutrition and Metabolic Care, 13*(4), 377-381.

Kovacs, P., Harper, I., Hanson, R. L., Infante, A. M., Bogardus, C., Tataranni, P. A., & Baier, L. J. (2004). A novel missense substitution (Val1483Ile) in the fatty acid synthase gene (FAS) is associated with percentage of body fat and substrate oxidation rates in nondiabetic Pima Indians. *Diabetes, 53*(7), 1915-1919.

Krishnan, V., Bryant, H. U., & MacDougald, O. A. (2006). Regulation of bone mass by Wnt signaling. *The Journal of Clinical Investigation, 116*(5), 1202-1209.

Kudo, N., Arai, Y., Suhara, Y., Ishii, T., Nakayama, T., & Osakabe, N. (2015). A single oral administration of theaflavins increases energy expenditure and the expression of metabolic genes. *PloS One, 10*(9), e0137809.

Kumar, M. V., Shimokawa, T., Nagy, T. R., & Lane, M. D. (2002). Differential effects of a centrally acting fatty acid synthase inhibitor in lean and obese mice. *Proceedings of the National Academy of Sciences of the United States of America, 99*(4), 1921-1925.

Kuo, P.-C., Damu, A. G., Lee, K.-H., & Wu, T.-S. (2004). Cytotoxic and antimalarial constituents from the roots of Eurycoma longifolia. *Bioorganic and Medicinal Chemistry, 12*(3), 537-544.

Kwon, J. Y., Seo, S. G., Yue, S., Cheng, J.-X., Lee, K. W., & Kim, K.-H. (2012). An inhibitory effect of resveratrol in the mitotic clonal expansion and insulin signaling pathway in the early phase of adipogenesis. *Nutrition Research, 32*(8), 607-616.

Kwon, S. M., Kim, S., Song, N. J., Chang, S. H., Hwang, Y. J., Yang, D. K., . . . Park, K. W. (2016). Antiadipogenic and proosteogenic effects of luteolin, a major dietary flavone, are mediated by the induction of DnaJ (Hsp40) Homolog, Subfamily B, Member 1. *The Journal of Nutritional Biochemistry, 30*, 24-32.

Lage, R., Diéguez, C., Vidal-Puig, A., & López, M. (2008). AMPK: a metabolic gauge regulating whole-body energy homeostasis. *Trends in Molecular Medicine, 14*(12), 539-549.

Lahrita, L., Hirosawa, R., Kato, E., & Kawabata, J. (2017). Isolation and lipolytic activity of eurycomanone and its epoxy derivative from Eurycoma longifolia. *Bioorganic and Medicinal Chemistry, 25*(17), 4829-4834.

Lahrita, L., Kato, E., & Kawabata, J. (2015). Uncovering potential of Indonesian medicinal plants on glucose uptake enhancement and lipid suppression in 3T3-L1 adipocytes. *Journal of Ethnopharmacology, 168*, 229-236.

Lai, C. S., Tsai, M. L., Badmaev, V., Jimenez, M., Ho, C. T., & Pan, M. H. (2012). Xanthigen suppresses preadipocyte differentiation and adipogenesis through down-regulation of PPARgamma and C/EBPs and modulation of SIRT-1, AMPK, and FoxO pathways. *Journal of Agricultural and Food Chemistry, 60*(4), 1094-1101.

Lakhan, S. E., & Kirchgessner, A. (2011). Anti-inflammatory effects of nicotine in obesity and ulcerative colitis. *Journal of Translational Medicine, 9*, 129.

Lass, A., Zimmermann, R., Oberer, M., & Zechner, R. (2011). Lipolysis – A highly regulated multi-enzyme complex mediates the catabolism of cellular fat stores. *Progress in Lipid Research, 50*(1-4), 14-27.

Lecka-Czernik, B., Moerman, E. J., Grant, D. F., Lehmann, J. r. M., Manolagas, S. C., & Jilka, R. L. (2002). Divergent effects of selective peroxisome proliferator-activated receptor-γ2 ligands on adipocyte versus osteoblast differentiation. *Endocrinology, 143*(6), 2376-2384.

Lee, B.-C., & Lee, J. (2014). Cellular and molecular players in adipose tissue inflammation in the development of obesity-induced insulin resistance. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1842*(3), 446-462.

Lee, H., Shaw, Y.-T., Chiou, S.-T., Chang, W.-C., & Lai, M.-D. (1991). The effects of glucocorticoid hormone on the expression of c-jun. *FEBS Letters, 280*(1), 134-136.

Lee, J. H., Jung, H. A., Kang, M. J., Choi, J. S., & Kim, G. D. (2017). Fucosterol, isolated from Ecklonia stolonifera, inhibits adipogenesis through modulation of FoxO1 pathway in 3T3-L1 adipocytes. *Journal of Pharmacy and Pharmacology, 69*(3), 325-333.

Lefterova, M. I., & Lazar, M. A. (2009). New developments in adipogenesis. *Trends in Endocrinology and Metabolism, 20*(3), 107-114.

Leite, C. E., Mocelin, C. A., Petersen, G. O., Leal, M. B., & Thiesen, F. V. (2009). Rimonabant: an antagonist drug of the endocannabinoid system for the treatment of obesity. *Pharmacological Reports, 61*(2), 217-224.

Lenhard, J. M. (2011). Lipogenic enzymes as therapeutic targets for obesity and diabetes. *Current Pharmaceutical Design, 17*(4), 325-331.

Leonardini, A., Laviola, L., Perrini, S., Natalicchio, A., & Giorgino, F. (2010). Cross-Talk between PPAR and Insulin Signaling and Modulation of Insulin Sensitivity. *PPAR Research, 2009*.

Li, Z., Maglione, M., Tu, W., Mojica, W., Arterburn, D., Shugarman, L. R., . . . Shekelle, P. G. (2005). Meta-analysis: pharmacologic treatment of obesity. *Annals of Internal Medicine, 142*(7), 532-546.

Lin, C.-S., Xin, Z.-C., Deng, C.-H., Ning, H., Lin, G., & Lue, T. F. (2010). Defining adipose tissue-derived stem cells in tissue and in culture. *Histology and Histopathology, 25*(4), 807.

Little, J. P., Jung, M. E., Wright, A. E., Wright, W., & Manders, R. J. (2014). Effects of high-intensity interval exercise versus continuous moderate-intensity exercise on postprandial glycemic control assessed by continuous glucose monitoring in obese adults. *Applied Physiology, Nutrition, and Metabolism, 39*(7), 835-841.

Liu, A. G., Arceneaux, K. P., 3rd, Chu, J. T., Jacob, G., Jr., Schreiber, A. L., Tipton, R. C., . . . Primeaux, S. D. (2015). The effect of caffeine and albuterol on body composition and metabolic rate. *Obesity, 23*(9), 1830-1835.

Liu, S., Xu, R., Gerin, I., Cawthorn, W. P., Macdougald, O. A., Chen, X. W., . . . Xu, B. (2014). SRA regulates adipogenesis by modulating p38/JNK phosphorylation and stimulating insulin receptor gene expression and downstream signaling. *PloS One, 9*(4), e95416.

Lobo, G. P., Amengual, J., Li, H. N. M., Golczak, M., Bonet, M. L., Palczewski, K., & Von Lintig, J. (2010). β, β-carotene decreases peroxisome proliferator receptor γ activity and reduces lipid storage capacity of adipocytes in a β, β-carotene oxygenase 1-dependent manner. *Journal of Biological Chemistry, 285*(36), 27891-27899.

Logan, C. Y., & Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annual Review of Cell and Developmental Biology, 20*, 781-810.

Longo, K. A., Wright, W. S., Kang, S., Gerin, I., Chiang, S.-H., Lucas, P. C., . . . MacDougald, O. A. (2004). Wnt10b inhibits development of white and brown adipose tissues. *Journal of Biological Chemistry, 279*(34), 35503-35509.

López, M., Lelliott, C. J., & Vidal‐Puig, A. (2007). Hypothalamic fatty acid metabolism: a housekeeping pathway that regulates food intake. *Bioessays, 29*(3), 248-261.

Low, B., Teh, C. H., Yuen, K. H., & Chan, K. L. (2011). Physico-chemical effects of the major quassinoids in a standardized Eurycoma longifolia extract (Fr 2) on the bioavailability and pharmacokinetic properties, and their implications for oral antimalarial activity. *Natural Product Communications, 6*(3), 337-341.

Low, B. S., Choi, S. B., Abdul Wahab, H., Das, P. K., & Chan, K. L. (2013). Eurycomanone, the major quassinoid in Eurycoma longifolia root extract increases spermatogenesis by inhibiting the activity of phosphodiesterase and aromatase in steroidogenesis. *Journal of Ethnopharmacology, 149*(1), 201-207.

Low, B. S., Das, P. K., & Chan, K. L. (2013). Standardized quassinoid-rich Eurycoma longifolia extract improved spermatogenesis and fertility in male rats via the hypothalamic-pituitary-gonadal axis. *Journal of Ethnopharmacology, 145*(3), 706-714.

Low, B. S., Das, P. K., & Chan, K. L. (2014). Acute, reproductive toxicity and two-generation teratology studies of a standardized quassinoid-rich extract of Eurycoma longifolia Jack in Sprague-Dawley rats. *Phytotherapy Research, 28*(7), 1022-1029.

Low, B. S., Ng, B. H., Choy, W. P., Yuen, K. H., & Chan, K. L. (2005). Bioavailability and pharmacokinetic studies of eurycomanone from Eurycoma longifolia. *Planta Medica, 71*(9), 803-807.

Luque, C. A., & Rey, J. A. (2002). The discovery and status of sibutramine as an anti-obesity drug. *European Journal of Pharmacology, 440*(2), 119-128.

Luvizotto Rde, A., Nascimento, A. F., Imaizumi, E., Pierine, D. T., Conde, S. J., Correa, C. R., . . . Ferreira, A. L. (2013). Lycopene supplementation modulates plasma concentrations and epididymal adipose tissue mRNA of leptin, resistin and IL-6 in diet-induced obese rats. *British Journal of Nutrition, 110*(10), 1803-1809.

Ma, S., Yu, H., Zhao, Z., Luo, Z., Chen, J., Ni, Y., . . . Zhu, Z. (2012). Activation of the cold-sensing TRPM8 channel triggers UCP1-dependent thermogenesis and prevents obesity. *Journal of Molecular Cell Biology, 4*(2), 88-96.

MacDougald, O. A., & Lane, M. D. (1995). Transcriptional regulation of gene expression during adipocyte differentiation. *Annual Review of Biochemistry, 64*(1), 345-373.

Mafauzy, M. (2006). Diabetes mellitus in Malaysia. *The Medical journal of Malaysia, 61*(4), 397.

Manchester, K. (1996). Use of UV methods for measurement of protein and nucleic acid concentrations. *Biotechniques, 20*(6), 968.

Mannucci, E., Dicembrini, I., Rotella, F., & Rotella, C. M. (2008). Orlistat and sibutramine beyond weight loss. *Nutrition, Metabolism and Cardiovascular Diseases, 18*(5), 342-348.

Martin-Rodriguez, E., Guillen-Grima, F., Martí, A., & Brugos-Larumbe, A. (2015). Comorbidity associated with obesity in a large population: The APNA study. *Obesity Research & Clinical Practice, 9*(5), 435-447.

Matsuzaki, H., Daitoku, H., Hatta, M., Aoyama, H., Yoshimochi, K., & Fukamizu, A. (2005). Acetylation of Foxo1 alters its DNA-binding ability and sensitivity to phosphorylation. *Proceedings of the National Academy of Sciences of the United States of America, 102*(32), 11278-11283.

Mattson, M. P. (2010). Perspective: does brown fat protect against diseases of aging? *Ageing Research Reviews, 9*(1), 69-76.

Mayer, M. A., Hocht, C., Puyó, A., & Taira, C. A. (2009). Recent advances in obesity pharmacotherapy. *Current Clinical Pharmacology, 4*(1), 53-61.

Mayoral, R., Osborn, O., McNelis, J., Johnson, A. M., Izquierdo, C. L., Chung, H., . . . Pessentheiner, A. R. (2015). Adipocyte SIRT1 knockout promotes PPARγ activity, adipogenesis and insulin sensitivity in chronic-HFD and obesity. *Molecular metabolism, 4*(5), 378-391.

McLaughlin, T., Lamendola, C., Liu, A., & Abbasi, F. (2011). Preferential fat deposition in subcutaneous versus visceral depots is associated with insulin sensitivity. *The Journal of Clinical Endocrinology & Metabolism, 96*(11), E1756-E1760.

McNulty, S. J., Ur, E., & Williams, G. (2003). A randomized trial of sibutramine in the management of obese type 2 diabetic patients treated with metformin. *Diabetes Care, 26*(1), 125-131.

Mi, L., Chen, Y., Zheng, X., Li, Y., Zhang, Q., Mo, D., & Yang, G. (2015). MicroRNA-139-5p Suppresses 3T3-L1 Preadipocyte Differentiation Through Notch and IRS1/PI3K/Akt Insulin Signaling Pathways. *Journal of Cellular Biochemistry, 116*(7), 1195-1204.

Miki, H., Yamauchi, T., Suzuki, R., Komeda, K., Tsuchida, A., Kubota, N., . . . Kadowaki, T. (2001). Essential role of insulin receptor substrate 1 (IRS-1) and IRS-2 in adipocyte differentiation. *Molecular and Cellular Biology, 21*(7), 2521-2532.

Miles, J. M., Leiter, L., Hollander, P., Wadden, T., Anderson, J. W., Doyle, M., . . . Klein, S. (2002). Effect of orlistat in overweight and obese patients with type 2 diabetes treated with metformin. *Diabetes Care, 25*(7), 1123-1128.

Miller, L. H., Howard, R. J., Carter, R., Good, M. F., Nussenzweig, V., & Nussenzweig, R. S. (1986). Research toward malaria vaccines. *Science, 234*(4782), 1349-1356.

Milone, M., Lupoli, R., Maietta, P., Di Minno, A., Bianco, P., Ambrosino, P., . . . Musella, M. (2015). Lipid profile changes in patients undergoing bariatric surgery: a comparative study between sleeve gastrectomy and mini-gastric bypass. *International Journal of Surgery, 14*, 28-32.

Mohamed, G. A., Ibrahim, S. R., Elkhayat, E. S., & El Dine, R. S. (2014). Natural anti-obesity agents. *Bulletin of Faculty of Pharmacy, Cairo University, 52*(2), 269-284.

Mohd Effendy, N., Mohamed, N., Muhammad, N., Naina Mohamad, I., & Shuid, A. N. (2012). Eurycoma longifolia: Medicinal Plant in the Prevention and Treatment of Male Osteoporosis due to Androgen Deficiency. *Evidence-based complementary and alternative medicine : eCAM, 2012*, 125761-125761.

Monteiro, P. A., Chen, K. Y., Lira, F. S., Saraiva, B. T., Antunes, B. M., Campos, E. Z., & Freitas, I. F., Jr. (2015). Concurrent and aerobic exercise training promote similar benefits in body composition and metabolic profiles in obese adolescents. *Lipids in Health and Disease, 14*, 153.

Mori, T., Sakaue, H., Iguchi, H., Gomi, H., Okada, Y., Takashima, Y., . . . Kasuga, M. (2005). Role of Kruppel-like factor 15 (KLF15) in transcriptional regulation of adipogenesis. *Journal of Biological Chemistry, 280*(13), 12867-12875.

Moseti, D., Regassa, A., & Kim, W. K. (2016). Molecular Regulation of Adipogenesis and Potential Anti-Adipogenic Bioactive Molecules. *International Journal of Molecular Sciences, 17*(1), 124.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, 65*(1-2), 55-63.

Mozaffarian, D., Hao, T., Rimm, E. B., Willett, W. C., & Hu, F. B. (2011). Changes in diet and lifestyle and long-term weight gain in women and men. *New England Journal of Medicine, 364*(25), 2392-2404.

Murphy, J. C., McDaniel, J. L., Mora, K., Villareal, D. T., Fontana, L., & Weiss, E. P. (2011). Preferential reductions in intermuscular and visceral adipose tissue with exercise-induced weight loss compared with calorie restriction. *Journal of Applied Physiology, 112*(1), 79-85.

Nakae, J., Kitamura, T., Kitamura, Y., Biggs III, W. H., Arden, K. C., & Accili, D. (2003). The forkhead transcription factor Foxo1 regulates adipocyte differentiation. *Developmental Cell, 4*(1), 119-129.

Nandi, A., Kitamura, Y., Kahn, C. R., & Accili, D. (2004). Mouse models of insulin resistance. *Physiological Reviews, 84*(2), 623-647.

Naowaboot, J., Chung, C. H., Choi, R., & Pannangpetch, P. (2014). Umbelliferone increases the expression of adipocyte-specific genes in 3T3-L1 adipocyte. *Phytotherapy Research, 28*(11), 1671-1675.

Naowaboot, J., Piyabhan, P., Munkong, N., Parklak, W., & Pannangpetch, P. (2016). Ferulic acid improves lipid and glucose homeostasis in high-fat diet-induced obese mice. *Clinical and Experimental Pharmacology and Physiology, 43*(2), 242-250.

Narsete, T., Narsete, M., Buckspan, R., & Ersek, R. (2012). Large-volume liposuction and prevention of type 2 diabetes: a preliminary report. *Aesthetic Plastic Surgery, 36*(2), 438-442.

Nishimura, S., Manabe, I., Nagasaki, M., Hosoya, Y., Yamashita, H., Fujita, H., . . . Nagai, R. (2007). Adipogenesis in obesity requires close interplay between differentiating adipocytes, stromal cells, and blood vessels. *Diabetes, 56*(6), 1517-1526.

Norris, A. W., Chen, L., Fisher, S. J., Szanto, I., Ristow, M., Jozsi, A. C., . . . Gonzalez, F. J. (2003). Muscle-specific PPARγ-deficient mice develop increased adiposity and insulin resistance but respond to thiazolidinediones. *The Journal of Clinical Investigation, 112*(4), 608-618.

Oh, S., Tanaka, K., Tsujimoto, T., So, R., Shida, T., & Shoda, J. (2014). Regular exercise coupled to diet regimen accelerates reduction of hepatic steatosis and associated pathological conditions in nonalcoholic fatty liver disease. *Metabolic Syndrome and Related Disorders, 12*(5), 290-298.

Oliveira, A. G., Carvalho, B. M., Tobar, N., Ropelle, E. R., Pauli, J. R., Bagarolli, R. A., . . . Saad, M. J. (2011). Physical exercise reduces circulating lipopolysaccharide and TLR4 activation and improves insulin signaling in tissues of DIO rats. *Diabetes, 60*(3), 784-796.

Ono, M., & Fujimori, K. (2011). Antiadipogenic effect of dietary apigenin through activation of AMPK in 3T3-L1 cells. *Journal of Agricultural and Food Chemistry, 59*(24), 13346-13352.

Organization, W. H. (2017). BMI classification 2017. 2017. *URL: http://apps. who. int/bmi/index. jsp*.

Otto, T. C., & Lane, M. D. (2005). Adipose development: from stem cell to adipocyte. *Critical Reviews in Biochemistry and Molecular Biology, 40*(4), 229-242.

Padwal, R. S., & Majumdar, S. R. (2007). Drug treatments for obesity: orlistat, sibutramine, and rimonabant. *Lancet, 369*(9555), 71-77.

Pantovic, A., Krstic, A., Janjetovic, K., Kocic, J., Harhaji-Trajkovic, L., Bugarski, D., & Trajkovic, V. (2013). Coordinated time-dependent modulation of AMPK/Akt/mTOR signaling and autophagy controls osteogenic differentiation of human mesenchymal stem cells. *Bone, 52*(1), 524-531.

Park, H. J., Chung, B. Y., Lee, M.-K., Song, Y., Lee, S. S., Chu, G. M., . . . Cho, J.-H. (2012). Centipede grass exerts anti-adipogenic activity through inhibition of C/EBPβ, C/EBPα, and PPARγ expression and the AKT signaling pathway in 3T3-L1 adipocytes. *BMC Complementary and Alternative Medicine, 12*(1), 230.

Perry, L. M., & Metzger, J. (1980). *Medicinal plants of east and southeast Asia: attributed properties and uses*: MIT press.

Perseghin, G., Petersen, K., & Shulman, G. I. (2003). Cellular mechanism of insulin resistance: potential links with inflammation. *International Journal of Obesity and Related Metabolic Disorders, 27 Suppl 3*, S6-11.

Pi-Sunyer, F. X. (2002). The obesity epidemic: pathophysiology and consequences of obesity. *Obesity, 10*(S12).

Picard, F., Kurtev, M., Chung, N., Topark-Ngarm, A., Senawong, T., Machado De Oliveira, R., . . . Guarente, L. (2004). Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. *Nature, 429*(6993), 771-776.

Pongchaidecha, A., Lailerd, N., Boonprasert, W., & Chattipakorn, N. (2009). Effects of curcuminoid supplement on cardiac autonomic status in high-fat-induced obese rats. *Nutrition, 25*(7-8), 870-878.

Preiss-Landl, K., Zimmermann, R., Hammerle, G., & Zechner, R. (2002). Lipoprotein lipase: the regulation of tissue specific expression and its role in lipid and energy metabolism. *Current Opinion in Lipidology, 13*(5), 471-481.

Qu, P., Wang, L., Min, Y., McKennett, L., Keller, J. R., & Lin, P. C. (2016). Vav1 regulates mesenchymal stem cell differentiation decision between adipocyte and chondrocyte via Sirt1. *Stem Cells, 34*(7), 1934-1946.

Rahman, N., Jeon, M., & Kim, Y. S. (2016). Delphinidin, a major anthocyanin, inhibits 3T3‐L1 pre‐adipocyte differentiation through activation of Wnt/β-catenin signaling. *Biofactors, 42*(1), 49-59.

Ramji, D. P., & Foka, P. (2002). CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochemical Journal, 365*(Pt 3), 561.

Ranganathan, G., Unal, R., Pokrovskaya, I., Yao-Borengasser, A., Phanavanh, B., Lecka-Czernik, B., . . . Kern, P. A. (2006). The lipogenic enzymes DGAT1, FAS, and LPL in adipose tissue: effects of obesity, insulin resistance, and TZD treatment. *Journal of Lipid Research, 47*(11), 2444-2450.

Rangwala, S. M., & Lazar, M. A. (2000). Transcriptional control of adipogenesis. *Annual Review of Nutrition, 20*(1), 535-559.

Rehman, S. U., Choe, K., & Yoo, H. H. (2016). Review on a traditional herbal medicine, Eurycoma longifolia Jack (Tongkat Ali): its traditional uses, chemistry, evidence-based pharmacology and toxicology. *Molecules, 21*(3), 331.

Reichert, M., & Eick, D. (1999). Analysis of cell cycle arrest in adipocyte differentiation. *Oncogene, 18*, 459.

Ren, D., Collingwood, T. N., Rebar, E. J., Wolffe, A. P., & Camp, H. S. (2002). PPARγ knockdown by engineered transcription factors: exogenous PPARγ2 but not PPARγ1 reactivates adipogenesis. *Genes and Development, 16*(1), 27-32.

Reyes-Escogido Mde, L., Gonzalez-Mondragon, E. G., & Vazquez-Tzompantzi, E. (2011). Chemical and pharmacological aspects of capsaicin. *Molecules, 16*(2), 1253-1270.

Rock, C. L., Flatt, S. W., Pakiz, B., Taylor, K. S., Leone, A. F., Brelje, K., . . . Sherwood, N. E. (2014). Weight loss, glycemic control, and cardiovascular disease risk factors in response to differential diet composition in a weight loss program in type 2 diabetes: a randomized controlled trial. *Diabetes Care, 37*(6), 1573-1580.

Roden, M., Price, T. B., Perseghin, G., Petersen, K. F., Rothman, D. L., Cline, G. W., & Shulman, G. I. (1996). Mechanism of free fatty acid-induced insulin resistance in humans. *The Journal of Clinical Investigation, 97*(12), 2859-2865.

Rondini, E. A., Mladenovic-Lucas, L., Roush, W. R., Halvorsen, G. T., Green, A. E., & Granneman, J. G. (2017). Novel Pharmacological Probes Reveal ABHD5 as a Locus of Lipolysis Control in White and Brown Adipocytes. *Journal of Pharmacology and Experimental Therapeutics, 363*(3), 367-376.

Rosen, E. D. (2005). The transcriptional basis of adipocyte development. *Prostaglandins, Leukotrienes, and Essential Fatty Acids, 73*(1), 31-34.

Rosen, E. D., Hsu, C.-H., Wang, X., Sakai, S., Freeman, M. W., Gonzalez, F. J., & Spiegelman, B. M. (2002). C/EBPα induces adipogenesis through PPARγ: a unified pathway. *Genes and Development, 16*(1), 22-26.

Rosen, E. D., & MacDougald, O. A. (2006). Adipocyte differentiation from the inside out. *Nature Reviews: Molecular Cell Biology, 7*(12), 885-896.

Rosen, E. D., Sarraf, P., Troy, A. E., Bradwin, G., Moore, K., Milstone, D. S., . . . Mortensen, R. M. (1999). PPARγ is required for the differentiation of adipose tissue in vivo and in vitro. *Molecular Cell, 4*(4), 611-617.

Ross, S. E., Erickson, R. L., Gerin, I., DeRose, P. M., Bajnok, L., Longo, K. A., . . . MacDougald, O. A. (2002). Microarray analyses during adipogenesis: understanding the effects of Wnt signaling on adipogenesis and the roles of liver X receptor alpha in adipocyte metabolism. *Molecular and Cellular Biology, 22*(16), 5989-5999.

Ross, S. E., Hemati, N., Longo, K. A., Bennett, C. N., Lucas, P. C., Erickson, R. L., & MacDougald, O. A. (2000). Inhibition of adipogenesis by Wnt signaling. *Science, 289*(5481), 950-953.

Rossmeisl, M., Rim, J. S., Koza, R. A., & Kozak, L. P. (2003). Variation in type 2 diabetes-related traits in mouse strains susceptible to diet-induced obesity. *Diabetes, 52*(8), 1958-1966.

Ruderman, N. B., Saha, A. K., Vavvas, D., & Witters, L. A. (1999). Malonyl-CoA, fuel sensing, and insulin resistance. *American Journal of Physiology, 276*(1 Pt 1), E1-e18.

Sabin, M. A., & Kiess, W. (2015). Childhood obesity: Current and novel approaches. *Best Practice & Research: Clinical Endocrinology & Metabolism, 29*(3), 327-338.

Sahebkar, A., Simental-Mendía, L. E., Reiner, Ž., Kovanen, P. T., Simental-Mendía, M., Bianconi, V., & Pirro, M. (2017). Effect of orlistat on plasma lipids and body

weight: A systematic review and meta-analysis of 33 randomized controlled trials. *Pharmacological Research, 122*, 53-65.

Salans, L. B., Horton, E. S., & Sims, E. A. (1971). Experimental obesity in man: cellular character of the adipose tissue. *The Journal of Clinical Investigation, 50*(5), 1005-1011.

Sale, E., Atkinson, P., & Sale, G. (1995). Requirement of MAP kinase for differentiation of fibroblasts to adipocytes, for insulin activation of p90 S6 kinase and for insulin or serum stimulation of DNA synthesis. *The EMBO Journal, 14*(4), 674-684.

Sarjeant, K., & Stephens, J. M. (2012). Adipogenesis. *Cold Spring Harbor Perspectives in Biology, 4*(9), a008417.

Sartoretto, J., Michel, T., & Gray, S. (2008). *KLF15 knockout mice are protected against high-fat diet induced insulin resistance.* Paper presented at the Diabetes.

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C_T method. *Nature Protocols, 3*(6), 1101.

Schneider, J., Kreuzer, J., Hamann, A., Nawroth, P. P., & Dugi, K. A. (2002). The proline 12 alanine substitution in the peroxisome proliferator--activated receptor-gamma2 gene is associated with lower lipoprotein lipase activity in vivo. *Diabetes, 51*(3), 867-870.

Schoonjans, K., Peinado-Onsurbe, J., Fruchart, J. C., Tailleux, A., Fievet, C., & Auwerx, J. (1999). 3-Hydroxy-3-methylglutaryl CoA reductase inhibitors reduce serum triglyceride levels through modulation of apolipoprotein C-III and lipoprotein lipase. *FEBS Letters, 452*(3), 160-164.

Schoonjans, K., Peinado-Onsurbe, J., Lefebvre, A. M., Heyman, R. A., Briggs, M., Deeb, S., . . . Auwerx, J. (1996). PPARalpha and PPARgamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *The EMBO Journal, 15*(19), 5336-5348.

Semenkovich, C. F., Wims, M., Noe, L., Etienne, J., & Chan, L. (1989). Insulin regulation of lipoprotein lipase activity in 3T3-L1 adipocytes is mediated at posttranscriptional and posttranslational levels. *Journal of Biological Chemistry, 264*(15), 9030-9038.

Seoane-Collazo, P., Martinez de Morentin, P. B., Ferno, J., Dieguez, C., Nogueiras, R., & Lopez, M. (2014). Nicotine improves obesity and hepatic steatosis and ER stress in diet-induced obese male rats. *Endocrinology, 155*(5), 1679-1689.

Shaham, O., Wei, R., Wang, T. J., Ricciardi, C., Lewis, G. D., Vasan, R. S., . . . Mootha, V. K. (2008). Metabolic profiling of the human response to a glucose challenge reveals distinct axes of insulin sensitivity. *Molecular Systems Biology, 4*(1), 214.

Shanik, M. H., Xu, Y., Škrha, J., Dankner, R., Zick, Y., & Roth, J. (2008). Insulin resistance and hyperinsulinemia: is hyperinsulinemia the cart or the horse? *Diabetes Care, 31*(Supplement 2), S262-S268.

Sharma, B. R., Kim, H. J., Kim, M. S., Park, C. M., & Rhyu, D. Y. (2017). Caulerpa okamurae extract inhibits adipogenesis in 3T3-L1 adipocytes and prevents high-fat diet–induced obesity in C57BL/6 mice. *Nutrition Research, 47*, 44-52.

Shen, W.-J., Sridhar, K., Bernlohr, D. A., & Kraemer, F. B. (1999). Interaction of rat hormone-sensitive lipase with adipocyte lipid-binding protein. *Proceedings of the National Academy of Sciences, 96*(10), 5528-5532.

Smith, S. M., Meyer, M., & Trinkley, K. E. (2013). Phentermine/topiramate for the treatment of obesity. *Annals of Pharmacotherapy, 47*(3), 340-349.

Sobri, H., Rusli, I., & Kiong, L. (2007). A summary of reported chemical constituents and medicinal uses of Eurycoma longifolia. *Journal of Tropical Medicinal Plants, 8*(1), 103-110.

Solomon, M. C., Erasmus, N., & Henkel, R. R. (2014). In vivo effects of Eurycoma longifolia Jack (Tongkat Ali) extract on reproductive functions in the rat. *Andrologia, 46*(4), 339-348.

Son, M. J., Rico, C. W., Nam, S. H., & Kang, M. Y. (2010). Influence of oryzanol and ferulic acid on the lipid metabolism and antioxidative status in high fat-fed mice. *Journal of Clinical Biochemistry and Nutrition, 46*(2), 150-156.

Song, Y., Park, H. J., Kang, S. N., Jang, S. H., Lee, S. J., Ko, Y. G., . . . Cho, J. H. (2013). Blueberry peel extracts inhibit adipogenesis in 3T3-L1 cells and reduce high-fat diet-induced obesity. *PloS One, 8*(7), e69925.

Spalding, K. L., Arner, E., Westermark, P. O., Bernard, S., Buchholz, B. A., Bergmann, O., . . . Britton, T. (2008). Dynamics of fat cell turnover in humans. *Nature, 453*(7196), 783.

Spiegelman, B. M., & Flier, J. S. (1996). Adipogenesis and obesity: rounding out the big picture. *Cell, 87*(3), 377-389.

Strutt, D. (2003). Frizzled signalling and cell polarisation in Drosophila and vertebrates. *Development, 130*(19), 4501-4513.

Student, A. K., Hsu, R. Y., & Lane, M. D. (1980). Induction of fatty acid synthetase synthesis in differentiating 3T3-L1 preadipocytes. *Journal of Biological Chemistry, 255*(10), 4745-4750.

Subramanian, S., & Chait, A. (2012). Hypertriglyceridemia secondary to obesity and diabetes. *Biochimica et Biophysica Acta, 1821*(5), 819-825.

Sullivan, J. E., Brocklehurst, K. J., Marley, A. E., Carey, F., Carling, D., & Beri, R. K. (1994). Inhibition of lipolysis and lipogenesis in isolated rat adipocytes with AICAR, a cell-permeable activator of AMP-activated protein kinase. *FEBS Letters, 353*(1), 33-36.

Susic, D., & Varagic, J. (2017). Obesity: A Perspective from Hypertension. *Medical Clinics of North America, 101*(1), 139-157.

Szabo, K., Gesztelyi, R., Lampe, N., Kiss, R., Remenyik, J., Pesti-Asboth, G., . . . Juhasz, B. (2018). Fenugreek (Trigonella Foenum-Graecum) seed flour and diosgenin preserve endothelium-dependent arterial relaxation in a rat model of early-stage metabolic syndrome. *International Journal of Molecular Sciences, 19*(3).

Talbott, S. M. (2007). Weight loss compositions using citrus peel extract and eurycoma longfolia: *U.S. Patent Application No. 11/690,754*.

Tamori, Y., Masugi, J., Nishino, N., & Kasuga, M. (2002). Role of peroxisome proliferator-activated receptor-γ in maintenance of the characteristics of mature 3T3-L1 adipocytes. *Diabetes, 51*(7), 2045-2055.

Tanaka, T., Yoshida, N., Kishimoto, T., & Akira, S. (1997). Defective adipocyte differentiation in mice lacking the C/EBPβ and/or C/EBPδ gene. *The EMBO Journal, 16*(24), 7432-7443.

Tang, Q., Gronborg, M., Huang, H., Kim, J., Otto, T., & Pandey, A. (2005). Sequential phosphorylation of C/EBP b by MAPK and GSK3 b is required for adipogenesis. *Proceedings of the National Academy of Sciences of the United States of America, 102*, 9766-9771.

Tchoukalova, Y. D., Sarr, M. G., & Jensen, M. D. (2004). Measuring committed preadipocytes in human adipose tissue from severely obese patients by using adipocyte fatty acid binding protein. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 287*(5), R1132-R1140.

Tee, B. H., Hoe, S. Z., Cheah, S. H., & Lam, S. K. (2016). First report of Eurycoma longifolia Jack root extract causing relaxation of aortic rings in rats. *BioMed Research International, 2016*, 1361508.

Teh, C.-H., Abdulghani, M., Morita, H., Shiro, M., Hussin, A. H., & Chan, K.-L. (2011). Comparative X-Ray and Conformational Analysis of a New Crystal of 13α, 21-Dihydroeurycomanone with Eurycomanone from Eurycoma longifolia and Their Anti-Estrogenic Activity Using the Uterotrophic Assay. *Planta Medica, 77*(02), 128-132.

Teh, C.-H., Morita, H., Shirota, O., & Chan, K.-L. (2010). 2, 3-Dehydro-4α-hydroxylongilactone, a novel quassinoid and two known phenyl propanoids from Eurycoma longifolia Jack. *Food Chemistry, 120*(3), 794-798.

Teh, C.-H., Murugaiyah, V., & Chan, K.-L. (2011). Developing a validated liquid chromatography–mass spectrometric method for the simultaneous analysis of five bioactive quassinoid markers for the standardization of manufactured batches of Eurycoma longifolia Jack extract as antimalarial medicaments. *Journal of Chromatography A, 1218*(14), 1861-1877.

Tian, L., Song, Z., Shao, W., Du, W. W., Zhao, L. R., Zeng, K., . . . Jin, T. (2017). Curcumin represses mouse 3T3-L1 cell adipogenic differentiation via inhibiting miR-17-5p and stimulating the Wnt signalling pathway effector Tcf7l2. *Cell Death & Disease, 8*(1), e2559.

Tiraby, C., Tavernier, G., Lefort, C., Larrouy, D., Bouillaud, F., Ricquier, D., & Langin, D. (2003). Acquirement of brown fat cell features by human white adipocytes. *Journal of Biological Chemistry*, 278(35), 33370-33376.

Tong, K. L., Chan, K. L., AbuBakar, S., Low, B. S., Ma, H. Q., & Wong, P. F. (2015). The in vitro and in vivo anti-cancer activities of a standardized quassinoids composition from Eurycoma longifolia on LNCaP human prostate cancer cells. *PloS One, 10*(3), e0121752.

Tontonoz, P., Hu, E., Graves, R. A., Budavari, A. I., & Spiegelman, B. M. (1994). mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. *Genes and Development, 8*(10), 1224-1234.

Tontonoz, P., & Spiegelman, B. M. (2008). Fat and beyond: the diverse biology of PPARγ. *Annual Review of Biochemistry, 77*, 289-312.

Tran, T. V. A., Malainer, C., Schwaiger, S., Atanasov, A. G., Heiss, E. H., Dirsch, V. M., & Stuppner, H. (2014). NF-κB inhibitors from Eurycoma longifolia. *Journal of Natural Products, 77*(3), 483-488.

Trayhurn, P. (2007). Adipocyte biology. *Obesity Reviews, 8*(s1), 41-44.

Tseng, P. C., Hou, S. M., Chen, R. J., Peng, H. W., Hsieh, C. F., Kuo, M. L., & Yen, M. L. (2011). Resveratrol promotes osteogenesis of human mesenchymal stem cells by upregulating RUNX2 gene expression via the SIRT1/FOXO3A axis. *Journal of Bone and Mineral Research, 26*(10), 2552-2563.

Turner, J., & Crossley, M. (1999). Basic Krüppel-like factor functions within a network of interacting haematopoietic transcription factors. *The International Journal of Biochemistry & Cell Biology, 31*(10), 1169-1174.

Uchida, S., Tanaka, Y., Ito, H., Saitoh-Ohara, F., Inazawa, J., Yokoyama, K. K., . . . Marumo, F. (2000). Transcriptional regulation of the CLC-K1 promoter by myc-associated zinc finger protein and kidney-enriched Krüppel-like factor, a novel zinc finger repressor. *Molecular and Cellular Biology, 20*(19), 7319-7331.

Uemura, T., Goto, T., Kang, M. S., Mizoguchi, N., Hirai, S., Lee, J. Y., . . . Kawada, T. (2011). Diosgenin, the main aglycon of fenugreek, inhibits LXRalpha activity in HepG2 cells and decreases plasma and hepatic triglycerides in obese diabetic mice. *Journal of Nutrition, 141*(1), 17-23.

Unger, R. H., Clark, G. O., Scherer, P. E., & Orci, L. (2010). Lipid homeostasis, lipotoxicity and the metabolic syndrome. *Biochimica et Biophysica Acta, 1801*(3), 209-214.

Valet, P., Tavernier, G., Castan-Laurell, I., Saulnier-Blache, J. S., & Langin, D. (2002). Understanding adipose tissue development from transgenic animal models. *Journal of Lipid Research, 43*(6), 835-860.

van der Heide, L. P., & Smidt, M. P. (2005). Regulation of FoxO activity by CBP/p300-mediated acetylation. *Trends in Biochemical Sciences, 30*(2), 81-86.

van Marken Lichtenbelt, W. D., Vanhommerig, J. W., Smulders, N. M., Drossaerts, J. M., Kemerink, G. J., Bouvy, N. D., . . . Teule, G. J. (2009). Cold-activated brown adipose tissue in healthy men. *New England Journal of Medicine, 360*(15), 1500-1508.

Veeman, M. T., Axelrod, J. D., & Moon, R. T. (2003). A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. *Developmental Cell, 5*(3), 367-377.

Veerkamp, J., & Van Moerkerk, H. (1993). Fatty acid-binding protein and its relation to fatty acid oxidation. *Molecular and Cellular Biochemistry, 123*(1-2), 101-106.

Vermaak, I., Viljoen, A. M., & Hamman, J. H. (2011). Natural products in anti-obesity therapy. *Natural Product Reports, 28*(9), 1493-1533.

Villena, J. A., Viollet, B., Andreelli, F., Kahn, A., Vaulont, S., & Sul, H. S. (2004). Induced adiposity and adipocyte hypertrophy in mice lacking the AMP-activated protein kinase-α2 subunit. *Diabetes, 53*(9), 2242-2249.

Vogel Hertzel, A., & Bernlohr, D. A. (2000). The mammalian fatty acid-binding protein multigene family: molecular and genetic insights into function. *Trends in Endocrinology and Metabolism, 11*(5), 175-180.

Vu, D., Ong, J. M., Clemens, T. L., & Kern, P. A. (1996). 1,25-Dihydroxyvitamin D induces lipoprotein lipase expression in 3T3-L1 cells in association with adipocyte differentiation. *Endocrinology, 137*(5), 1540-1544.

Wadden, T. A., Webb, V. L., Moran, C. H., & Bailer, B. A. (2012). Lifestyle modification for obesity: new developments in diet, physical activity, and behavior therapy. *Circulation, 125*(9), 1157-1170.

Wang, F., & Tong, Q. (2009). SIRT2 suppresses adipocyte differentiation by deacetylating FOXO1 and enhancing FOXO1's repressive interaction with PPARγ. *Molecular Biology of the Cell, 20*(3), 801-808.

Wang, G.-L., Shi, X., Salisbury, E., Sun, Y., Albrecht, J. H., Smith, R. G., & Timchenko, N. A. (2006). Cyclin D3 maintains growth-inhibitory activity of C/EBPα by stabilizing C/EBPα-cdk2 and C/EBPα-Brm complexes. *Molecular and Cellular Biology, 26*(7), 2570-2582.

Wang, S., Moustaid-Moussa, N., Chen, L., Mo, H., Shastri, A., Su, R., . . . Shen, C.-L. (2014). Novel insights of dietary polyphenols and obesity. *The Journal of Nutritional Biochemistry, 25*(1), 1-18.

Wang, S., Zhang, Q., Zhang, Y., Shen, C., Wang, Z., Wu, Q., . . . Qiao, Y. (2016). Agrimol B suppresses adipogenesis through modulation of SIRT1-PPAR gamma signal pathway. *Biochemical and Biophysical Research Communications, 477*(3), 454-460.

Wang, T., Choi, R. C., Li, J., Li, J., Bi, C. W., Zang, L., . . . Tsim, K. W. (2010). Antihyperlipidemic effect of protodioscin, an active ingredient isolated from the rhizomes of Dioscorea nipponica. *Planta Medica, 76*(15), 1642-1646.

Wang, Z., Ka, S. O., Lee, Y., Park, B. H., & Bae, E. J. (2017). Butein induction of HO-1 by p38 MAPK/Nrf2 pathway in adipocytes attenuates high-fat diet induced adipose hypertrophy in mice. *European Journal of Pharmacology, 799*, 201-210.

Ward, A. S., Comer, S. D., Haney, M., Fischman, M. W., & Foltin, R. W. (1999). Fluoxetine-maintained obese humans: effect on food intake and body weight. *Physiology and Behavior, 66*(5), 815-821.

Wehrli, N. E., Bural, G., Houseni, M., Alkhawaldeh, K., Alavi, A., & Torigian, D. A. (2007). *Determination of age-related changes in structure and function of skin, adipose tissue, and skeletal muscle with computed tomography, magnetic resonance imaging, and positron emission tomography.* Paper presented at the Seminars in Nuclear Medicine.

White, M. F. (2002). IRS proteins and the common path to diabetes. *American Journal of Physiology-Endocrinology and Metabolism, 283*(3), E413-E422.

White, U. A., & Stephens, J. M. (2010). Transcriptional factors that promote formation of white adipose tissue. *Molecular and Cellular Endocrinology, 318*(1-2), 10-14.

Who, W. (2013). WHO Traditional Medicine Strategy 2014–2023. *World Health Organization*.

Wing, R. R., Goldstein, M. G., Acton, K. J., Birch, L. L., Jakicic, J. M., Sallis, J. F., . . . Surwit, R. S. (2001). Behavioral science research in diabetes. *Diabetes Care, 24*(1), 117.

Winzell, M. S., & Ahren, B. (2004). The high-fat diet-fed mouse: a model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes. *Diabetes, 53*(suppl 3), S215-219.

Wolfram, S., Wang, Y., & Thielecke, F. (2006). Anti-obesity effects of green tea: From bedside to bench. *Molecular Nutrition & Food Research, 50*(2), 176-187.

Wong, P. F., Cheong, W. F., Shu, M. H., Teh, C. H., Chan, K. L., & AbuBakar, S. (2012). Eurycomanone suppresses expression of lung cancer cell tumor markers, prohibitin, annexin 1 and endoplasmic reticulum protein 28. *Phytomedicine, 19*(2), 138-144.

Wu, Z., Rosen, E. D., Brun, R., Hauser, S., Adelmant, G., Troy, A. E., . . . Spiegelman, B. M. (1999). Cross-regulation of C/EBPα and PPARγ controls the transcriptional pathway of adipogenesis and insulin sensitivity. *Molecular Cell, 3*(2), 151-158.

Wu, Z., & Wang, S. (2013). Role of kruppel-like transcription factors in adipogenesis. *Developmental Biology, 373*(2), 235-243.

Xiong, X. Q., Chen, D., Sun, H. J., Ding, L., Wang, J. J., Chen, Q., . . . Zhu, G. Q. (2015). FNDC5 overexpression and irisin ameliorate glucose/lipid metabolic derangements and enhance lipolysis in obesity. *Biochimica et Biophysica Acta, 1852*(9), 1867-1875.

Xu, Y., Zhang, M., Wu, T., Dai, S., Xu, J., & Zhou, Z. (2015). The anti-obesity effect of green tea polysaccharides, polyphenols and caffeine in rats fed with a high-fat diet. *Food & Function, 6*(1), 297-304.

Yang, J. Y., Della-Fera, M. A., Nelson-Dooley, C., & Baile, C. A. (2006). Molecular mechanisms of apoptosis induced by ajoene in 3T3-L1 adipocytes. *Obesity (Silver Spring), 14*(3), 388-397.

Yang, T., & Sauve, A. A. (2006). NAD metabolism and sirtuins: metabolic regulation of protein deacetylation in stress and toxicity. *The AAPS Journal, 8*(4), E632.

Ye, J. (2007). Role of insulin in the pathogenesis of free fatty acid-induced insulin resistance in skeletal muscle. *Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders), 7*(1), 65-74.

Ye, J. (2008). Regulation of PPARγ function by TNF-α. *Biochemical and Biophysical Research Communications, 374*(3), 405-408.

Ye, J. (2013). Mechanisms of insulin resistance in obesity. *Frontiers of Medicine, 7*(1), 14-24.

Yeh, W.-C., Cao, Z., Classon, M., & McKnight, S. L. (1995). Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. *Genes and Development, 9*(2), 168-181.

Yin, W., Mu, J., & Birnbaum, M. J. (2003). Role of AMP-activated protein kinase in cyclic AMP-dependent lipolysis in 3T3-L1 adipocytes. *Journal of Biological Chemistry, 278*(44), 43074-43080.

Yoon, M. S., Zhang, C., Sun, Y., Schoenherr, C. J., & Chen, J. (2013). Mechanistic target of rapamycin controls homeostasis of adipogenesis. *Journal of Lipid Research, 54*(8), 2166-2173.

Zakaria, Y., Rahmat, A., Pihie, A. H., Abdullah, N. R., & Houghton, P. J. (2009). Eurycomanone induce apoptosis in HepG2 cells via up-regulation of p53. *Cancer Cell International, 9*, 16.

Zaki, M., Robaayah, Z., Chan, S., Vadivale, M., & Lim, T. (2010). Malaysia Shape of the Nation (MySoN): a primary care based study of abdominal obesity in Malaysia. *Medical Journal of Malaysia, 65*(Suppl A), 143-149.

Zang, K., Wang, J., Dong, M., Sun, R., Wang, Y., Huang, Y., . . . Yu, M. (2013). Brd2 inhibits adipogenesis via the ERK1/2 signaling pathway in 3T3-L1 adipocytes. *PloS One, 8*(10), e78536.

Zang, L., Xue, B., Lu, Z., Li, X., Yang, G., Guo, Q., . . . Mu, Y. (2013). Identification of LRP16 as a negative regulator of insulin action and adipogenesis in 3T3-L1 adipocytes. *Hormone and Metabolic Research, 45*(5), 349-358.

Zhang, Gu, M., Cai, W., Yu, L., Feng, L., Zhang, L., . . . Chen, H. (2016). Dietary component isorhamnetin is a PPARγ antagonist and ameliorates metabolic disorders induced by diet or leptin deficiency. *Scientific Reports, 6*.

Zhang, H., Zhang, S., Jiang, C., Li, Y., Xu, G., Xu, M., & Wang, X. (2016). Intermedin/adrenomedullin 2 polypeptide promotes adipose tissue browning and reduces high-fat diet-induced obesity and insulin resistance in mice. *International Journal of Obesity, 40*(5), 852-860.

Zhang, H. H., Huang, J., Duvel, K., Boback, B., Wu, S., Squillace, R. M., . . . Manning, B. D. (2009). Insulin stimulates adipogenesis through the Akt-TSC2-mTORC1 pathway. *PloS One, 4*(7), e6189.

Zhang, H. Q., Chen, S. Y., Wang, A. S., Yao, A. J., Fu, J. F., Zhao, J. S., . . . Bao, Y. P. (2016). Sulforaphane induces adipocyte browning and promotes glucose and lipid utilization. *Molecular Nutrition & Food Research, 60*(10), 2185-2197.

Zhang, J., Fu, M., Cui, T., Xiong, C., Xu, K., Zhong, W., . . . Chen, Y. E. (2004). Selective disruption of PPARgamma 2 impairs the development of adipose tissue and insulin sensitivity. *Proceedings of the National Academy of Sciences of the United States of America, 101*(29), 10703-10708.

Zhang, W., Zhang, X., Wang, H., Guo, X., Li, H., Wang, Y., . . . Huo, Y. (2012). AMP-activated protein kinase alpha1 protects against diet-induced insulin resistance and obesity. *Diabetes, 61*(12), 3114-3125.

Zhao, J.-X., Yue, W.-F., Zhu, M.-J., & Du, M. (2011). AMP-activated protein kinase regulates β-catenin transcription via histone deacetylase 5. *Journal of Biological Chemistry*, *286*(18), 16426-16434.

Zhu, Y., Qi, C., Korenberg, J. R., Chen, X.-N., Noya, D., Rao, M. S., & Reddy, J. K. (1995). Structural organization of mouse peroxisome proliferator-activated receptor gamma (mPPAR gamma) gene: alternative promoter use and different splicing yield two mPPAR gamma isoforms. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(17), 7921-7925.

LIST OF PUBLICATIONS AND PAPERS PRESENTED

PUBLICATION

Original research article

Balan D, Chan K-L, Murugan D, AbuBakar S, Wong P-F. Antiadipogenic effects of a standardized quassinoids-enriched fraction and eurycomanone from *Eurycoma longifolia*. *Phytotherapy Research*. 2018;32:1332–1345. https://doi.org/10.1002/ptr.6065

CONFERENCE ATTENDED

Poster presentation

Balan D, Chan K-L, Murugan D, AbuBakar S, Wong P-F. Anti-adipogenic effect of a standardized quassinoids-enriched fraction of *Eurycoma longifolia*. 3rd Pan-Asian Biomedical Science Conference 2016, 7-8 December 2016, Premiera Hotel Kuala Lumpur, Malaysia.