EFFECTS OF MONOTERPENES ON GLUCOSE UPTAKE AND LIPID METABOLISM IN 3T3-L1 ADIPOCYTES

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ABSTRACT

Various strategies have been adopted to combat complications caused by Type 2 diabetes mellitus (T2DM) and diet control is one of them. Monoterpenes, the major constituents of essential oils, are synthesized and widely used as artificial food flavours. The aims of the present study were to assess the effects of twelve monoterpenes on antioxidant capacity, glucose uptake and lipid metabolism. Antioxidant capacity of monoterpenes were assessed using to 2, 2-diphenyl-2-picrylhydrazyl (DPPH) and 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assays. *In vitro* alpha-amylase and alpha-glucosidase inhibitory assays were used to assess the potential anti-hyperglycaemic property of monoterpenes. The 3T3-L1 cellular model was used to assess the effects of monoterpenes on adipocyte differentiation, lipid accumulation, glucose uptake and lipolysis. Gene expression study was conducted to determine the potential mechanisms involved in the above mentioned processes. Results showed that the monoterpenes used in the current study exerted low DPPH and ABTS radical scavenging activity even at high concentrations. Some monoterpenes inhibited alpha-glucosidase activities and stimulated glucose uptake and lipolysis in 3T3-L1 adipocytes. (R)-(+)-Limonene stimulated both glucose uptake (17.4%) and lipolysis (17.7%); in parallel, the mRNA expressions of glucose transporter 1 (GLUT1) was upregulated, glucose transporter 4 (GLUT4) was unaffected, and mRNA expression of adipose triglyceride lipase (ATGL) was suppressed. Oil Red O quantitative results showed that low level oxidative stress induced by glucose oxidase was able to stimulate both adipogenesis and lipid accumulation in 3T3-L1 adipocytes though mRNA expression of glutathione peroxidase (GPx) remained unaffected. The effect of glucose oxidase in adipogenesis and lipogenesis was not affected by the addition of monoterpenes. Taken together, the selected monoterpenes may not confer strong

protection against free radicals but nevertheless, their positive influence on lipid and glucose metabolism may have potential in the control of obesity and T2DM.

ABSTRAK

Pelbagai strategi telah digunakan untuk memperbaikkan komplikasi-komplikasi yang disebabkan oleh kencing manis jenis 2 dan pemakanan terkawal merupakan salah satu strategi yang lazim digunakan. Monoterpena, salah satu elemen utama dalam minyak pati, telah disintesis dan biasa digunakan sebagai perisa tiruan dalam makanan. Objektif kajian ini adalah menyiasat kesan-kesan dua belas monoterpena dalam kapasiti antioksida, pengambilan glukosa dan lipolysis. Kapasiti antioksida dinilai melalui ujian penghapusan radikal-radikal 2, 2-difenil-2-pikril hidrazil (DPPH) and 2, 2'-azinobis-(3-etilbenzotiazolina-6-asid sulfonik) (ABTS). Potensi monoterpena dalam anti-hiperglisemia dinilai melalui ujian perencatan aktiviti α-amilase dan aktiviti α-glukosida secara *in vitro.* Untuk menilai kesan-kesan monoterpena dalam pembezaan adiposit, pengumpulan lipid, pengambilan glukosa dan lipolisis, sel model 3T3-L1 telah digunakan. Ujian ekspresi gen telah digunakan memahami mekanisme yang terlibat dalam proses-proses yang dibincangkan di atas. Monoterpena-monoterpena telah menunjukkan kesan yang lemah dalam menghapuskan radikal-radikal DPPH and ABTS walaupun pada kepekatan yang tinggi. Selain itu, beberapa monoterpena telah menunjukkan potensi mereka dalam perencatan aktiviti aktiviti α-glukosida secara *in vitro*, serta perangsangan pengambilan glukosa dan lipolisis dalam adiposity 3T3-L1. Monoterpena seperti (R)-(+)-limonina dapat merangsangkan pengambilan glukosa (17.4%) dan lipolisis (17.7%) dalam adiposit; pada masa yang sama, ekspresi mRNA Pengangkut Glukosa 1 (GLUT1) juga meningkat tetapi ekspresi mRNA Pengangkut Glukosa 4 (GLUT4) tidak terjejas, dan ekspresi mRNA Adipos Trigliserida Lipase (ATGL) juga mengurang. Selain itu, kajian tentang kesan-kesan tekanan oksidatif (gred rendah) dalam proses adipogenesis dan metabolisme lipid juga telah dijalankan. Keputusan Ujian Kuantitatif Oil Red O menunjukkan bahawa tekanan oksidatif (gred rendah) yang dihasilkan oleh glukosa oksidase dapat merangsangkan adipogenesis dan

pengumpulan lipid dalam adiposit 3T3-L1, walaupun ekspresi mRNA glutation peroksidase (GPx) tidak terjejas. Seterusnya, kami mendedahkan adiposit 3T3-L1 kepada beberapa monoterpena yang terpilih dan glukosa oksidase secara serentak. Hasil kajian menunjukkan bahawa kesan glukosa oksidase dalam adipogenesis dan lipogenesis adalah dominan berbanding dengan monoterpena, mungkin disebabkan oleh kapasiti antioksida monoterpena yang lemah. Secara umumnya, monoterpena-monoterpena yang terpilih untuk projek penyelidikan ini tidak dapat memberikan perlindungan yang kuat terhadap radikal bebas, namun, pengaruh positif mereka dalam metabolime glukosa dan lipid menunjukkan potensi mereka dalam pengawalan obesity dan kencing manis jenis 2.

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbol/Abbreviation	Description
•OH	hydroxyl
°C	degree Celsius
%	percentage
α	alpha
β	beta
µ	micro
µg	microgram
µl	microliter
µm	micrometer
2DPD	two days prior to induction of differentiation
ABI	Applied Biosystems
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ADA	American Diabetes Association
ADD-1/SREBP-1	adipocyte determination- and differentiation-dependent factor-1/sterol regulatory element-binding protein-1
AdPLA	adipose-specific phospholipase A_2
AKT	protein kinase B
ATGL	adipose triglyceride lipase
ATCC	American Type Culture Collection

LIST OF SYMBOLS AND ABBREVIATIONS

Symbol/abbreviation	Description
ATP	adenosine triphosphate
BMI	body mass index
BSA	bovine serum albumin
C/EBP	CCAAT/enhancer binding proteins
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CGI-58	comparative gene identification-58
cm^2	square centimeter
CO_2	carbon dioxide
day 0	the day induction of differentiation took place
day 2	two days after induction of differentiation
ddH_2O	double distilled water
DEX	dexamethasone
DGs	diacyglycerols
DM	diabetes mellitus
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNS	3,5-dinitrosalicylic acid
DPP-4	dipeptidyl-peptidase 4
DPPH	2,2-diphenyl-2-picrylhydrazyl hydrate

LIST OF SYMBOLS AND ABBREVIATIONS

Symbol/Abbreviation	Description
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
FBS	fetal bovine serum
FDA	Food and Drug Administration
Fe^{2+}-TPTZ	ferrous-2,4,6-tri(2-pyridyl)-1,3,5-triazine
Fe^{3+}-TPTZ	ferric-2,4,6-tri(2-pyridyl)-1,3,5-triazine
$FeCl_3 \bullet 6H_2O$	ferric chloride hexahydrate
$FeSO_4 \bullet 7H_2O$	ferrous sulphate heptahydrate
FRAP	ferric reducing antioxidant power
g	gram
g	gravitational acceleration
GDM	gestational diabetes mellitus
GLP-1	glucagon-like peptide 1
GLUT1	glucose transporter-1
GLUT4	glucose transporter-4
GLUTs	glucose transporters
GSH	glutathione
GSSG	glutathione disulfide
h	hour
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid

LIST OF SYMBOLS AND ABBREVIATIONS

Symbol/Abbreviation	Description
HOO•	hydroperoxyl
HSL	hormone sensitive lipase
IBMX	3-isobutyl-1-methylxanthine
IGF-1	insulin-like growth factor 1
IL-6	interleukin 6
IR	insulin receptor
IRS	insulin receptor substrate
$K_2O_8S_2$	potassium persulfate
LPL	lipoprotein lipase
M	molar
MEM	Eagle's Minimum Essential Medium
mg	milligram
MGL	monoacylglycerol lipase
MGs	monoacylglycerols
min	minute
ml	milliliter
mM	millimolar
mm	millimeter
mm^3	cubic millimeter
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na_2HPO_4	disodium phosphate (sodium hydrogen phosphate)

LIST OF SYMBOLS AND ABBREVIATIONS

Symbol/Abbreviation	Description
NaH_2PO_4	monosodium phosphate (sodium dihydrogen phosphate)
ng	nanogram
nm	nanometer
nM	nanomolar
NO•	nitric oxide
NO_2•	nitrogen dioxide
$O2•^-$	superoxide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDE3B	phosphodiesterase 3B
PGE2	prostaglandin E2
PI3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol (4,5) bisphosphate
PIP_3	phosphatidylinositol (3,4,5) trisphosphate
PKA	protein kinase A
pM	picomolar
PNPG	4-nitrophenyl-α-D-glucopyranoside
PPARγ	peroxisome proliferator-activatedreceptor γ
ppm	parts-per-million
PTP	protein-tyrosine phosphatase
RIN	RNA Integrity number

LIST OF SYMBOLS AND ABBREVIATIONS

Symbol/Abbreviation	Description
RNA	ribonucleic acid
RNS	reactive nitrogen species
RO•	alkoxyl
ROS	reactive oxygen species
rpm	revolutions per minute
SCFAs	short-chain fatty acids
sec	second
SOD	superoxide dismutase
T1DM	Type 1 diabetes mellitus
TGs	triacylglycerols
TNF	tumor-necrosis factor
TNFα	Tumor-necrosis factor α
TPTZ	2,4,6-tri(2-pyridyl)-1,3,5-triazine
TZD	thiazolidinedione
U	unit
UNG	uracil-N-glycosylase
v/v	volume over volume
WHO	World Health Organization

LIST OF APPENDICES

CHAPTER ONE: INTRODUCTION

1.1 RESEARCH BACKGROUND

1.1.1 PREVALANCE AND MEDICAL COST OF OBESITY

Overweight or obesity is defined as the abnormal or excessive accumulation of fat which poses risks of morbidity and mortality in an individual. Globally, overweight and obesity are categorized as the fifth most common risk factors for death (Kakkar & Dahiya, 2015). In general, World Health Organization (WHO) monitors the prevalence of global obesity through the Global Database using body mass index (BMI) (Nguyen & El-Serag, 2010).

In 2014, globally over 1.9 billion adults with a minimum age of 18 years old were overweight and more than 600 million of them were obese. Worldwide, there were approximately 39% and 13% adults who were overweight (BMI $\geq$ 25 kg/m^2) and obese (BMI $\geq$ 30 kg/m^2) respectively. From 1980 to 2014, the global prevalence of obesity had been more than doubled (WHO, 2015a). The obesity epidemic has expanded from developed countries to developing countries, with approximately three times increase in the number of overweight and obese adults between the years 1980 and 2008, exceeding 900 million adults (Kakkar & Dahiya, 2015). In addition to that, prevalence of overweight or obesity in children had been consistently increasing with at least 42 million children below the age of 5 being diagnosed as overweight or obese in 2013 (WHO, 2015a). In 2011, Mohamud et al. (2011) reported that the prevalence of overweight and obesity in Malaysian adults were approximately 33.6% and 19.5% respectively, with a higher prevalence in females over males. Prevalence of obesity was also found to be highest among the Indians, followed by the Malays and Chinese.

Overweight and obesity have become the growing epidemics that increase the risk of global noncommunicable diseases, such as cardiovascular diseases, musculoskeletal disorders, some cancers and diabetes mellitus. At present, majority of the world's populations live in countries where overweight and obesity cause a higher mortality rate compared to underweight (WHO, 2015a). Besides depletion of quality of life, overweight and obesity also lead to economic burden. In 2008, an estimate of $113.9 billion was spent on the medical cost of both overweight and obesity in the United States (Tsai, Williamson, & Glick, 2011).

1.1.2 PREVALENCE AND MEDICAL COST OF TYPE 2 DIABETES MELLITUS (T2DM)

Type 2 Diabetes mellitus (T2DM) is one of the metabolic disorders characterized by chronic hyperglycemia due to impaired insulin production, insulin function, or both (WHO, 2015b). T2DM is a global epidemic with growing prevalence. In 2013, an estimate of 382 million people worldwide had T2DM and the number was projected to rise to 592 million by 2035 (Guariguata et al., 2014). It is also predicted that the prevalence of T2DM in adults, aged between 45 and 64 years, will increase in the next 20 years, particularly in developing countries (Olokoba, Obateru, & Olokoba, 2012). Prevalence of T2DM in children has been increasing as well (WHO, 2015b).

In 2012, approximately 1.5 million deaths were a direct result of DM and over 80% of these deaths occured in low- and middle-income countries (WHO, 2015b). In 2014, 16.6% of Malaysian adults had DM, which were equivalent to approximately 3.2 million cases of DM, implying the severity of the disease (International Diabetes Federation, 2015).

T2DM is associated with complications such as retinopathy, nephropathy, peripheral neuropathy, cardiovascular complications and sexual dysfunctions that deplete the quality of life and directly imposes substantial economic burden on all nations, particularly in developing countries (American Diabetes Association [ADA], 2014a; Mazzola, 2012). Between 1997 and 2007, the actual expenditure on anti-diabetic drugs had increased by 87% (from $6.7 billion to $12.5 billion) in the United States (Mazzola, 2012). Approximately $376 billion was spent on medical care of diabetes globally, in 2010 (Zhang et al., 2010).

1.1.3 DIETARY COMPONENTS IN THE MANAGEMENT OF OBESITY AND T2DM

Several effective interventions have been implemented over the past decades for the effective control of obesity and diabetes, such as changes in diet and exercise. In recent years, there is emerging evidence which indicates that quality and type of food are highly associated with the risk factors of T2DM. For instance, whole-grain diets with high fiber and polyunsaturated fat are associated with a lower risk of T2DM whereas diets with high *trans* fat increase the risk of T2DM (Hu, 2011). More interestingly, diet can induce changes in the composition of gut microbiota which then alters the metabolism of short-chain fatty acids (SCFAs). SCFAs such as butyrate have been shown to improve insulin resistance in diet-induced obese mice (Shen, Obin, & Zhao, 2013). Collectively, these findings suggest that components in diet play important roles in the development of insulin resistance and T2DM. This necessitates further studies for the better understanding on food components.

Traditional medicine has been used as a primary health care in approximately 80% of the world's population. Such intervention involves the use of plant extracts or their bioactive compounds, such as terpenoids (Santos et al., 2011). Therefore, in the current study, one of the main constituents of essential oils, monoterpenes were investigated.

Monoterpenes were shown to have numerous biological effects such as antioxidant, anti-inflammatory and anti-tumor properties (Bakkali, Averbeck, Averbeck, & Idaomar, 2008). Choi et al. (2000) showed that some of the monoterpenes such as γ-terpinene, terpinolene and citral exerted greater DPPH radical scavenging activity than Trolox. Evidence from the phase I clinical trials had also shown that oral consumption of d-limonene, a monoterpene, in advanced cancer patients helped in the control of breast cancer and colorectal carcinoma (Sun, 2007).

On the other hand, several animal studies showed that monoterpenes such as limonene and linalool having the potential to improve obesity and T2DM. For instance, Jing et al. (2013) showed that limonene lowered fasting plasma glucose, serum triglyceride and low-density lipoprotein (LDL) cholesterol levels in high-fat diet-fed mice. In addition, More, Kulkarni, Nalawade, & Arvindekar (2014) also reported that both linalool and limonene caused attenuation of glucose uptake in streptozotocin-induced diabetic rat. Another study by Santiago, Jayachitra, Shenbagam, & Nalini (2012) also demonstrated that high-fat-diet-fed rat supplemented with limonene showing reduced plasma glucose level and improved insulin sensitivity. Taken together, they suggested that monoterpenes have the potential in the control of obesity and T2DM. However, studies of other monoterpenes on anti-obesity and anti-diabetic effects are limited. Therefore, in the current study, the biological effects of monoterpenes were assessed and their roles on glucose uptake and lipid metabolism in 3T3-L1 adipocytes were investigated. 3T3-L1 adipocytes were used as they are one of the most common cellular models used in the study of obesity and T2DM due to their roles on glucose uptake, lipogenesis and lipolysis which contribute directly to the control of energy balance (Arsenijevic, Gregoire, Delforge, Delporte, & Perret, 2012). By understanding the biological effects of food components including food additives such as monoterpenes, diet can be more effectively planned for a better control of obesity and T2DM.

1.2 AIMS AND JUSTIFICATIONS OF THE STUDY

The aim of this study was to assess the biological effects of monoterpenes, namely their effects on glucose uptake and lipid metabolism. By understanding their roles with regards to the above stated properties, diet can be well-tailored for a better control of obesity and T2DM. In addition, if monoterpenes have the potential in the management of obesity and T2DM, they can be further studied and developed as pharmacotherapies to treat obesity- and T2DM-related complications.

The current study was divided into two parts: the first part of the study focused primarily on the biological effect of monoterpenes involving investigations on their radical scavenging capacity, ferric reducing power, α-amylase and α-glucosidase inhibitory effect; the second part of the study was conducted *in vitro* using murine 3T3-L1 cell line to evaluate the effect of monoterpenes on 3T3-L1 adipocyte proliferation, differentiation, lipid accumulation, glucose uptake and lipolysis, in the presence or absence of oxidative stress.

Monoterpenes with high antioxidant capacity were speculated to aid in the amelioration of oxidative stress level and hence, related disease complications (Huynh et al., 2014). Meanwhile, monoterpenes that stimulated 3T3-L1 adipocyte differentiation, lipid accumulation and glucose uptake could serve as potential insulin-like agents. Monoterpenes that decreased 3T3-L1 adipocyte proliferation, differentiation, glucose uptake but enhanced lipolysis could potentially serve as anti-obesity/weight reducing agents.

To date, effective drugs for the treatment of obesity and T2DM are limited with potential adverse events. Therefore, the efforts to explore and investigate new alternatives are essential to explore and provide outcomes that can aid in the finding of new alternatives and more effective interventions for obesity and T2DM in the future.

Since monoterpenes are widely present in edible plants, the influence of these compounds on lipid and glucose metabolism may help in the control of obesity and T2DM.

1.3 OBJECTIVES OF THE STUDY

1. To evaluate the radical scavenging capacity and ferric reducing power of monoterpenes.

2. To examine the effect of monoterpenes on the inhibition of α-amylase and α-glucosidase.

3. To assess the effect of monoterpenes and glucose oxidase induced oxidative stress on 3T3-L1 pre-adipocyte viability, adipocyte differentiation and lipid accumulation.

4. To investigate the effect of monoterpenes on glucose uptake and lipolysis in 3T3-L1 adipocytes.

5. To study the effect of selected monoterpene on gene expression pertinent to the above mentioned properties.

CHAPTER TWO: LITERATURE REVIEW

2.1 OBESITY

Overweight or obesity is defined as the abnormal or excessive fat accumulation which may result in increased morbidity and mortality rate in an individual (WHO, 2015a). Obesity is characterized by hyperplasia and/or hypertrophy of adipocytes (due to rapid adipocyte differentiation) accompanied by chronic low-grade inflammation which can alter adipocyte metabolism (Inadera, 2008; Masoodi, Kuda, Rossmeisl, Flachs, & Kopecky, 2015).

Body mass index (BMI) is one of the most useful epidemiological measures used to classify overweight and obesity in adults. Individuals with BMI more than 30 are considered obese (WHO, 2015a). In recent years, new guidelines and cut-off points of BMI have been established for Asian populations which help to predict disease risks more efficiently (Table 2.1).

$$\textbf{Body mass index (BMI)} = \frac{\text{Weight (kg)}}{\text{Height (m) x Height (m)}}$$

Table 2.1: Conventional and newly established Asian BMI cut-off points (WHO, 2004).

Classification	WHO BMI (kg/m^2)	Asian BMI (kg/m^2)	Health Risk (Cardiovascular Diseases)
Underweight	< 18.5	< 18.5	Low
Normal Range	18.5 – 24.9	18.5 – 22.9	Average
Overweight	25.0 – 29.9	23.0 – 27.4	Increased
Obese	≥ 30.0	≥ 27.5	High

2.1.1 RISK FACTORS

The main factor of overweight and obesity is imbalance between caloric intake and energy expenditure (WHO, 2015a). Due to the rapid urbanization, growth in economy and improvement of technology, a shift towards reduction of physical activity and high-caloric-diet lacking in micronutrients (e.g. vitamins and minerals) is believed to contribute predominantly to overweight and obesity (Kakkar & Dahiya, 2015). Other factors include gene mutations related to glucose and lipid metabolism and drugs (Nguyen & El-Serag, 2010; WHO, 2015a). For instance, thiazolidinedione (TZD), used in the treatment of T2DM, enhances insulin sensitivity in adipocytes which then stimulates the glucose uptake and adipogenesis leading to obesity (Evans, Barish, & Wang, 2004; Nguyen & El-Serag, 2010).

2.1.2 COMPLICATIONS OF OBESITY

BMI is positively correlated with the risk of numerous diseases. Comorbidities that are closely associated with obesity include cardiovascular disease, stroke, hypertension, dyslipidemia, T2DM, hyperglycemia, musculoskeletal disorder (especially osteoarthritis), asthma, sleep apnoea, and some cancers (e.g. endometrial, breast and colon cancers). In children, obesity increases the risk of premature death and disability (Inadera, 2008; WHO, 2015a).

2.1.3 MANAGEMENT OF OBESITY

Main treatments for obesity are diet, exercise and medication. The main mechanisms of these non-invasive treatments revolve around reduction of caloric intake and increased energy expenditure via exercise. In general, through intensive lifestyle intervention, loss of 5% to 10% of initial weight is achievable. Such weight loss has been shown to reduce the risk and development of cardiovascular diseases, T2DM and improve obesity-related complications. Greater weight loss is negatively correlated to the cardio-metabolic risk. During clinical trials, intensive lifestyle intervention helped to reduce 7% to 10% of initial weights in majority of the obese participants within the first year. However, weight maintenance in the long run is hard to achieve, even for the most motivated patients who had lost significant body weight (Yanovski & Yanovski, 2014). Bariatric surgery is another option for patients with BMI over 40 who suffer serious health threat. This intervention can help to maintain weight loss in majority of the patients for at least 5 years but bariatric surgery puts patients at risks of peri-operative mortality and operative complications in addition to its expensive medical cost and the needs of lifelong monitoring (Picot et al., 2009). In this regards, adjunctive therapies such as pharmacotherapy are necessary to help these patients to sustain weight loss to minimize and improve obesity-related comorbidities (Yanovski & Yanovski, 2014).

In United States, there are five drugs that are currently approved by the Food and Drug Administration (FDA) for the control of body weight in obese patients with BMI $\geq$ 30 kg/m^2 or overweight patients with BMI $\geq$ 27 kg/m^2 and obesity-related comorbidity: orlistat, lorcaserin, phentermine/topiramate extended release, naltrexone/bupropion extended release and liraglutide. In the past, several promising pharmacotherapies for obesity such as sibutramine, aminorex and mazindol have been withdrawn from the market owing to the unacceptable safety concerns, mostly

associating to the cardiovascular and psychiatric adverse events (Kakkar & Dahiya, 2015). Due to the concern with regards to the safety and efficacy, orlistat is the only approved pharmacological drug for the weight management in obese patients in Europe (Barja-Fernandez, Leis, Casanueva, & Seoane, 2014; Yanovski & Yanovski, 2014).

Orlistat is a synthetic hydrogenated derivative of lipstatin (an endogenous lipase inhibitor). Orlistat is potent with long reversible inhibitory effect on pancreatic and gastric lipases which hydrolyze dietary fat into monoacylglycerols (MGs) and free fatty acids, resulting in the reduction of systemic absorption of dietary ingested fats by approximately 30%. The main adverse effects of orlistat are gastrointestinal related complications such as flatus with discharge, fecal urgency, fecal incontinence, oily fecal spotting or evacuation, and soft stools. As orlistat interferes with dietary fat absorption, it also reduces the systemic absorption of fat-soluble vitamins (e.g. vitamin A, D, E and K, and beta-carotene). In response to this, patients on orlistat treatment are advised to routinely take multivitamin supplements containing fat soluble vitamins. Rare (thirteen reported cases) but severe adverse events such as severe liver injury, hepatocellular necrosis and acute hepatic failure were reported with the use of orlistat (Kakkar & Dahiya, 2015).

Due to the limited pharmacological choices for the management of weight control, it is crucial to discover new safe anti-obesity drugs with high drug efficacy.

2.2 METABOLIC SYNDROME

Metabolic syndrome (MetS) is characterized by a group of underlying risk factors such as central obesity, insulin resistance, elevated fasting serum triglyceride level (VLDL triglyceride), decreased fasting serum high-density lipoprotein (HDL) cholesterol level, raised blood pressure, and elevated fasting plasma glucose level that increase the risk of T2DM and cardiovascular diseases. An individual is diagnosed to have MetS if three or more of these components are exhibited concurrently (Kaur, 2014; O'Neill & O'Driscoll, 2015). International Diabetes Federation estimates that approximately 25% of the global population has MetS. Nevertheless, other studies reported that global prevalence of MetS can range from <10% to 84% due to the differences in region (urban or rural), composition (age, gender, ethnicity and race), and the diagnostic definition of MetS (Kaur, 2014).

2.2.1 RISK FACTORS OF METABOLIC SYNDROME

The main risk factors of MetS include decreased physical activity and high caloric food consumption which lead to increased risk of central obesity and insulin resistance (O'Neill & O'Driscoll, 2015). National Health and Nutrition Examination Survey's report also showed that the prevalence of MetS in normal weight, overweight and obese individuals is 5%, 22% and 60% respectively suggesting obesity plays important roles in the development of MetS. Further, MetS increases with age with 45% in individual aged 60-69 compared to 10% in individuals aged 20-29. Other risk factors of MetS include insulin resistance, genetic, smoking, post-menopause, stress, sleep disruption, drugs and excessive alcohol consumption (Kaur, 2014).

2.2.2 COMPLICATIONS OF METABOLIC SYNDROME

MetS confers an increased risk of T2DM and cardiovascular diseases in the following 5 to 10 years by fivefold and threefold respectively. In addition, patients with MetS showed an increased risk of stroke and myocardial infaction by 2- to 4-fold and 3- to 4-fold respectively and the risk of dying from such medical complications increases by 2-fold regardless of previous medical history of cardiovascular diseases (Kaur, 2014). Recent studies have also shown that MetS is associated with the increased risk of cancers (Esposito, Chiodini, Colao, Lenzi, & Giugliano, 2012). Other common associated medical conditions include microalbuminuria, fatty liver, acanthosis nigricans, erectile dysfunction and polycystic ovarian syndrome (Kaur, 2014).

2.2.3 MANAGEMENT OF METABOLIC SYNDROME

Management of MetS targets mainly on lifestyle changes with an increase of physical activity and a healthy diet with a lower caloric intake. A pharmacological approach is considered when the lifestyle changes do not result in significant improvement of MetS. To date, as there is no recognized single drug that can treat the entire syndrome, each component of MetS is treated separately (Kaur, 2014).

2.3 DIABETES MELLITUS

Diabetes mellitus (DM) was first reported in an Egyptian manuscript about 3000 years ago, making it one of the oldest diseases known to human (Olokoba et al., 2012). DM is one of the metabolic disorders characterized by chronic hyperglycemia due to impaired insulin production, insulin function, or both (ADA, 2014a). This causes a reduction in glucose uptake by the liver, muscle cells, and fat cells, resulting in hyperglycemia, a physiological condition in which blood glucose levels exceed the normal range (Olokoba et al., 2012). There are several readily available tests for the diagnosis of T2DM as shown in Table 2.2.

Table 2.2: Diagnostic tests of diabetes (ADA, 2014b).

Diagnostic Test	Normal	Prediabetes	Diabetes
Glycated Hemoglobin (A1C)	< 5.7%	5.7 to 6.4%	≥ 6.5%
Fasting Plasma Glucose (FPG)	< 5.6 mmol/l	5.6 to 6.9 mmol/l	≥ 7.0 mmol/l
Oral Glucose Tolerance Test (OGTT)	< 7.8 mmol/l	7.8 to 11.0 mmol/l	≥ 11.1 mmol/l

In general, there are two major forms of DM, which are Type 1 diabetes mellitus (T1DM) and Type 2 diabetes mellitus (T2DM). T1DM, previously known as insulin-dependent or juvenile-onset diabetes, is primarily due to deficiency of insulin synthesis and secretion owing to the autoimmune response-mediated destruction of β cells in the islets of Langerhans (ADA, 2014a; Govers, 2014; WHO, 2015b). T1DM accounts for 5% to 10% of diabetic patients. Onset of immune-mediated diabetes can take place anytime including childhood and adolescence, even in elders who are in the late stage of life (80 to 90 years old). The risk factors of autoimmune response-mediated destruction of β cells include genetic predisposition and environmental factors that are still not fully understood. In this type of diabetes, the rate of β cell destruction varies among the individual, which usually is rapid in infants and children; and slow in adults. Main

manifestations of T1DM are modest fasting hyperglycemia with/without ketoacidosis. T1DM patients are usually not obese. At the later stage of T1DM, due to insufficient or absence of insulin secretion, the patients will eventually be dependent on insulin to survive (ADA, 2014a; WHO, 2015b).

T2DM, previously known as non-insulin-dependent or adult onset diabetes, is due to insulin resistance as a result of impaired insulin production, insulin function, or both. T2DM accounts for 90% to 95% of diabetes, with the increasing prevalence in adolescent lifetime (ADA, 2014a; Govers, 2014; WHO, 2015b). In T2DM, a degree of hyperglycemia is adequate to result in functional changes in different target tissues, such as alteration of carbohydrate metabolism and impaired glucose tolerance. However, it is possible that clinical symptoms are undetectable for a long period of time before T2DM is diagnosed. Patients with T2DM may not need insulin treatment to survive in the initial stages of the disease, or even throughout their lifetime (ADA, 2014a; WHO, 2015b). Due to the high prevalence of T2DM, the latter part of the discussion in this thesis will mainly focus on T2DM. Complications and treatment of T2DM will be discussed in Section 2.3.2 and Section 2.3.3 respectively.

Gestational diabetes mellitus (GDM) is defined as any degree of impaired glucose tolerance during pregnancy in previously non-diabetic women. Approximately 9% of pregnant women have GDM (ADA, 2013b). GDM usually resolves with delivery but it is also possible that T2DM develops right after that, or later in life. GDM is mostly diagnosed through prenatal screening (ADA, 2013c; ADA; 2014a; WHO, 2015b).

2.3.1 RISK FACTORS OF T2DM

Risk factors of T2DM include obesity (particularly central visceral/abdominal obesity), dyslipidemia (abnormal level of cholesterol and triglyceride levels), injury of pancreas, genetic (mutations of pancreatic cells and/or insulin receptor), endocrinopathies (e.g., epinephrine, cortisol, glucagon and growth hormone), age, inadequate exercise, cigarette smoking, stress, drugs and infections (ADA, 2014a; Govers, 2014; WHO, 2015b). Increased prevalence of T2DM is also observed in women with prior GDM. On the other hand, in non-obese T2DM, an increased body fat percentage, predominantly in the abdominal region, was observed (ADA, 2014a; WHO, 2015b).

Obesity contributes to about 55% of T2DM, making it the main contributing factor of T2DM (Olokoba et al., 2012). Obesity predisposes an individual to insulin resistance leading to the development of T2DM. In the state of insulin resistance, insulin-responsive cells such as liver, muscle and adipocytes do not respond efficiently upon insulin stimulation causing elevated blood sugar levels. Consequently, more insulin is produced by the pancreatic β cells to compensate for the elevated blood sugar levels, leading to hyperinsulinemia. Prolonged hyperinsulinemia exhausts the β cells and apoptosis takes place, which in turn reduces the insulin secretion. Reduced insulin level is not sufficient to counteract the effect of insulin resistance in insulin-responsive cells, resulting in T2DM (ADA, 2014a; Govers, 2014).

Apart from that, hereditary factors are closely associated with the development of T2DM. T2DM risk in the children increases in a family with history of T2DM children. Another possible risk factor of T2DM is the use of drugs. For instance, blood glucose level of a diabetic patient resulted from high doses of exogenous steroids may return to the normal range after the steroid is discontinued. However, T2DM may

develop years later due to relapsing episodes of pancreatitis (ADA, 2014a). Recent studies also show a weak positive correlation between the urine concentration of bisphenol A, a constituent of plastics, and T2DM (Olokoba et al., 2012).

2.3.2 COMPLICATIONS OF DIABETES MELLITUS

Diabetic patients with chronic hyperglycemia commonly suffer from long-term damage and dysfunction of organs, particularly kidney, eye, heart, blood vessels, and nerves. Symptoms of prominent hyperglycemia include polyuria, polydipsia, weight loss, polyphagia (occasionally), and blurred vision. Rarer events such as growth impairment and increased susceptibility to certain infections are observed in some chronic hyperglycemia cases as well. Ketoacidosis is also rare in T2DM (ADA, 2014a).

Uncontrolled diabetes can cause acute and life-threatening consequences: ketoacidosis due to hyperglycemia and non-ketotic hyperosmolar syndrome. Other long-term complications are cardiovascular diseases, myocardial infarction, retinopathy (potentially lead to loss of vision), nephropathy leading to chronic renal failure, peripheral neuropathy (with the risk of foot ulcers/diabetic foot and amputation), autonomic neuropathy (with the risk of gastrointestinal and genitourinary dysfunction), and sexual dysfunction. The severity of the complications can progress, regress or remain the same (ADA, 2014a; Govers, 2014).

2.3.3 MANAGEMENT OF DIABETES MELLITUS

T2DM can be prevented by a change of lifestyle. Studies have shown that maintenance of BMI of ≤ 25 kg/m^2 and lifestyle modification including regular exercise, restraining of smoking, moderate alcohol consumption, and diet with high fiber, unsaturated fat, low in saturated and *trans* fats can significantly reduce the incidence of T2DM (Olokoba et al., 2012). On the other hand, in some diabetic patients, interventions such as weight reduction, exercise, and/or oral glucose-lowering agents are adequate to achieve the glycemic control but are seldom restored to normal (ADA, 2014a).

Other than insulin, there are several drugs currently used for the management of T2DM. During the selection of pharmacological drugs for the treatment of T2DM, the choices of drugs are based on several factors: effective in glycemic control, safe, easy to use, and reasonable costs (Mazzola, 2012). Overview of several drugs that are currently used in the management of diabetes is shown in Table 2.3.

Over the years, there have been efforts in search for drugs that are effective in the management of diabetes, with the hope of a cure. Several effective drugs have been discovered and are currently in use but some were withdrawn from the market due to safety issue, such as troglitazone and rosiglitazone owing to liver failure and cardiovascular events respectively (Mazzola, 2012; Olokoba et al., 2012). Therefore, the search for effective and safe therapeutic drugs is ongoing.

Table 2.3: Current drugs used in the treatment of diabetes (Olokoba et al., 2012; Van Gaal & Scheen, 2015).

Drug Class	Mechanism of Actions	Drawbacks
Insulin	Activation of insulin signaling	Hypoglycemia, weight gain
Biguanides (metformin, most commonly and only biguanide used for the treatment for T2DM)	Repression of: - hepatic glucose production - glucose production -intestinal absorption of glucose Enhancement of insulin sensitivity by stimulating peripheral glucose uptake and utilization Increase in fatty acid oxidation	Low risk of lactic acidosis and hypoglycemia
Sulfonylureas	Enhancement of insulin secretion	High risk of severe hypoglycemia and potential weight gain with some sulfonylureas
Meglitinides	Stimulation of insulin secretion (short-term action)	Low risk of hypoglycemia
Thiazolidinediones	Serve as insulin sensitizer, ligand for peroxisomes proliferator-activated gamma (PPARγ)	Weight gain and fluid retention, low risk of hypoglycemia, cardiovascular disorders, renal impairment and increased subcutaneous adiposity
α-Glucosidase inhibitors	Reversible inhibition of membrane-bound intestinal α-glucoside hydrolase enzymes Glucose absorption reduction GLP-1 secretion enhancement	Diarrhoea and flatulence (very common), less effective in lowering blood glucose level

Table 2.3, continued: Current drugs used in the treatment of diabetes (Olokoba et al., 2012; Van Gaal & Scheen, 2015).

Drug Class	Mechanism of Actions	Drawbacks
Amylin mimetics	Induction of satiety Slowing of gastric emptying Suppression of postprandial glucagon secretion, thus, reduction in hepatic glucose output	Increased risk of severe hypoglycemia
Glucagon-like peptide 1 (GLP-1) agonists	Activation of human GLP-1R Enhancement of glucose-dependent insulin secretion by the pancreatic β cell Stimulation of intracellular cyclic adenosine monophosphate (cAMP) resulting in increased insulin release when blood glucose level increases. Reduction in gastric emptying Induction of satiety	Rapid inactivation by DPP-4
Dipeptidyl-peptidase 4 (DPP-4) inhibitors	Increase in and prolonging active incretin levels Enhancement of insulin release Suppression of glucagon levels in a glucose-dependent manner	Low risk of hypoglycemia and expensive

2. 4 ADIPOCYTES

Adipocytes, also known as fat cells, are specialized cells that play crucial roles in sustaining energy balance and energy metabolism. Adipocytes are mainly distributed in subcutaneous and visceral parts of the body. As the largest energy storage depot, approximately 95% of the body fat is stored as triacylglycerols (TGs) inside cytosolic vesicles of white adipocytes (Large, Peroni, Letexier, Ray, & Beylot, 2004). However, excessive accumulation of TGs can lead to obesity, insulin resistance and T2DM (Ahmadian, Wang, & Sul, 2010).

In general, there are two types of adipocytes, namely, white adipocytes and brown adipocytes. White adipocytes are unilocular cells with lipid droplets and predominantly found in normal human adults. White adipocytes mainly serve as an inert storage depot of triglycerides (stored in a single large lipid droplet) that will be rapidly hydrolyzed by lipases into free fatty acids (a process known as lipolysis) and mobilized to the target tissues to be oxidized in mitochondria to generate energy when energy deprivation occur (Guilherme, Virbasius, Puri, & Czech, 2008; Niemelä, Miettinen, Sarkanen, & Ashammakhi, 2008). White adipocytes account for 5% to 60% of total body weight. Number of adipocyte remains static in adult humans regardless of significant changes in body weight. Previous study proposed that the number of adipocytes is determined during childhood and adolescence with only about 10% of adult human adipocytes are renewed every year. Metabolic syndrome is closely correlated with visceral fat accumulation, characterized by upper-body obesity (Masoodi et al., 2015). Other than energy storage depot, white adipose tissues also serve as an endocrine organ that secretes adipokines which will be further discussed in section 2.5.

On the contrary, brown adipocytes are multilocular cells and relatively scarce in human adults (Masoodi et al., 2015; Niemelä et al., 2008). Brown adipocytes play primary roles in the non-shivering thermogenesis, an adaptation to cold climate as well as producing body heat in the newborn babies. Thereby, brown adipocytes are rich in mitochondria (which gives the brown color) and characterized with multiple small lipid droplets to enable accessibility of rapid hydrolysis of triglycerides and in turn, rapid oxidation of the fatty acids to produce heat. Brown adipocytes decrease in number as the newborns grow up and are gradually replaced with white adipocytes in human. However, recent findings suggest that dispersed brown adipocytes might be present in human adults (Masoodi et al., 2015).

2. 5 ADIPOKINES

Adipocytes play important roles as a regulator that controls whole-body lipid flux, which in turn modulates glucose and lipid homeostasis in humans. Recent years, after the discovery of leptin, a cytokine secreted by adipocytes, also known as adipokine, focus has been subjected to adipocytes as an endocrine organ and their roles in the etiology of metabolic disorders: mainly on obesity, insulin resistance and T2DM. Other than energy storage depot, white adipose tissues, serving as an endocrine organ, can secrete several signals known as adipokines, including hormones, cytokines, growth factors and non-peptide biologically active molecules such as activated lipids. These signals could exert autocrine, paracrine and endocrine effects on skeletal muscle, adipocytes, liver, pancreas, hypothalamus, immune cells and endothelium, influencing glucose and lipid metabolism, food intake (neuroendocrine pathways), inflammatory response, immunity and blood pressure (Guilherme et al., 2008; Masoodi et al., 2015; Pereira & Alvarez-Leite, 2014). A brief overview of extensively studied adipokines is presented in Table 2.4. On the other hand, studies on secretory functions of brown adipocytes are, however, limited and poorly understood.

Adipokines have been shown to exhibit pro- and anti-inflammatory effect and the imbalance of these adipokines can lead to insulin resistance. Dysregulation of adipokines can also lead to the excess in adiposity, dysfunction of adipocytes and inflammation, especially in individuals with abdominal obesity, linking to the pathogenesis of insulin resistance and metabolic disorders. Nevertheless, such morbidities are not demonstrated in some healthy obese individuals, suggesting the protective effect of adipokines. Unraveling the underlying mechanisms of adipokines in the control of inflammation, insulin resistance and metabolic disorders is important in

the discovery of therapeutic agents that aid in the growing epidemic of obesity and related comorbidities (Pereira & Alvarez-Leite, 2014).

Table 2.4: Adipokines and their functions (Inadera, 2008; Pereira & Alvarez-Leite, 2014).

Adipokine	Distribution	Main Functions	Changes Observed In Obese Individuals
Leptin	Expressed mainly in subcutaneous white adipose tissue. Higher secretion of leptin in women than in men.	Reduce food intake Increase energy expenditure Possibly involved in the mediation of inflammation	Increased leptin expression and secretion Hyperleptinemia Leptin resistance Inflammatory cell activation
Adiponectin	Expressed primarily in subcutaneous white adipose tissue.	Increase: - insulin sensitivity - fatty acid oxidation - glucose uptake and adipogenesis in adipocytes - glucose metabolism in muscle Decrease: - free fatty acid uptake - glucose secretion in the liver Exhibit anti-diabetic, anti-inflammatory and anti-atherogenic effect	Decrease in: - adiponectin expression and secretion in adipocytes. - serum adiponectin
Tumor-necrosis factor α (TNFα)	Expression primarily in M1 macrophage from the stromal vascular fraction. Also expressed in white adipocytes.	Increase: - insulin resistance by inhibiting insulin signaling - lipolysis in adipocytes, by inhibiting lipoprotein lipase (LPL) in adipocytes - serum free fatty acids - inflammation Decrease: - 3T3-L1 adipocyte differentiation	Elevated TNFα expression and secretion in adipose tissue

2.6 ADIPOGENESIS IN 3T3-L1 ADIPOCYTES

Adipogenesis is the differentiation of pre-adipocytes into mature adipocytes until the adipocytes are capable of accumulating fat. In general, the 3T3-L1 cell line is widely used as an *in vitro* model for the study of adipocyte differentiation. Therefore, 3T3-L1 cell line was used in the current study and a brief overview on 3T3-L1 adipocyte differentiation is discussed in this section.

A standard differentiation process of 3T3-L1 pre-adipocytes will undergo three distinct stages, which are cell-contact inhibition, mitotic clonal expansion and terminal differentiation. Committed pre-adipocytes have to exit the cell cycle before the differentiation of adipocytes takes place. When 3T3-L1 pre-adipocytes are grown to confluence, cells will experience a growth arrest due to cell-contact inhibition. Cell-contact inhibition is essential for the differentiation of 3T3-L1 adipocytes and according to standard protocol, which usually takes place for 48 h after 3T3-L1 cells are grown to confluence (Guo et al., 2009; Moreno-Navarrete & Fernández-Real, 2012; Niemelä et al., 2008). Recent findings have shown that extensive epigenetic modifications take place during contact-inhibition in 3T3-L1 adipocytes and in part, contribute to the commencement of adipocyte differentiation (Guo et al., 2009).

These post-confluent cells are then induced to differentiate with a differentiation cocktail consisting of 3-isobutyl-1-methylxanthine (IBMX), dexamethasone and insulin. Dexamethasone serves to stimulate the glucocorticoid receptor pathway; IBMX serves to stimulate the cAMP-dependent protein kinase pathway; and insulin serves to stimulate the insulin/insulin-like growth factor 1 (IGF-1) signalling pathway (Niemelä et al., 2008). Following the induction, these quiescent cells re-enter the cell cycle and undergo the process of mitotic clonal expansion, a process in which the cells will undergo at least one round of DNA replication and division of cells, prior to the

differentiation (Guo et al., 2009). Mitotic clonal expansion was believed to unwind DNA to allow the access of transcription factors to regulatory response elements in genes that are responsible for adipocyte differentiation. An unusual growth arrest takes place upon the completion of mitotic clonal expansion. Growth-arrested cells undergo terminal differentiation by expressing late markers including lipogenic and lipolytic enzymes and proteins which are responsible for the mature phenotype of adipocytes (Guo et al., 2009; Moreno-Navarrete & Fernández-Real, 2012; Niemelä et al., 2008).

2.7 GENE EXPRESSION DURING 3T3-L1 ADIPOCYTE DIFFERENTIATION

The gene expression regulation that occurs during adipocyte differentiation is illustrated in Fig. 2.1.

At confluence, cell-contact inhibition in 3T3-L1 pre-adipocytes mediates the expression of early markers of adipocyte differentiation such as LPL. LPL, expressed predominantly in adipocytes, serves to hydrolyze TGs that are associated with capillary endothelial surfaces (Ntambi & Young-Cheul, 2000).

Within 1 h after the induction of differentiation (with adipogenic differentiation cocktail), elevated mRNA expression of c-fos, c-jun, and c-myc with mitogenic properties is observed, possibly involves in mitotic clonal expansion. In differentiating pre-adipocytes, expression of c-myc has been shown to mediate mitogenesis. However, another study shows that overexpression of the c-myc oncogene can block adipocyte differentiation. These findings suggest that the transient c-myc expression is in part, responsible for directing the growth arrested cells (confluent cells) toward cell cycle in the mitotic clonal expansion prior to terminal differentiation. The expression of c-fos, c-myc and c-jun dissipates within 2 to 6 h after the induction of differentiation (Ntambi & Young-Cheul, 2000).

At the same time, within the first hour after the induction of differentiation, the expressions of the first transcription factors, CCAAT/enhancer binding proteins (C/EBP) β and δ are mediated. Both C/EBPβ or C/EBPδ are responsive to hormonal inducers during the differentiation process: C/EBPβ responds primarily to dexamethasone, whereas C/EBPδ responds primarily to IBMX. After the withdrawal of dexamethasone and IBMX from the culture medium, C/EBPδ expression dissipates gradually in the

next 48 h, whereas C/EBPβ expression dissipates by day 8 (Niemelä et al., 2008; Ntambi & Young-Cheul, 2000).

Collective data from several studies suggest that C/EBPβ and δ mediate the expression of peroxisome proliferator-activated receptor γ (PPARγ), the regulator of adipocyte differentiation. Endogenous PPARγ ligand is absent in 3T3-L1 pre-adipocytes and is produced in the course of differentiation (Niemelä et al., 2008; Ntambi & Young-Cheul, 2000). Rising adipocyte determination- and differentiation-dependent factor-1/sterol regulatory element-binding protein-1 (ADD-1/SREBP-1) expression during adipocyte differentiation is thought to mediate the generation of endogenous PPARγ (Niemelä et al., 2008). Expression of PPARγ is observed on day 1 and reaches maximum by day 3 to d 4. In parallel, C/EBPβ and C/EBPδ also mediates C/EBPα expression, which rises from day 2 and peaks at day 5. After that, PPARγ and C/EBPα expression serves to maintain each other expression in spite of the dissipation C/EBPβ and C/EBPδ. The expression of PPARδ and C/EBPα mediates the transcription of many adipocyte genes that encode for proteins and enzymes that are responsible for the phenotypes and functions of adipocytes. The C/EBPα is shown to have antimitotic properties and prevents mitotic clonal expansion (Niemelä et al., 2008; Ntambi & Young-Cheul, 2000).

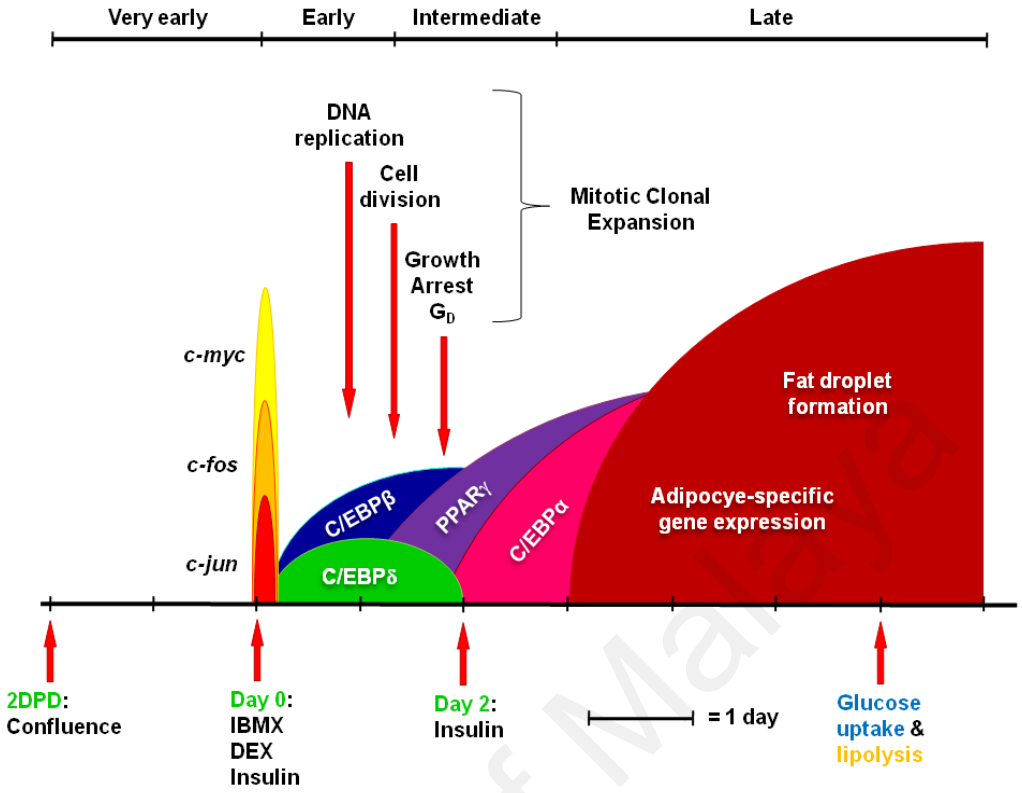


Figure 2.1: Gene regulation during adipocyte differentiation (Ntambi & Young-Cheul, 2000).

2.8 GLUCOSE UPTAKE

After a high caloric intake, elevated blood glucose levels are normalized rapidly; whereas during a long term starvation, blood sugar levels are maintained at slightly lower than the normal levels only. The major cellular mechanism that involves the glucose disposal is via the insulin-stimulated glucose transport into muscle and adipocytes. Glucose taken up by the cells will either be oxidized to produce energy or stored as TGs in lipid droplets. The control of blood glucose levels is important in the prevention of hyperglycemia and hypoglycemia that can lead to severe cellular dysfunctions (Huang & Czech, 2007).

Glucose uptake in cells is mediated by glucose transporters (GLUTs), GLUT1 to GLUT14, a family of integral membrane proteins that mediate sodium-independent, facilitated-diffusion of hexoses across plasma membrane (Govers, 2014; Mueckler & Thorens, 2013). Two main glucose transporter isoforms, namely, GLUT1 and GLUT4 are responsible for the glucose uptake in insulin-responsive tissues such as skeletal muscles and adipocytes. Predominantly, GLUT1 is expressed and found on the plasma membrane and intracellular membranes of both pre-adipocytes and adipocytes, mainly responsible for the basal glucose uptake (Herman & Kahn, 2006). On the other hand, GLUT4 is predominantly expressed in skeletal muscles, cardiomyocytes and adipocytes, and largely accounts for glucose transport into these tissues upon insulin stimulation. In adipocytes, GLUT4 is expressed during the course of adipocyte differentiation and sequestered in intracellular vesicles and translocated to the plasma membrane upon insulin stimulation. GLUT4 in muscles cells can also be activated upon muscle contraction or insulin stimulation via distinct mechanisms. The cellular regulation of GLUT4 upon insulin stimulation is however highly identical in both muscle cells and adipocytes (Govers, 2014; Herman & Kahn, 2006).

The insulin-induced glucose uptake by muscle cells and adipocytes is mainly mediated by GLUT4. Therefore, the mechanism involved in glucose uptake in GLUT4 is briefly discussed here. In the basal state (absence of insulin), GLUT4 is sequestered in intracellular vesicles in the cytosol, which prevents GLUT4 reaching the plasma membrane and stimulating the glucose transport when blood glucose level is low. Conversely, elevated blood glucose levels stimulate the release of insulin from the β-langerhan cells (Govers, 2014; Herman & Kahn, 2006; Huang & Czech, 2007). Insulin signaling pathway is triggered when insulin binds to its receptors and induces the autophosphorylation of insulin receptor (IR) tyrosine kinase. Receptor activation result in the phosphorylation of tyrosine residues in insulin receptor substrate (IRS) protein, which in turn results in the activation of phosphoinositide 3-kinase (PI3K) that phosphorylates phosphatidylinositol (4,5) bisphosphate (PIP_2) into phosphatidylinositol (3,4,5) trisphosphate (PIP_3). PIP3 activates AKT (protein kinase B) and leads to downstream activities including the stimulation of glucose uptake (Huang & Czech, 2007). Therefore, as a result of insulin signaling cascades upon insulin stimulation, GLUT4 vesicles translocate from cytosol to fuse with plasma membrane facilitating the glucose transport in both adipocytes and muscle cells resulting in the control of blood glucose level (Govers, 2014; Herman & Kahn, 2006; Huang & Czech, 2007).

Though adipose tissue accounts for only 15% of the blood glucose uptake and the remaining 85% blood glucose is attributed to muscle, knockout of GLUT4 in adipocytes results in glucose intolerance, hyperinsulinemia and insulin resistance (in adipose tissues, as well as muscle and liver cells). In addition, obesity, characterized by increase number and size of adipocytes, is found to impair the insulin sensitivity of the cells, leading to insulin resistance. Taken together, other than muscle, it is suggested that adipocytes play important roles in the control of glucose homeostasis and insulin resistance in the etiology of diabetes mellitus (Govers, 2014).

2.9 LIPOLYSIS

Lipolysis is a catabolic process that hydrolyzes TGs stored in lipid droplets liberating glycerols and fatty acids which serve as energy substrates during energy deprivation. Neutral hydrolysis of TGs involves three distinct enzymes; adipose triglyceride lipase (ATGL), the major TG lipase, initiates lipolysis by hydrolyzing TGs to diacyglycerols (DGs); hormone sensitive lipase (HSL) converts DGs into monoacylglycerols (MGs), which in turn, acted by monoacylglycerol lipase (MGL) releasing glycerol and fatty acids. In adipose tissue, ATGL and HSL account for at least 90% of the hydrolysis of TGs (Zechner et al., 2012).

Lipolysis is strongly modulated by hormonal control. During the fasted state, glucocorticoid level increases which then induces the upregulation of ATGL transcription. In parallel, catecholamines bind to Gα5-coupled β-adrenergic receptors and initiate a signaling cascade that leads to the elevation of cAMP levels and in turn, activation of protein kinase A (PKA). PKA phosphorylates HSL and phosphorylated HSL translocates from the cytosol to the lipid droplet to catalyze the hydrolysis of DAG. Perilipin, a lipid droplet associated protein that prevents the activation of ATGL under the state of desphosphorylation, is also phosphorylated by PKA during the fasted state to allow the activation of ATGL. On the contrary, during the fed state, insulin initiates the insulin signaling cascades that phosphorylate and activate phosphodiesterase 3B (PDE3B) which reduces cAMP level and thus, inhibition of lipolysis (Ahmadian et al., 2010).

Other than energy substrates, fatty acids also play important roles in the synthesis of most lipids (e.g. membrane lipids) and modulation of signaling cascades. However, excessive nonesterified fatty acids can lead to lipotoxicity symptoms of which includes disruptions of biological membrane integrity, alteration of cellular acid-base

homeostasis, inflammation, dysfunctions of mitochondria and cell death. To counter the detrimental effect of excessive fatty acids, nonesterified fatty acids can be re-esterified with glycerol to produce TGs (Zechner et al., 2012).

Elevated plasma fatty acid levels caused by increased lipolysis has been shown to be closely related to the development of insulin resistance, which in turn, results in T2DM. In relation to that, lipolysis inhibition has been used as one of the therapeutic approaches to improve insulin resistance in T2DM patients. Having said that, recent studies using mouse models showed that elevated lipolysis does not necessarily lead to the increase in serum fatty acid levels. Rather, fatty oxidation within adipocytes and leaner mouse were observed (Ahmadian et al., 2010; Zechner et al., 2012). These findings further stimulate the interest in the research of lipolysis which shows the potential as the key player to the enhancement of obesity, insulin resistance and diabetes mellitus.

2.10 FREE RADICALS AND OXIDATIVE STRESS

A free radical is an atom or molecular species with one or more unpaired electron(s) which are highly reactive (Kabel, 2014). Free radicals can be generated from endogenous or exogenous sources. Endogenous free radicals result from normal cellular metabolism, immune response, inflammation, infection, ischemia, aging and excessive exercise. Exogenous free radicals can be produced by cigarette smoke, alcohol, cooking, heavy metals and radiation (Kabel, 2014; Rahal et al., 2014; Shafaq, 2012). Free radicals include reactive oxygen species (ROS) and reactive nitrogen species (RNS). Some examples of ROS are superoxide ($O2^{\bullet -}$), hydroxyl (•OH), hydroperoxyl (HOO•) and alkoxyl (RO•) whereas examples of radical RNS are nitric oxide (NO•) and nitrogen dioxide (NO_2•). Other than free radicals, there are substances that are not free radicals but able to induce oxidative stress by the generation of free radicals, namely, oxidants. Examples of oxidants are hydrogen peroxide, singlet oxygen, ozone, lipid peroxide, nitrous acid, peroxynitrite and dinitrogen trioxide (Kabel, 2014; Rahal et al., 2014; Shafaq, 2012).

ROS and RNS are constantly generated in our body playing both toxic and beneficial effects. Free radicals are important in the modulation of biological activities (second messengers in signaling cascades), defense mechanism against harmful substance and cell survival (apoptosis and necrosis). Free radicals and their reactive metabolites are selectively inactivated by the antioxidant defense system. Due to their high reactivity, they can possibly alter the structures of the molecules (e.g. DNA damage and lipid peroxidation of membrane) and hence alter the cellular functions. Therefore, balance between free radicals and antioxidants are crucial in cell survival (Kabel, 2014; Rahal et al., 2014; Shafaq, 2012).

Oxidative stress is a condition in which the generation and neutralization of free radicals are imbalanced with increased production of free radicals or decreased antioxidant levels (Baynes, 1991). Oxidative stress can lead to tissue damage and it plays important roles in the pathogenesis of aging and many diseases including cancer, hypertension, atherosclerosis, diabetes mellitus, autoimmune disorders and neurodegenerative diseases (Kabel, 2014). Oxidative stress can impair the glucose uptake in the muscle and fat and reduce the secretion of insulin by pancreatic β cells (Lenzen, 2008). At the same time, oxidative stress predisposes an individual to hypertension and atherosclerosis by affecting endothelial cells directly (Li, Horke, & Förstermann, 2013). In the previous study, it was reported that increased fat accumulation in mice increased oxidative stress due to increased ROS in adipose tissue accompanied by increased expression of NADPH oxidase and decreased expression of antioxidant enzymes. In the same study, increased fatty acids in cultured pre-adipocytes also increased oxidative stress by elevated expression of NADPH oxidase (Furukawa et al., 2004). Oxidative stress can also cause dysregulation of production of adipokines which play important roles in the regulation of lipid metabolism, such as adiponectin, TNF and IL-6.The dysregulation of adipokines was improved after the reduction of ROS in adipose tissue production by treatment with NADPH oxidase inhibitor which also enhanced the condition of diabetes, hyperlipidemia and hepatic streatosis (Furukawa et al., 2004; Guilherme et al., 2008). Emerging evidences have shown that oxidative stress plays an important role in the etiology of obesity, insulin resistance and T2DM, and related complications. Recent studies demonstrated that antioxidant therapies are potential in the improvement of such complications (Huynh, Bernardo, McMullen, & Ritchie, 2014; Kabel, 2014).

2.11 ANTIOXIDANT

An antioxidant is a substance capable of delaying or preventing the oxidation of the substrate, when the substance is present at a low concentration compared to the oxidizable substrate. Free radicals that are produced from the oxidation reactions can initiate chain reactions that damage or/and kill cells. Antioxidant plays important roles in the terminations of these chain reactions via the removal of radical intermediates and inhibition of auto-oxidations (Kabel, 2014; Shafaq, 2012).

Antioxidants can be divided into different categories based on their activity: primary antioxidants (prevention of oxidation), secondary antioxidants (scavenging of free radicals), and tertiary antioxidants (repair of oxidized molecules). The cellular antioxidant defense system includes enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants, produced *in situ*, eliminate free radicals by participating in cellular defense mechanism. Primary enzymatic antioxidants such as superoxide dismutase (SOD), catalase and glutathione peroxidase function mainly to inactivate the free radicals into intermediates. For instance, SOD hydrolyzes superoxide anion into oxygen and hydrogen peroxides whereas catalase decomposes hydrogen peroxides into water and oxygen (Kabel, 2014; Shafaq, 2012).

On the other hand, secondary enzymatic antioxidants such as glutathione reductase, glutathione-S-transferase, glucose-6-phosphate dehydrogenase, and ubiquinone serve to reduce the peroxides in the system and supply NADPH and glutathione to the primary antioxidants to maintain their functions (Shafaq, 2012).

Non-enzymatic antioxidants are mainly obtained from dietary sources. Non-enzymatic antioxidants, generally reducing agents, can be further classified into hydrophilic antioxidants that react with oxidants in the plasma and cytosol (e.g. ascorbic acid, glutathione, uric acid, albumin and bilirubin); and lipophilic antioxidants that

inhibit lipid peroxidation in cell membrane (e.g. carotenes and α-tocopherol) (Kabel, 2014; Shafaq, 2012).

Interestingly, recent studies reported that some flavonoids with antioxidant capacity could behave as prooxidants in the presence of transition metal (copper-initiated), though the *in vivo* effect was less significant (Rahal et al., 2014). These findings suggest that antioxidants may act as prooxidants under certain circumstances and more in depth studies are needed to unravel the underlying mechanism. Numerous studies have shown that bioactive compounds from herbs, vegetables and fruits which are high in phytochemicals such as flavonoids, terpenoids and alkaloids have high antioxidant properties and biological benefits, potentially help in the complications caused by obesity and T2DM due to oxidative stress (Huynh et al., 2014; Shafaq, 2012).

2.12 MONOTERPENES

Monoterpenes are amongst the major constituents of essential oils and abundantly found in citrus fruits, vegetables, spice and herbs. Monoterpenes are a class of terpenes, consisting of two isoprene units in the structures, which can either be in the form of acyclic (linear) or cyclic (ring). Monoterpenes are volatile compounds and characterized by strong odors responsible for the fragrance in many plants and fruits, but they have no nutritional value. Monoterpenes which are well known for their fragrance have been commercially used as artificial flavors in food, sanitary, cosmetic and perfume industries (Bakkali, Averbeck, Averbeck, & Idaomar, 2008). In the past decades, increasing evidence showing biological effects of monoterpenes, such as antioxidant, anti-phlogistic, anti-tumor, antibacterial, antifungal, antiviral, anti-spasmodic, and anti-nociceptive properties have intensified the usage and consumption of monoterpenes (Bakkali, Averbeck, Averbeck, & Idaomar, 2008; Choi, Song, Ukeda, & Sawamura, 2000; Kamatou, Vermaak, Viljoen, & Lawrence, 2013; Santos et al., 2011). Dietary intake of monoterpenes in human is fairly high. For instance, concentration of d-limonene, a fragrance additive with a lemon-like odor in candy, ice cream, orange juice and chewing gum is 49 ppm, 68 ppm, 100 ppm, and 2300 ppm, respectively. The United States per capita dietary consumption of both naturally occurring and artificial d-limonene is 0.27 mg/kg body weight/day. By this large scale consumption of monoterpenes, significant beneficial biological effects could possibly be reflected on human health in the long run. Evidence from the phase I clinical trials had shown that oral consumption of d-limonene in advanced cancer patients helped in the control of breast cancer and colorectal carcinoma which further strengthens the benefits of dietary monoterpenes in human health (Sun, 2007).

There have been numerous studies focused primarily on anti-cancer and anxiolytic properties of monoterpenes but very scarce on their potential in the management of obesity and T2DM. Until recently, there has been arising animal studies show that monoterpenes such as limonene and linalool help to improve obesity- and T2DM-related complications such as control of body weight, enhanced insulin sensitivity, ameliorated plasma glucose level and improved lipid profile (Jing et al., 2013; More, Kulkarni, Nalawade, & Arvindekar, 2014; Santiago, Jayachitra, Shenbagam, & Nalini, 2012). These findings suggest that monoterpenes might hold the potential in the control of obesity and T2DM. Thus, in this current study, the biological effects of twelve monoterpenes (Table 2.5) and their roles on glucose uptake and lipid metabolism were investigated. Twelve of these monoterpenes were selected because they are of the common monoterpenes found in vegetables, fruits, spice, and herbs and their synthetic forms are commercially available.

In addition, since oxidative stress is closely associated with complications caused by obesity and T2DM, the effect of monoterpenes on lipid accumulation in 3T3-L1 adipocytes under oxidative stress was also studied.

Table 2.5: Twelve monoterpenes that were evaluated in this study.

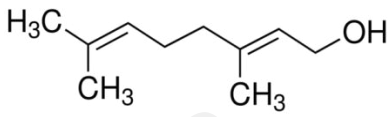
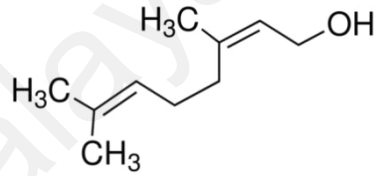
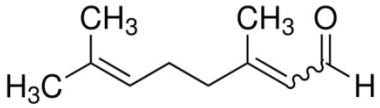
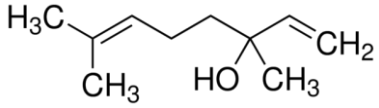
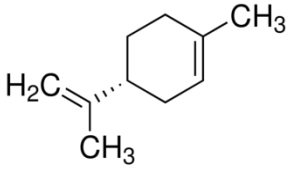
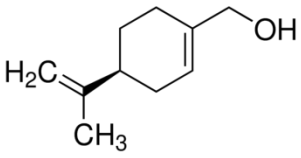
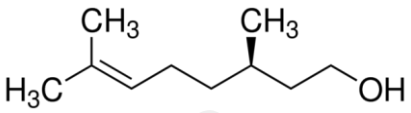
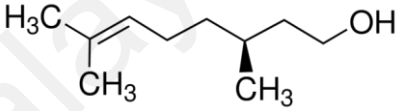
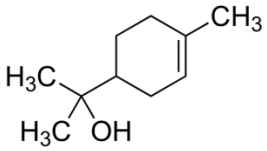
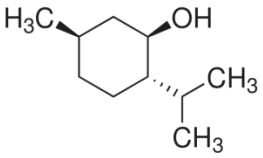
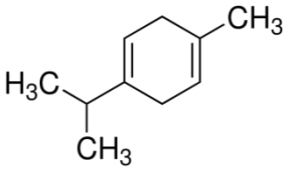
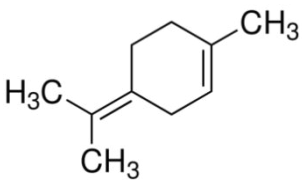
Monoterpenes	Molecular Weight (g/mol)	Molecular Structure
Geraniol (2E)-3,7-dimethylocta-2,6-dien-1-ol	154.25	
Nerol (2Z)-3,7-dimethylocta-2,6-dien-1-ol	154.25	
Citral (2E)-3,7-dimethylocta-2,6-dienal	152.23	
(-)-Linalool (3R)-3,7-dimethylocta-1,6-dien-3-ol	154.25	
(R)-(+)-Limonene (4R)-1-methyl-4-prop-1-en-2-ylcyclohexene	136.23	
(S)-(-)-Perillyl alcohol [(4S)-4-prop-1-en-2-ylcyclohexen-1-yl]methanol	152.23	

Table 2.5, continued: Twelve monoterpenes that were evaluated in this study.

Monoterpenes	Molecular Weight (g/mol)	Molecular Structure
(R)-(+)-β-Citronellol (3R)-3,7-dimethyloct-6-en-1-ol	156.27	
(S)-(-)- β-Citronellol (3S)-3,7-dimethyloct-6-en-1-ol	156.27	
α-Terpineol 2-[(1S)-4-methylcyclohex-3-en-1-yl]propan-2-ol	154.25	
L-Menthol (1R,2S,5R)-5-methyl-2-propan-2-ylcyclohexan-1-ol	156.27	
γ-Terpinene 1-methyl-4-propan-2-ylcyclohexa-1,4-diene	136.23	
Terpinolene 1-methyl-4-propan-2-ylidenecyclohexene	136.23	

CHAPTER THREE:MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 MONOTERPENES AND GLUCOSE OXIDASE

Monoterpenes such as geraniol (PubChem CID: 637566), nerol (PubChem CID: 643820), citral (PubChem CID: 638011), (R)-(-)-linalool (PubChem CID: 443158), (R)-(+)-limonene (PubChem CID: 440917), (S)-(-)-perillyl alcohol (PubChem CID: 369312), (R)-(+)-β-citronellol (PubChem CID: 101977), (S)-(-)-β-citronellol (PubChem CID: 7793), α-terpineol (PubChem CID: 443162), l-menthol (PubChem CID: 16666), γ-terpinene (PubChem CID: 7461) and terpinolene (PubChem CID: 11463) were purchased from Sigma-Aldrich (USA). Glucose oxidase from *Asperfillus niger*, Type II, was also purchased from Sigma-Aldrich (USA).

3.1.2 3T3-L1 CELL LINE

The 3T3-L1 cell line was purchased from American Type Culture Collection (ATCC) (Manassas, USA).

3.1.3 CHEMICALS, ASSAY KITS AND CONSUMABLES

The source of chemicals, assay kits, consumables and instruments used in the current study were listed in Appendix A, B and C.

3.2 METHODS

3.2.1 PREPARATION OF MONOTERPENES AND GLUCOSE OXIDASE

Monoterpenes were respectively solubilized in absolute EtOH and diluted to 1% EtOH using double distilled water (ddH_2O). Monoterpenes were further diluted to the desired concentrations using different solvent or medium containing 1% (v/v) EtOH according to the requirements of each assay. In radical scavenging assays and ferric reducing power assay, due to the solubility issue, the highest concentration of monoterpenes prepared was 100 mM. In enzymatic assays and tissue culture, the final concentration of EtOH in monoterpenes was maintained at 1% (v/v) to prevent impaired enzymatic activities and undesirable cell damage or cytotoxicity. Solvent or medium containing 1% EtOH was used as the negative control in accordance to the requirements of different assays. Monoterpenes were freshly prepared in each assay.

In the current study, glucose oxidase (powder form) from *Aspergillus niger*, Type II, was solubilized in medium comprising Dulbecco's Modified Eagle's Medium (DMEM) with 10% (v/v) fetal bovine serum (FBS). Glucose oxidase was then aliquoted into microfuge tubes and stored at -20 °C until use. Frozen glucose oxidase was thawed at room temperature prior to use. Glucose oxidase solution was diluted to desired concentrations using DMEM/10% (v/v) FBS. DMEM/10% (v/v) FBS was used as the negative control.

3.2.2 RADICAL SCAVENGING EFFECT AND REDUCING POWER OF MONOTERPENES

3.2.2.1 2,2-DIPHENYL-2-PICRYLHYDRAZYL HYDRATE (DPPH) RADICAL SCAVENGING ASSAY

3.2.2.1.1 PRINCIPLE

DPPH radical scavenging activity of monoterpenes was assessed as described by Tan et al. (2015) with slight modifications. In the presence of antioxidants, DPPH radicals are reduced by donating its hydrogen, which is then accompanied by the discolouration of the violet colour of the solution as the number of DPPH radicals decreases. After the reduction of DPPH radicals, pale yellow colour solution is observable due to the picryl group. The colour change in DPPH solution can be measured spectrophotometrically at 517 nm.

3.2.2.1.2 GENERAL PROCEDURE

In the current study, 10 µl of test sample was mixed with 290 µl of 0.1 mM DPPH/100% EtOH and incubated for 3 h in the dark at room temperature prior to measuring the absorbance at 517 nm. Based on the optimisation, concentrations of monoterpenes used in the current assay were determined to be 10 mM and 100 mM as most monoterpenes were shown to have no significant DPPH radical scavenging activity lower concentrations. Ascorbic acid was freshly prepared and used as a positive control. A standard graph of DPPH radical scavenging activity (%) versus ascorbic acid concentration (0 - 30 µM) was plotted (Appendix D). The radical-scavenging activity of test sample was expressed in percentage of inhibition.

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{OD of blank} - \text{OD of test compound}}{\text{OD of blank}} \times 100$$

3.2.2.2 2,2'-AZINOBIS-(3-ETHYLBENZOTHIAZOLINE-6-SULFONIC ACID) (ABTS) RADICAL SCAVENGING ASSAY

3.2.2.2.1 PRINCIPLE

The ABTS radical scavenging activity of monoterpenes was assessed as described by Tan et al. (2015) with slight modifications. This assay is a radical scavenging discoloration assay similar to DPPH radical scavenging assay. However, it can assess both lipophilic and hydrophilic antioxidants (Re *et al.*, 1999). ABTS radicals are greenish blue chromophore produced from the reaction between ABTS and potassium persulfate ($K_2O_8S_2$). In the presence of antioxidants, ABTS radical in the solution will be reduced resulting in the discolourization of ABTS solution, which can be measured spectrometrically at 734 nm.

3.2.2.2.2 GENERAL PROCEDURE

In this assay, ABTS radical monocations were generated by mixing 5 ml of 7 mM ABTS solution with 89 µl of 2.45 mM potassium persulphate. The solution was incubated for 12 to 16 h at room temperature in the dark before use. The working solution was prepared by diluting the stock solution with absolute EtOH to obtain an absorbance of 0.7 ± 0.02 at a wavelength of 734 nm. Following that, 10 µl of the test sample was added to 90 µl of the working solution in a 96-well plate and incubated for 2 h before the absorbance was taken. Based on the optimisation, concentrations of monoterpenes used in the current assay were determined to be 1 mM, 10 mM, and 100 mM as most monoterpenes were shown to have no significant ABTS radical scavenging activity at lower concentrations. Trolox was used as a positive control. A standard graph of ABTS radical scavenging activity (%) versus Trolox concentration (0 - 100 µM) was plotted (Appendix E).

The radical-scavenging activity of the test sample was expressed in percentage of inhibition.

$$\text{ABTS radical scavenging activity (\%)} = \frac{\text{OD of blank} - \text{OD of test compound}}{\text{OD of blank}} \times 100$$

3.2.2.3 FERRIC REDUCING ANTIOXIDANT POWER (FRAP)

3.2.2.3.1 PRINCIPLE

Reducing power of monoterpenes was evaluated as described by Tan et al. (2015) with slight modifications. Reducing power of a compound can be assessed by reducing ferric-2,4,6-tri(2-pyridyl)-1,3,5-triazine (Fe^{3+}-TPTZ) into an intense blue-coloured ferrous-2,4,6-tri(2-pyridyl)-1,3,5-triazine (Fe^{2+}-TPTZ) complex at low pH, which can be measured spectrophotometrically at 593 nm. Compounds having high FRAP value indicate that they have a high reducing power which may serve as a good antioxidant by donating their electrons to eliminate the free radicals.

3.2.2.3.1 GENERAL PROCEDURE

Firstly, a 300 mM acetate buffer (pH 3.6) was prepared by adding 16 ml of glacial acetic acid to 3.1 g of sodium acetate trihydrate and topped up to 1 litre using ddH_2O. After that, FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ)/40 mM hydrochloric acid (HCl) and 20 mM ferric chloride ($FeCl_3 \cdot 6H_2O$) in a ratio of 10:1:1. The resulting orange-coloured FRAP reagent was incubated at 37 °C before use.

To evaluate the reducing power of the test sample, 10 µl of test sample was added to 290 µl of FRAP reagent for 3 hours. The absorbance of the mixture was measured at 593 nm. Ferrous sulphate ($FeSO_4 \cdot 7H_2O$) was used as a positive control. A standard graph of absorbance versus ferrous sulphate concentration (0 - 100 µM) was plotted (Appendix F). The results were expressed as FRAP value (mol $FeSO_4 \cdot 7H_2O$/mol test compound) based on the standard graph established by ferrous sulphate.

3.2.3 α-GLUCOSIDASE AND α-AMYLASE INHIBITORY EFFECT OF MONOTERPENES

3.2.3.1 α-GLUCOSIDASE INHIBITORY ACTIVITY

α-Glucosidase inhibitory activity of monoterpenes was evaluated based on the modified method by Manaharan et al. (2011). Firstly, 0.1 M sodium phosphate buffer pH 6.8 was prepared by mixing 490 ml of 0.1 M disodium phosphate (Na_2HPO_4) to 510 ml of 0.1 M monosodium phosphate (NaH_2PO_4). After that, α-glucosidase solution was prepared by dissolving α-glucosidase from *S. cerevisiae* in 0.1 M sodium phosphate buffer pH 6.8 supplemented with 0.2% bovine serum albumin (BSA) to give a concentration of 0.4 U/ml. Then, equal volumes (20 µl) of test compound, 0.4 U/ml α-glucosidase solution and 6 mM 4-nitrophenyl-α-D-glucopyranoside (PNPG) were mixed and allowed to stand at 37 °C for 15 min. To terminate the reaction, 80 µl of 0.2 M sodium carbonate was added to the reaction mixture and the absorbance was measured at 400 nm. Acarbose was used as a positive control. A standard graph of α-glucosidase inhibitory activity (%) versus acarbose concentration (log µM) was plotted (Appendix G). The α-glucosidase inhibitory activity of monoterpenes was expressed in percentage of inhibition.

$$\text{α-Glucosidase inhibitory activity (\%)} = \frac{\text{OD of control} - \text{OD of test compound}}{\text{OD of control}} \times 100$$

3.2.3.2 α-AMYLASE INHIBITORY ACTIVITY

α-Amylase inhibition assay was conducted according to previously described method with slight modifications (Manaharan et al., 2011). At first, 0.1 M sodium phosphate buffer pH 6.9 was prepared by mixing 550 ml of 0.1 M disodium phosphate (Na_2HPO_4) to 450 ml of 0.1 M monosodium phosphate (NaH_2PO_4). α-Amylase solution (2 U/ml) was prepared by dissolving porcine pancreatic α-amylase (Sigma Type IV-B) in ice-cold distilled water. Next, potato soluble starch solution (1%) was prepared by boiling starch in 20 mM phosphate buffer pH 6.9 until the solution became fully transparent. Then, 80 µl of the test sample was added to 40 µl of α-amylase solution. After 10 min incubation at room temperature, 40 µl of starch solution was added to the reaction mixture and allowed to stand at 37 °C for 10 min. To terminate the reaction, 80 µl of DNS solution consisting of 1 g 3,5-dinitrosalicylic acid (DNS) and 30 g sodium potassium tartrate dehydrate (dissolved in 100 ml of 2 M sodium hydroxide) was added and incubated at 95 °C for 10 min. The absorbance was measured at 540 nm. Acarbose was used as a positive control. A standard graph of α-amylase inhibitory activity (%) versus acarbose concentration (log µM) was plotted (Appendix H).The α-amylase inhibitory activity of monoterpenes was expressed in percentage of inhibition.

$$\text{α-Amylase inhibitory activity (\%)} = \frac{\text{OD of control} - \text{OD of test compound}}{\text{OD of control}} \times 100$$

3.2.4 CELL CULTURE

3.2.4.1 ASEPTIC TECHNIQUES

Cell culture was conducted in a Class II biohazard safety cabinet. The working area inside the cabinet was disinfected under UV lamp for 20 minutes before use. All items were sterilized with 70 % (v/v) alcohol before transferring into the cabinet. Material used for cell culture such as pipette tips, microfuge tubes, PCR tubes, 15 ml tubes and 50 ml tubes were autoclaved and dried in an oven (60 ºC) before use.

3.2.4.2 3T3-L1 PRE-ADICPOCYTE PROLIFERATION AND DIFFERENTIATION

Murine 3T3-L1 pre-adipocytes were grown and induced to differentiate into mature adipocytes as described by Kanagasabapathy, Chua, Malek, Vikineswary, & Kuppusamy (2014) with slight modifications. 3T3-L1 pre-adipocytes were grown in a growth medium comprising Dulbecco's Modified Eagle's Medium (DMEM) with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin (10,000 U/ml penicillin and 10,000 µg/ml streptomycin) and 4 mM L-glutamine at 37 ºC in 95% air and 5% CO_2. The medium was replaced at an interval of 2 days to supply the cells with sufficient nutrients and to remove the metabolic by-products. Upon the replacement of a new growth medium, the adhering cells were rinsed twice with phosphate buffered saline (PBS) after the spent medium was discarded. The 3T3-L1 pre-adipocytes (approximately 70% to 80% confluent) were subjected to subculturing before they reach the state of confluence (section 3.2.4.3).

To induce adipocyte differentiation, 3T3-L1 pre-adipocytes were grown to 100% confluent and 2-day post-confluent 3T3-L1 pre-adipocytes (day 0) were treated with a growth medium supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 µM dexamethasone (DEX) and 5 µg/ml insulin. On day 2, the medium was replaced

with a growth medium supplemented with 5 µg/ml insulin solely. On day 4, this medium was then replaced and maintained with the initial growth medium used for cell growth. This growth medium was discarded and replenished at a 2-day interval thereafter until analysis. Differentiating adipocytes were more delicate compared to pre-adipocytes. Hence, extra care was needed upon the changing of the medium to prevent the detachment of the cell layers. All culture media used in the cell culture were brought to 37 ºC in a water bath before adding to the cells. Morphologies of 3T3-L1 adipocytes when cells were pre-confluent, confluent, differentiating and maturing were shown in Appendix I.

At indicated time intervals, 1 µM monoterpenes and various concentrations of glucose oxidase were introduced to the system to study their effects on the cell response. The studies of oxidative stress on 3T3-L1 adipocytes were made possible by introducing glucose oxidase to the system as it serves to generate hydrogen peroxide (H_2O_2) in a constant manner (Rudich et al., 1998).

Table 3.1: Cell Number and Volume of Culture Medium

Process / Assay	Culture platform	Cell number	Medium amount
3T3-L1 pre-adipocyte growth	25 cm^2 flask	75,000 cells/flask	5 ml
3T3-L1 pre-adipocyte growth	75 cm^2 flask	250,000 cells/flask	15 ml
MTT assay	96-well plate	1,000 cells/well	200 µl
ORO quantitative assay	24-well plate	25,000 cells/well	1 ml
Total glucose uptake assay	96-well plate	10,000 cells/well	200 µl
Lipolysis assay	96-well plate	10,000 cells/well	200 µl
Gene expression study	25 cm^2 flask	200,000 cells/flask	5 ml

3.2.4.3 SUBCULTURING

The 3T3-L1 pre-adipocytes were subcultured when the cells achieved 70% to 80% confluence, or reached a density of 5 to 6 x 10^4 cells/cm^2 of cultured flask. Upon the subculturing of the cells, the culture medium was removed and the cells were rinsed with PBS twice to remove the residues of serum that contained trypsin inhibitors. Then, the adhering cells in culture flask were subjected to 3 min to 5 min incubation with 2 ml to 3 ml of 0.25% trypsin-EDTA which was sufficient to cover the cell layer. During this process, cells were incubated at 37 °C in an incubator to facilitate the detachment process. Trypsin-EDTA was used to liberate the cells by dissolving fibronectin on the cell surface. Prolonged trypsinization and shaking of the flasks during the process of trypsinization were avoided as to prevent cell damage and cell clumping respectively. To inactivate the process of trypsinization, growth medium with serum that contained trypsin inhibitor was added. The cell suspension was then centrifuged at 100 x *g* unit for 10 min. The supernatant was removed and the pellet was gently resuspended in 1 ml of growth medium. The cells were further subcultured into culture flasks at the density of 2 to 3 x 10^3 cells/cm^2 (see Table 3.1 and section 3.2.4.4).

3.2.4.4 CELL COUNTING

After the cells were trypsinized and resuspended as described in section 3.2.4.3, 10 µl of the cell suspension was mixed with 10 µl of trypan blue. Then, 10 µl of the mixture was dispensed on a Neubour improved haemocytometer and viewed under an inverted microscope. Cell number was estimated by counting the viable cells in the 4 large squares with each having a volume of 0.1 mm^3, which is equivalent to 10^{-4} ml (Appendix J & K). Under the staining of trypan blue, viable cells will show a clear cytoplasm whereas nonviable cells will exhibit a blue cytoplasm (Appendix L). If the cells appeared to be clumping together under the microscope, the cell suspension was further diluted using growth medium and calculation was carried out based on the dilution factor. The number of viable cells in the suspension was calculated based on the formula shown below.

Cell number in 1 ml of suspension

$$= \frac{\text{mean of viable cells}}{10^{-4}\ \text{ml}} \times \text{dilution factor}$$

$$= \frac{(\text{total number of viable cells counted in 4 large squares}/4)}{10^{-4}\ \text{ml}} \times \text{dilution factor}$$

$$= \text{mean of viable cells} \times 10^4\ \text{ml} \times 2^*$$

*dilution factor was equivalent to 2 when 10 µl of the cell suspension was mixed with 10 µl of trypan blue

3.2.4.5 CRYO-PRESERVATION AND CRYO-RESUSCITATION

To store the cells for future use, sterile 3T3-L1 pre-adipocytes were preserved at an extreme low temperature by a process called cryo-preservation. In this process, harvested cells (as described in section 3.2.4.4) were resuspended in a growth medium supplemented with 10% dimethyl sulfoxide (DMSO) and transferred into a cryo-vial which was then kept in the liquid nitrogen for long term storage.

The cryo-preserved cells can be revived by a process called cryo-resuscitation. In order to obtain good cell viability, cryo-resuscitation has to be conducted rapidly. The frozen cell mixture in the cryo-vial was thawed at 37 °C in a water bath for 1 min. After the frozen cells had thawed, 10 ml of growth medium was added slowly to the cell suspension and mixed gently. After that, the mixture was subjected to centrifugation at 100 x *g* unit for 5 min. The supernatant was discarded and the pellet was resuspended in the growth medium and transferred into a cell culture flask. Cells were incubated at 37 ºC in 95% air and 5% CO_2. On the following day, the viable cells would adhere to the culture flask whereas the dead cells would remain floating in the solution. The growth medium was thus discarded and replenished with a new growth medium to remove the dead cells. The cells were gently rinsed with PBS prior to the replacement of the new growth medium. The medium was changed at a 2-day interval thereafter until analysis (as described in section 3.2.4.2).

3.2.5 CELL VIABILITY ASSAY

3.2.5.1 PRINCIPLE

Effect of monoterpenes on cell viability was evaluated using in vitro 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Kanagasabapathy et al. (2014) with slight modifications. MTT, a yellow-coloured compound with tetrazolium ring structure, will be reduced by active mitochondrial dehydrogenase in viable cells and break the tetrazolium ring forming insoluble purple formazan crystals (Appendix M).

3.2.5.2 PREPARATION OF MTT WORKING SOLUTION

MTT working solution was prepared by dissolving 5 mg/ml of MTT in PBS which was then filtered using a sterile filter (pore size: 0.2 µm). In the absence of contamination, MTT reagent is stable for 18 months at 4 ºC in the dark.

3.2.5.3 GENERAL PROCEDURE

Pre-confluent 3T3-L1 pre-adipocytes were seeded and cultured in 96-well plates at a density of 1000 cells/well (section 3.2.4.2). The cells were then incubated for 24 h at 37 ºC in 95% air and 5% CO_2 to allow the cells to adhere to the plates prior to the 48-hour treatment with monoterpenes (1 µM) and glucose oxidase (0.1 nU/ml – 10 µU/ml). Concentrations of monoterpenes and glucose oxidase used in the current assay were based on the assay optimisation. Higher concentrations of some monoterpenes and glucose oxidase showed strong inhibitory effect on cell growth whereas lower concentrations of most monoterpenes and glucose oxidase exerted no significant effect in some of the assays.

Following 48-hour incubation with monoterpenes and glucose oxidase, 0.5 mg/mL MTT was added to the cells and incubated in the dark for 4 hours at 37 ºC. The medium was aspirated and the purple formazan crystals were solubilized in DMSO. The absorbance of the solution was measured at 550 nm using a microplate reader.

$$\text{Cell viability (\% of control)} = \frac{\text{OD of treated cells}}{\text{OD of negative control}} \times 100$$

3.2.6 OIL RED O QUANTITATIVE ASSAY

3.2.6.1 PRINCIPLE

When the adipocytes were mature with large lipid droplets the lipid content in the cells was quantified using Oil Red O quantitative assay as described by Kanagasabapathy et al. (2014). Oil Red O is a dye that stains the lipid red. The amount of Oil Red O stained in the cells is directly proportional to the total amount of lipid accumulated in the cells. The stained lipid can be visualized microscopically (qualitative measurement) (Appendix N) or measured spectrophotometrically (quantitative measurement) at 510 nm. In the current study, quantification of lipid was carried out to assess the effect of monoterpenes and glucose oxidase on cell differentiation and lipogenic activity of the cells.

3.2.6.2 PREPARATION OF OIL RED O WORKING SOLUTION

Oil Red O stock solution was prepared by dissolving 0.5 g Oil Red O powder in 100 ml of 100% (v/v) isopropanol and was left overnight in the dark at room temperature. The solution was filtered using a filter paper (0.1 mm) to remove the undissolved particles. Next, four part of water was added to six part of the filtered Oil Red O stock solution forming the Oil Red O working solution. The supernatant was further filtered using a syringe filter (0.2 µm) to ensure the Oil Red O working solution was completely free of precipitate. This Oil Red O working solution was then ready to be used to stain the lipid droplets of adipocytes. However, the Oil Red O working solution was only stable approximately for 2 h in the dark.

3.2.6.3 GENERAL PROCEDURE

3T3-L1 adipocytes were cultured and differentiated as described in section 3.2.4.2. Based on the cell viability screening results, various concentrations of glucose oxidase (0.1 nU/ml – 1000 nU/ml) and monoterpenes (1 µM) were added to the culture medium and incubated for 48 h at three distinct time points in three separate experiments (Table 3.2).

Table 3.2: Time points at which monoterpenes and glucose oxidase were introduced to the cell culture to study their effects on total lipid accumulation.

Time point	Abbreviation	Incubation period	Negative control	Treatment
1. Two days prior to induction of differentiation	2DPD	48 h	Growth medium + Solvent	Growth medium + Monoterpenes
				Growth medium + Glucose oxidase
				Growth medium + Monoterpenes + Glucose oxidase
2. The day induction of differentiation was initiated	day 0	48 h	Differentiation cocktail + Solvent	Differentiation cocktail + Monoterpenes
				Differentiation cocktail + Glucose oxidase
				Differentiation cocktail + Monoterpenes + Glucose oxidase
3. Two days after induction of differentiation	day 2	48 h	Growth medium + Solvent	Growth medium + Insulin (Positive control)
				Growth medium + Monoterpenes
				Growth medium + Glucose oxidase
				Growth medium + Monoterpenes + Glucose oxidase

The adipocyte cultures which were ready for analysis were rinsed with PBS twice followed by 10% formalin fixation for 1 h at room temperature. Then, the cells

were rinsed twice with PBS to remove the formalin residue. To prevent the formation of precipitates when Oil Red O solution was added to the cells (Section 3.2.6.2), the cells were rinsed with 60% isopropanol and allowed to air dry. Complete absence of water was crucial. Subsequently, fixed cells were incubated in the dark with Oil Red O working solution for 1 h. After staining, used staining solution was discarded and the cells were rinsed with water several times (4 times) to remove the residue of Oil Red O working solution. The stained cells were allowed to air dry. Next, 1 ml of isopropanol (100% v/v) was added to each well of the 24-well plates that contained the stained cells to solubilize the Oil Red O dye in the lipid droplets. The plates were then covered and mixed at 200 rpm using a mixer for 5 min to ensure that the dye dissolved completely in the isopropanol. Isopropanol is volatile and evaporates rapidly. Evaporation of isopropanol would lead to the differences of absorbance and result in inaccuracy of the results. Thus, right after 5 min, 200 µl of the stained solution in each well was immediately transferred into a 96-well plate and the absorbance was measured spectrophotometrically at 510 nm. The total lipid accumulation by the cells was expressed in percentage of difference to the negative control.

$$\text{Total lipid accumulation (\% of difference)} = \frac{\text{OD of treated cells} - \text{OD of negative control}}{\text{OD of negative control}} \times 100$$

3.2.7 TOTAL GLUCOSE UPTAKE ASSAY

Total glucose uptake by the mature adipocytes was measured according to the method described by Cheng, Huang, Chang, Tsai, and Chou (2008) with slight modifications. 3T3-L1 pre-adipocytes were seeded in 96-well plates at a density of 20,000 cells/well and induced to differentiate into mature adipocytes (section 3.2.4.2). Mature 3T3-L1 adipocytes with lipid droplets were starved in serum-free DMEM for 2 h. After that, the medium was replaced with Eagle's Minimum Essential Medium (MEM). At the same time, various monoterpenes (1 µM) were added to the medium respectively and incubated for 5 h. Insulin (5 µg/ml) was used as a positive control. Incubations in this experiment were all conducted at 37 °C in 95 % air and 5 % CO_2. After 5-hour glucose uptake by adipocytes, remaining glucose in the medium was measured by adding 5 µl of the medium to 500 µL of Glucose GOD kit (DiaSys Diagnostic Systems GmbH, Germany) in a 24-well plate and incubated at room temperature for 20 min prior to the absorbance reading at 500 nm. A standard graph of absorbance was plotted against glucose solution concentration (0 – 100 µM) (Appendix O). The glucose uptake by the cells was expressed in percentage of difference.

$$\text{Glucose uptake (\% of difference)} = \frac{\text{OD of negative control} - \text{OD of treated cells}}{\text{OD of negative control}} \times 100$$

3.2.8 LIPOLYSIS ASSAY

The lipolytic effect of monoterpenes on 3T3-L1 adipocytes were quantified as described by Murosaki et al. (2007) with modifications. 3T3-L1 pre-adipocytes were seeded into 96-well plates at the density of 20,000 cells/well and induced to differentiate into mature adipocytes with lipid droplets (section 3.2.4.2). Mature adipocytes were starved in serum-free DMEM for 2 h before the medium was replaced with MEM. Next, various monoterpenes (1 µM) were added to the medium and incubated for another 5 h. Epinephrine (1 µM) was used as a positive control. Incubations in this experiment were carried out at 37 °C in 95 % air and 5 % CO_2. Subsequently, 20 µl of each sample medium were respectively transferred to a 96-well plate. Free glycerol released into the medium was then assayed using EnzyChrom™ Adipolysis Assay Kit (BioAssay Systems, USA) following the manufacturer's protocol.

The working solution for enzyme reaction contained 100 µl Assay Buffer, 2 µl Enzyme MIX, 1 µl ATP and 1 µl Dye Reagent. The working solution (100 µl) and 20 µl sample were transferred into each assay, mixed and incubated for 20 min at room temperature. Optical density was read at 570 nm. A standard graph of absorbance versus glycerol concentration (0 – 100 µM) was plotted (Appendix P). The free glycerol released by the cells was expressed in percentage of difference.

$$\text{Glycerol released by adipocytes (\% of difference)} = \frac{\text{OD of treated cells} - \text{OD of negative control}}{\text{OD of negative control}} \times 100$$

3.2.9 GENE EXPRESSION USING QUANTITATIVE REAL TIME PCR

3.2.9.1 RNA EXTRACTION

Based on the results from Oil Red O quantitative assay, total glucose uptake assay and lipolysis assay, (R)-(+)-limonene (1 µM) was selected to proceed with the gene expression study. Effect of glucose oxidase (100 nU/mL) on gene expression of 3T3-L1 adipocytes was investigated as well.

To study the effect of the (R)-(+)-limonene (1 µM) and glucose oxidase (1 µU/ml) on mRNA expression of genes pertinent to the properties mentioned above, 3T3-L1 pre-adipocytes were cultured and induced to differentiate as mentioned in section 3.2.4.2. Cells that were treated on 2DPD and day 0 were harvested on day 2 whereas cells that were treated on day 2 were harvested on day 4. Gene expression studies conducted on day 2 and day 4 were to investigate the regulation of different sets of genes involved in the differentiation of adipocytes and lipid metabolism, respectively. The total RNA from the adipocytes was isolated using an Ambion-RNAqueous Micro® kit from Applied Biosystems.

When the cells were ready for analysis, the spent medium was discarded and the cells were carefully rinsed twice with 5 ml of PBS. Next, 1 ml of PBS was dispensed into the flask where the cells were cultured and the cells were scrapped gently using a sterile cell scrapper. The cell suspension was then transferred into a sterile microfuge tube (1 ml) and centrifuged at 5000 x *g* unit. The supernatant was discarded and 350 µl of Lysis/Binding solution was added to the pellet. The cell mixture was then vortex-mixed until the cells were evenly distributed in the solution before equal volume of 64% (v/v) EtOH was added and mixed gently. After that, the lysate/ethanol mixture was transferred into a Filter Cartridge and centrifuged at 16000 x *g* unit for 60 sec. The flow-through was discarded and 700 µl of Wash Solution #1 was dispensed into the Filter Cartridge and centrifuged at 16000 x *g* unit for 60 sec. The flow-through was

discarded. The Filter Cartridge was then washed with 500 µl of Wash Solution #2/3 and centrifuged at 16000 x *g* unit for 60 sec. The flow-through was discarded and the washing with Wash Solution #2/3 was repeated. After the flow-through was discarded, the Filter Cartridge was centrifuged at 16000 x *g* unit for additional 60 sec to ensure all the solution was completely flown through to avoid downstream contamination. After the last centrifugation, the Filter Cartridge was left there for 30 sec. Subsequently, RNA was eluted by adding 40 µl of preheated Elution Solution (60 °C) to the cartridge and centrifuged at 16000 x *g* unit for 60 sec. To ensure higher yield of RNA, another 10 µl of Elution Solution was added to the cartridge and centrifuged at 16000 x *g* unit for another 60 sec. Isolated RNA was stored immediately at -80 °C until use.

3.2.9.2 RNA INTEGRITY CHECK

Isolated RNA was subjected to purity and integrity check using RNA 6000 Nano Assay according to manufacturer's protocol. Firstly, the chip priming station and working reagents were set up (Appendix Q). After that, 9 µl of gel-dye mix was pipetted into each of the indicated well and 5 µl of the Agilent RNA 6000 Nano marker was pipetted into the well marked with the ladder symbol and the other 12 sample wells. Unused wells were filled with 5 µl of the Agilent RNA 6000 Nano marker and 1 µl of the buffer which was used to dilute the sample.

Aliquots of ladder that was stored at -80 °C was heat denatured for 2 min at 70 °C before 1 µl of the ladder was loaded into the well marked with the ladder symbol on the chip. Following that, 1 µl of sample was loaded into each sample well. Samples were stored in ice prior to the loading to prevent RNA denaturation. The chip was then vortex-mixed for 60 sec at 2,400 rpm using a designated vortex mixer (MS2-S8/MS2-S9). After the vortex, the chip was placed in the Agilent 2100 Bioanalyzer and analysed using 2100 Expert Software.

3.2.9.3 cDNA SYNTHESIS

In the current study, a High Capacity cDNA Reverse Transcription Kit was used to synthesize single stranded complementary DNA (cDNA) from purified RNA (RNA Integrity number [RIN] values 8-10) (Appendix R). Each 20 µl reaction contained 10 µl of purified RNA (1 µg) and 10 µl of Reverse Transcription master mix. The 10 µl master mix was made up of 2 µl of 10X Reverse Transcription Buffer, 0.8 µl of 25X dNTP Mix (100 mM), 2 µl of 10x Reverse Transcription Random Primers, 1 µl of Multiscribe^{TM} Reverse Transcriptase and 4.2 µl of Nuclease-free H_2O. The mixing was conducted on ice.

After Reverse Transcription master mix was mixed with RNA in PCR tubes, they were then loaded into a PCR thermal cycler. Condition for reverse transcription was set as stated in Table 3.3. Synthesized cDNA was stored at -80 °C until use.

Table 3.3: Temperature and time required for high capacity cDNA reverse transcription.

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time (min)	10	120	5	∞

3.2.9.4 QUANTITATIVE REAL TIME PCR

Quantification of gene expression was conducted using an Applied Biosystmes (ABI) 7500 Real-Time PCR system. For each 20 µl reaction, 10 µl of 2X TaqMan® Fast Advanced Master Mix, 1 µl of TaqMan® Gene Expression Assay (20X) and 7 µl of nuclease-free water were added to 2 µl of cDNA template (10 ng). The reaction mixtures were incubated in a 96-well optical reaction plate and covered with an optical film. The plate was briefly centrifuged to settle down the contents and eliminate air bubbles. The PCR reaction plate was run using the ABI 7500 Real-Time PCR System Fast mode. The thermal cycling condition was shown in Table 3.4. The genes investigated in this study were depicted in Table 3.5. Gene expression was normalized using β-actin as a reference gene. Relative mRNA expression was calculated based on the comparative CT method, which is also known as $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Table 3.4: Thermal cycling condition.

Applied Biosystems Real-Time PCR System	Thermal-cycling profile				
	Parameter	UNG Incubation	Polymerase activation	PCR (40 cycles)	
		Hold	Hold	Denature	Anneal/extend
	Temperature (°C)	50	95	95	60
7500 Fast System	Time (min:sec)	02:00	00:20	00:03	00:30

Table 3.5: Selected genes used in the current gene expression study.

No	Gene	Abbreviation	Assay ID	Accession Number
1	Peroxisome proliferator activated-receptor-gamma	PPAR-γ	Mm 01184322_m1	NM_011146.3
2	Glucose transporter-1	GLUT1	Mm 00441480_m1	NM_011400.3
3	Glucose transporter-4	GLUT4	Mm 00436615_m1	NM_009204.2
4	Hormone sensitive lipase	HSL	Mm 00495359_m1	NM_001039507.2 NM_010719.5
5	Adipose triglyceride lipase	ATGL	Mm 00503040_m1	NM_025802.3 NM_001163689.1
6	Glutathione peroxidase	GPx	Mm 00492427_m1	NM_008161.3
7	Beta-actin	β-actin	Mm 00607939_s1	Mm_328431

Assay ID and Accession Number of the genes were obtained from Applied Biosystems and NCBI database. Assay ID refers to theTaqMan® Gene Expression Assay (20X) containing proprietary primer and TaqMan® probe mix. All TaqMan® Gene Expression Assays used in the current study were FAM/MGB probed.

3.2.10 STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism version 5 and Microsoft Excel 2010. Each experiment was conducted in triplicates. Data shown are expressed in mean ± SEM unless indicated otherwise. The significance of differences between means at a confidence limit of 95 %, set at $P < 0.05$, was assessed using one-way analysis of variance (ANOVA), followed by Tukey–Kramer test for multiple variable comparisons.

CHAPTER FOUR: RESULTS

4.1 DPPH RADICAL SCAVENGING ACTIVITY OF MONOTERPENES

At the concentration of 100 mM, γ-terpinene exhibited the strongest DPPH radical scavenging activity (83.0%) among the tested monoterpenes, followed by citral (77.9%) and terpinolene (75.3%). (R)-(+)-β-Citronellol exerted a weaker DPPH radical scavenging activity (44.0%). On the other hand, the radical scavenging effect of (R)-(+)-limonene, (R)-(-)-linalool, (S)-(-)-β-citronellol, nerol, geraniol, (S)-(-)-perillyl alcohol and α-terpineol were shown to be weak, ranging from 5.3% to 18.4%. L-Menthol was shown to have no significant DPPH radical scavenging activity. Conversely, in comparison to the positive control, ascorbic acid scavenged 77.6% of DPPH radicals at 25 μM, which was significantly more potent than monoterpenes (Fig. 4.1).

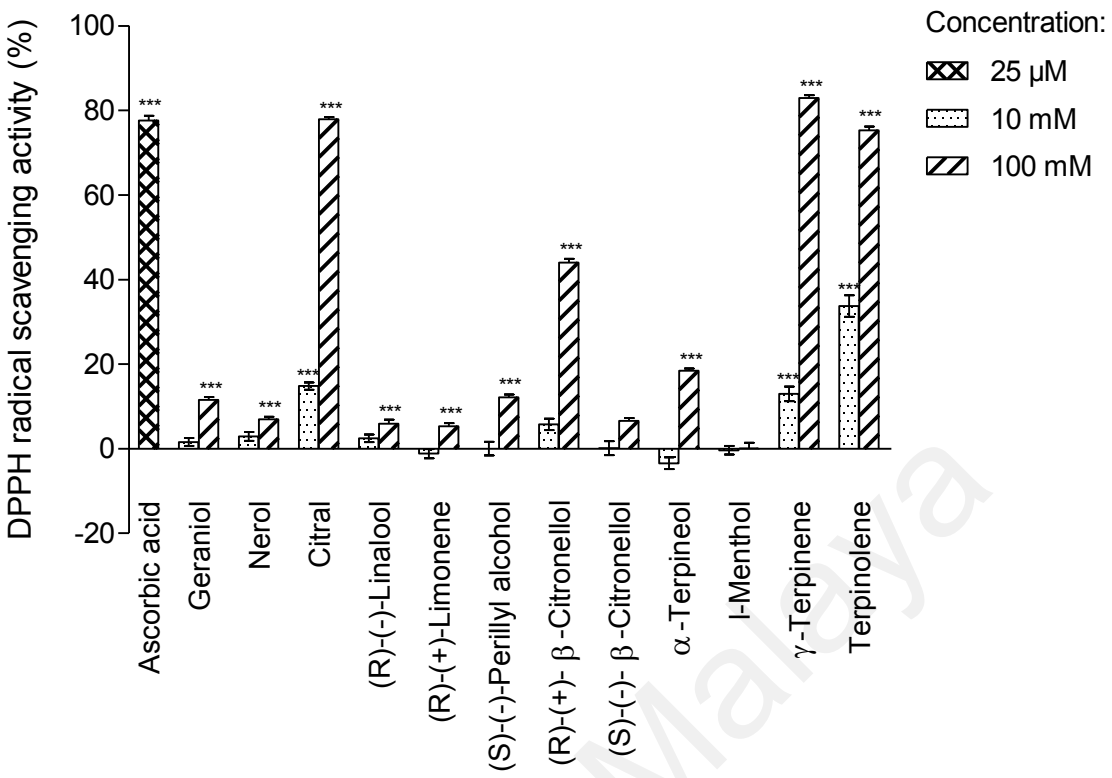


Figure 4.1: Scavenging effects of monoterpenes on the DPPH radicals. Results expressed are means ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to negative controls. *n* = 3 for each group.

4.2 ABTS RADICAL SCAVENGING ACTIVITY OF MONOTERPENES

Similar to DPPH radical scavenging activity, γ-terpinene (93.7%), terpinolene (89.5%) and citral (45.7%) exhibited the strongest ABTS radical scavenging activity at a concentration of 100 mM. Both terpinolene (59.5%) and γ-terpinene (32.7%) showed ABTS radical scavenging capacity at a lower concentration (1 mM) compared to DPPH radical scavenging (10 mM). ABTS radical scavenging activity of tested monoterpenes at 100 mM were followed by (S)-(-)-perillyl alcohol (34.9%), (R)-(+)-β-citronellol (16.8%), α-terpineol (11.2%) and (R)-(+)-limonene (10.4%). Geraniol, l-menthol, nerol, (R)-(-)-linalool and (S)-(-)-β-citronellol did not exert any significant ABTS radical scavenging activity (Fig. 4.2).

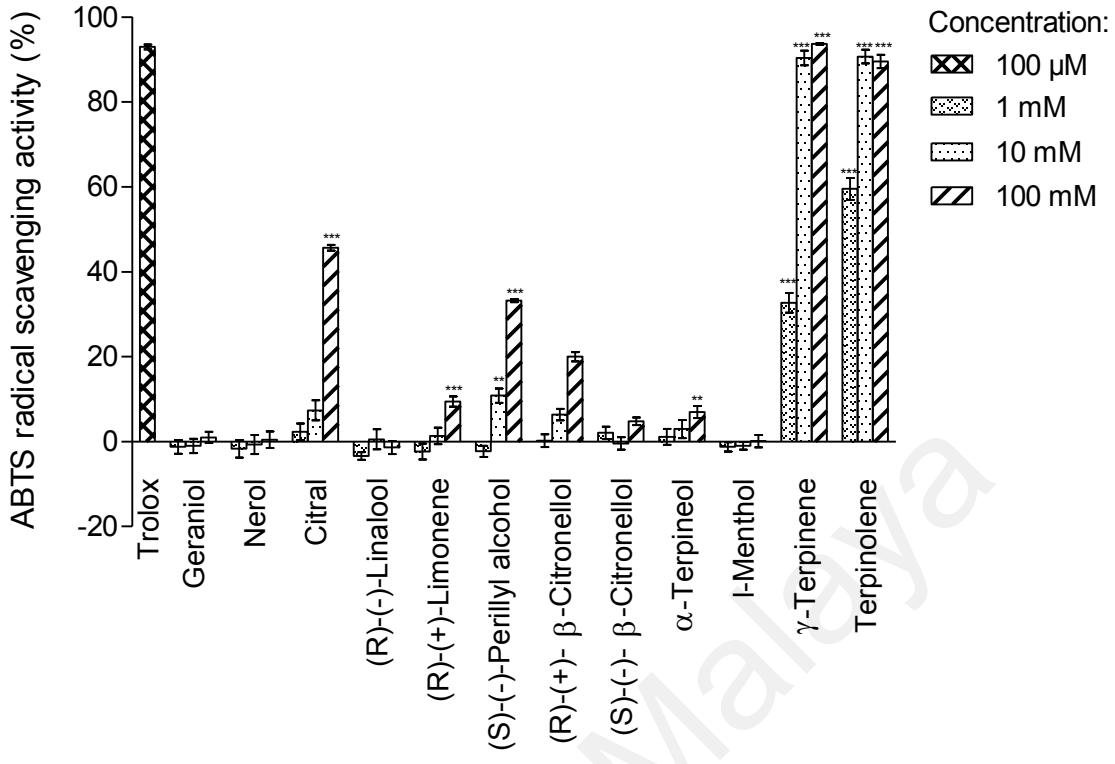


Figure 4.2: Scavenging effects of monoterpenes on the ABTS radicals. Results expressed are means ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to negative controls. *n* = 3 for each group.

4.3 FERRIC REDUCING POWER OF MONOTERPENES

In the ferric reducing assay, monoterpenes did not show any significant reducing power (Table 4.1).

Table 4.1: Reducing power of monoterpenes. Results expressed are means ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to negative controls. *n* = 3 for each group. FRAP value of ascorbic acid was adapted from a previous study by Wong, Cheung, Lau, Bolanos de la Torre, & Owusu-Apenten (2015).

Compound	**FRAP Value (mmol Fe 2^+ / mol sample)**
Ascorbic acid	1800 ± 108*
Geraniol	0.2 ± 0.0
Nerol	0.4 ± 0.1
Citral	0.8 ± 0.0
(R)-(-)-Linalool	0.3 ± 0.1
(R)-(+)-Limonene	0.1 ± 0.0
(S)-(-)-Perillyl alcohol	0.5 ± 0.1
(R)-(+)-β-Citronellol	0.1 ± 0.0
(S)-(-)- β-Citronellol	0.1 ± 0.0
α-Terpineol	0.1 ± 0.0
l-Menthol	0.1 ± 0.0
γ-Terpinene	0.7 ± 0.0
Terpinolene	6.3 ± 0.4

4.4 α-AMYLASE INHIBITORY EFFECT OF MONOTERPENES

Citral was the only monoterpene that showed α-amylase inhibitory effect of 45.7%, at a concentration of 10 mM as compared to the positive control, acarbose which inhibited 48.2% of α-amylase activity at a concentration of 10 µM, suggesting that citral was a weak α-glucosidase inhibitor (Fig. 4.3).

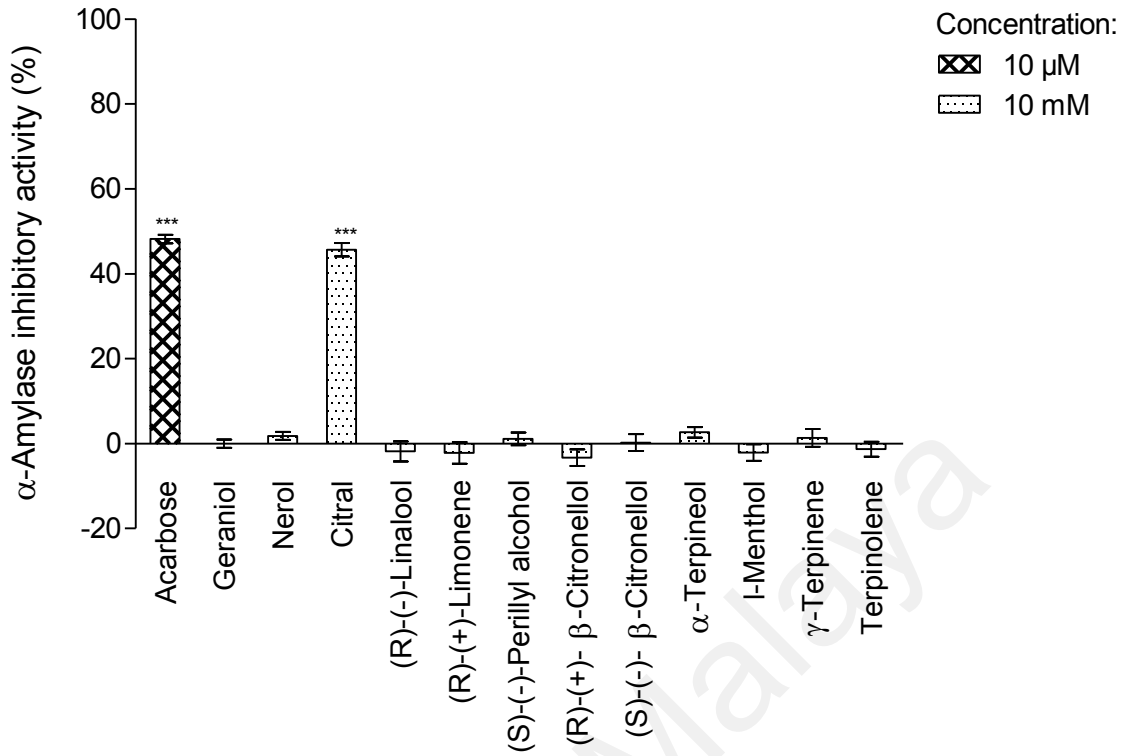


Figure 4.3: α-Amylase inhibitory activity of monoterpenes. Results expressed are means ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to negative controls. *n* = 3 for each group.

4.5 α-GLUCOSIDASE INHIBITORY EFFECT OF MONOTERPENES

(R)-(+)-Limonene and (S)-(-)-perillyl alcohol at 10 mM exhibited the strongest inhibitory effect on α-glucosidase activity, 21.3% and 21.1%, respectively, and this was followed by α-terpineol, 14.0%. (R)-(+)-β-Citronellol, terpinolene, citral, (R)-(-)-linalool, nerol, geraniol and (S)-(-)-β-citronellol exerted relatively weaker α-glucosidase inhibitory effect, ranging from 4.2-8.1%. L-Menthol and γ-terpinene did not show any significant α-glucosidase inhibitory activity. In comparison, the positive control, acarbose exerted α-glucosidase inhibitory effect with a much greater potency, which was 49.6% at a concentration of 5 μM (Figure 4.4).

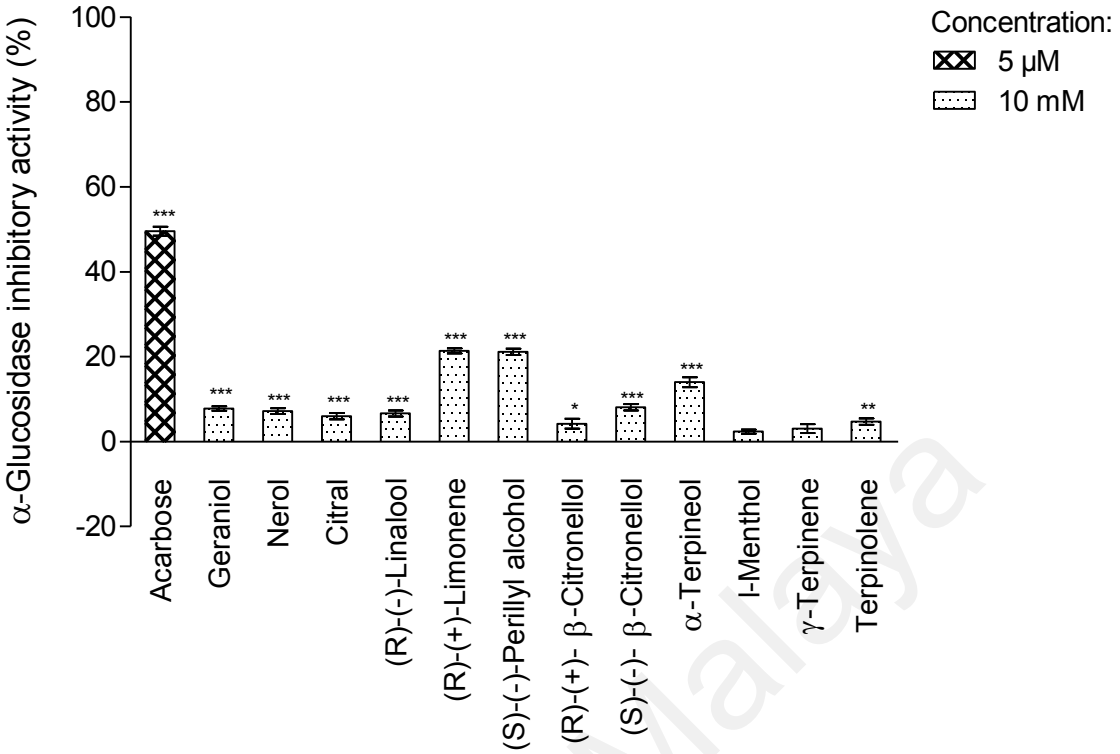


Figure 4.4: α-Glucosidase inhibitory activity of monoterpenes. Results expressed are means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to negative controls. n = 3 for each group.

4.6 EFFECT OF MONOTERPENES AND GLUCOSE OXIDASE ON VIABILITY 3T3-L1 PRE-ADIPOCYTES

4.6.1 TREATMENT WITH MONOTERPENES

Viability of pre-confluent 3T3-L1 pre-adipocytes after 48-hr exposure to various monoterpenes was determined using MTT assay. At a concentration of 1 μM, citral, geraniol and α-terpineol were found to suppress the proliferation of 3T3-L1 pre-adipocytes by 29.2%, 19.9% and 13.6%, respectively. In relation to this, Nerol, (R)-(-)-linalool, (R)-(+)-limonene, (S)-(-)-perillyl alcohol, (R)-(+)-β-citronellol, (S)-(-)-β-citronellol, l-menthol, γ-terpinene and terpinolene were found to have no negative proliferative effect on 3T3-L1 pre-adipocytes (Fig. 4.5A).

4.6.2 TREATMENT WITH GLUCOSE OXIDASE

On the other hand, 48-hour incubation with glucose oxidase from the concentration of 100 nU/ml to 10 μU/ml resulted in a dose-dependent reduction of growth in 3T3-L1 pre-adipocytes (11.7% to 89.1%). Glucose oxidase did not exert any effect on 3T3-L1 pre-adipocyte viability at concentrations of 10 nU/ml and below (Fig. 4.5B).

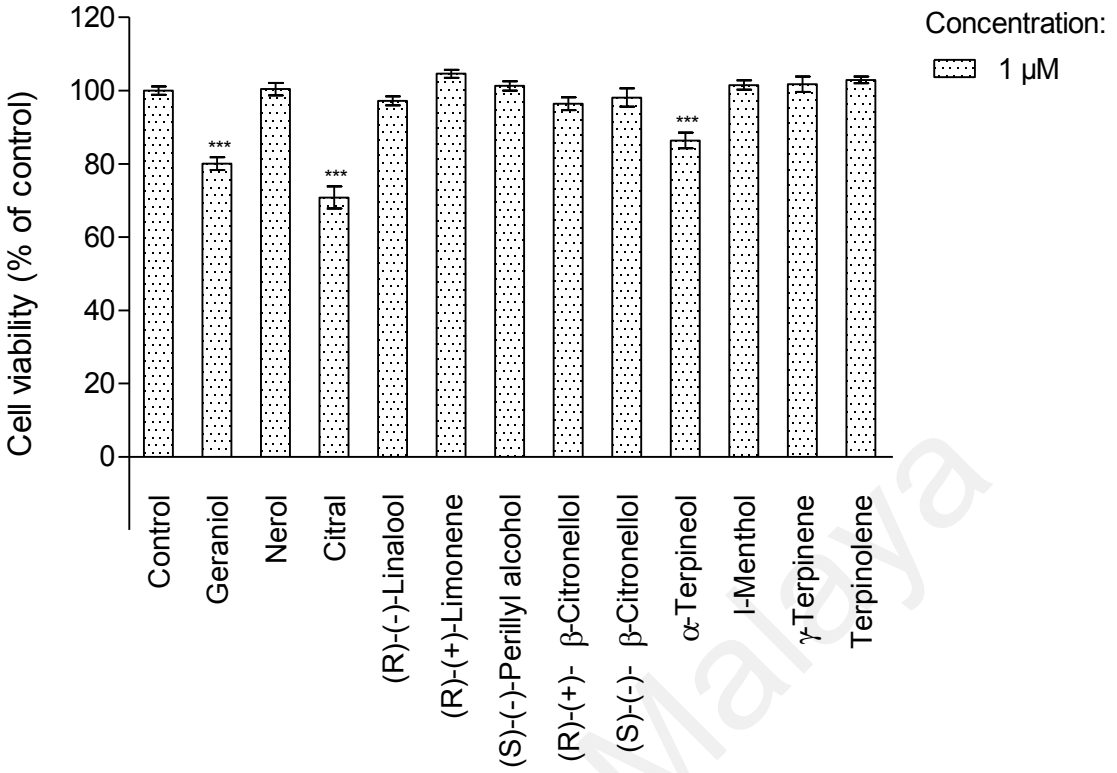


Figure 4.5A: Effect of monoterpenes on viability of 3T3-L1 pre-adipocytes. 3T3-L1 pre-adipocytes were exposed to various monoterpenes at concentration of 1 µM for 48 h prior to MTT assay. Results expressed are means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to negative controls. $n = 3$ for each group.

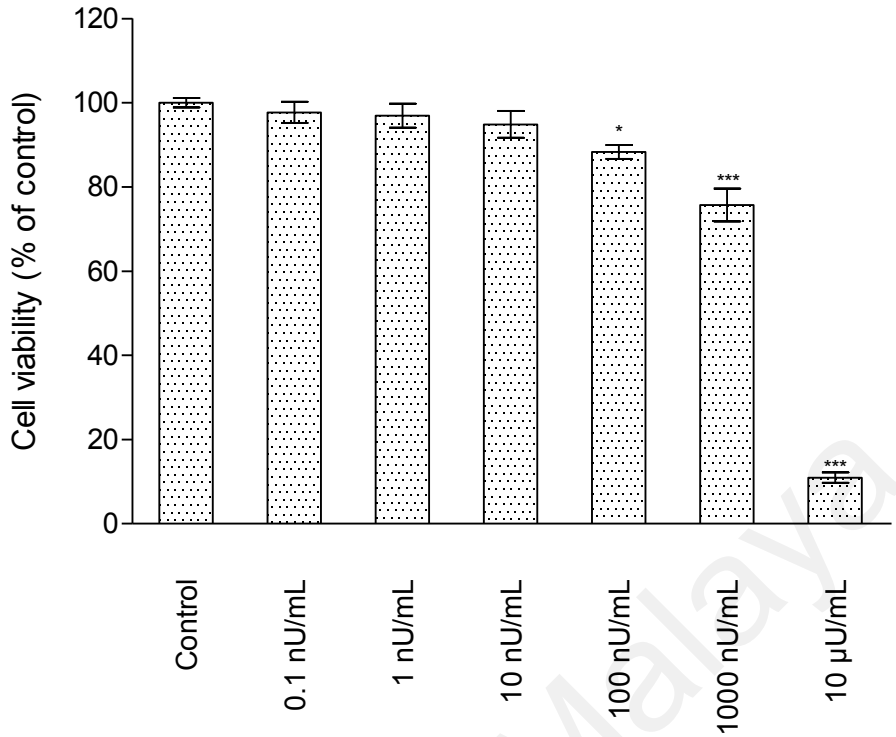


Figure 4.5B: Effect of glucose oxidase on viability of 3T3-L1 pre-adipocytes. 3T3-L1 pre-adipocytes were exposed to various concentrations of glucose oxidase for 48 h prior to MTT assay. Results expressed are means ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to negative controls. *n* = 3 for each group.

4.7 EFFECT OF MONOTERPENES AND GLUCOSE OXIDASE ON TOTAL LIPID ACCUMULATION IN 3T3-L1 ADIPOCYTES

4.7.1 TREATMENT WITH MONOTERPENES

To study the effect of monoterpenes on lipid accumulation in 3T3-L1 adipocytes, each monoterpene (1 μM) was introduced into the culture for 48 h at three different stages, which were 2DPD, day 0 and day 2, and were incubated for 48 h. When monoterpenes were introduced into the system during 2DPD, only α-terpineol (14.5%), geraniol (11.3%) and terpinolene (7.2%) showed stimulatory effect on total lipid accumulation in 3T3-L1 adipocytes [(Fig. 4.6A(i)].

Monoterpenes exhibited a more significant effect on total lipid accumulation in 3T3-L1 adipocytes when they were introduced into the system during day 0. At a concentration of 1 μM, terpinolene (43.0%) and γ-terpinene (42.5%) showed the strongest stimulatory effect on total lipid accumulation, followed by (R)-(-)-linalool (31.6%), α-terpineol (28.2%), citral (24.7%) and nerol (23.0%) [(Fig. 4.6A(ii)].

When monoterpenes were introduced into the system on day 2, geraniol enhanced total lipid accumulation by 19.8%, more significant in comparison to the positive control, 5 μg/ml insulin (14.6%). In addition, (S)-(-)-Perillyl alcohol and (R)-(+)-β-citronellol also stimulated total lipid accumulation by 11.9% and 10.7%. Amongst all the monoterpenes tested, only γ-terpinene showed inhibitory effect (19.0%) on the total lipid accumulation [(Fig. 4.6A(iii)].

4.7.2 TREATMENT WITH GLUCOSE OXIDASE

On the other hand, to study the effect of oxidative stress in 3T3-L1 adipocytes, glucose oxidase was also introduced into the culture system on 2DPD, day 0 and day 2, and incubated for 48 h. When glucose oxidase (1 nU/ml to 100 nU/ml) was added to the system on 2DPD, it significantly increased the total lipid accumulation in 3T3-L1 adipocytes in a dose-dependent manner (16.8% to 24.9%). However, at a higher concentration (1000 nU/ml), glucose oxidase did not induce any increment of total lipid accumulation.

When 3T3-L1 adipocytes were treated with glucose oxidase (10 nU/ml -1000 nU/ml) on day 0, a dose-dependent manner increase of total lipid accumulation in (7.9% to 19.8%) was observed. Lower concentrations of glucose oxidase did not result in any significant changes in the total lipid accumulation.

3T3-L1 adipocytes also responded to the treatment of glucose oxidase at concentrations of 10 nU/ml -1000 nU/ml when glucose oxidase was introduced into the system on day 2. An increase of 9.5%, 7.3% and 17.9% of total lipid accumulation were observed (Fig. 4.6B).

4.7.3 CO-TREATMENT WITH MONOTERPENES AND GLUCOSE OXIDASE

To study the effect of monoterpenes on total lipid accumulation in 3T3-L1 adipocytes under oxidative stress, selected monoterpenes (1 μM) were co-introduced with glucose oxidase on 2DPD, day 0 and day 2, and incubated for 48 h. Based on the earlier results on total lipid accumulation (Section 4.7.1 & Fig. 4.6A), three monoterpenes were selected to proceed with the experiment, namely geraniol, (R)-(+)-limonene and γ-terpinene. The concentration of glucose oxidase used was 100 nU/ml as it was the concentration that stimulated the most significant increase in lipid accumulation when introduced into the system on 2DPD, day 0 and day 2 (Section 4.7.2).

In another independent experiment, when 100 nU/ml glucose oxidase was introduced into the system on 2DPD, day 0 and day 2, the total lipid accumulation was increased by 11.4%, 9.2% and 7.9% respectively. However, co-incubation of 100 nU/ml glucose oxidase with 1 μM geraniol, 1 μM (R)-(+)-limonene and 1 μM γ-terpinene on 2DPD, day 0 and day 2 did not show any significant difference compared to the negative control (100 nU/ml glucose oxidase) (Fig. 4.6C). In view of these insignificant results, gene expression study on the combination effect of glucose oxidase and monoterpenes was not conducted.

(i) Monoterpenes incubated on 2DPD

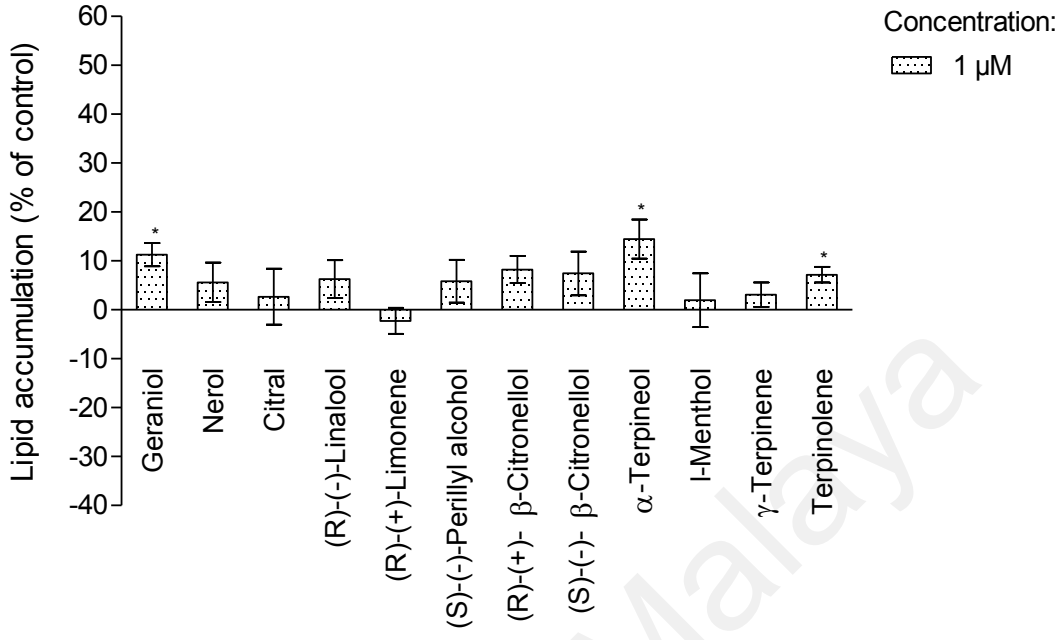


Figure 4.6A: Effect of monoterpenes on total lipid accumulation in 3T3-L1 adipocytes. 3T3-L1 adipocytes were exposed to various monoterpenes at concentration of 1 µM for 48 h on (i) 2DPD, (ii) day 0 and (iii) day 2. Mature adipocytes were subjected to ORO assay to quantify the total lipid accumulation. Results expressed are means ± SD. * $P <$ 0.05, ** $P < 0.01$, *** $P < 0.001$ compared to negative controls. $n = 3$ for each group.

(ii) Monoterpenes incubated on Day 0

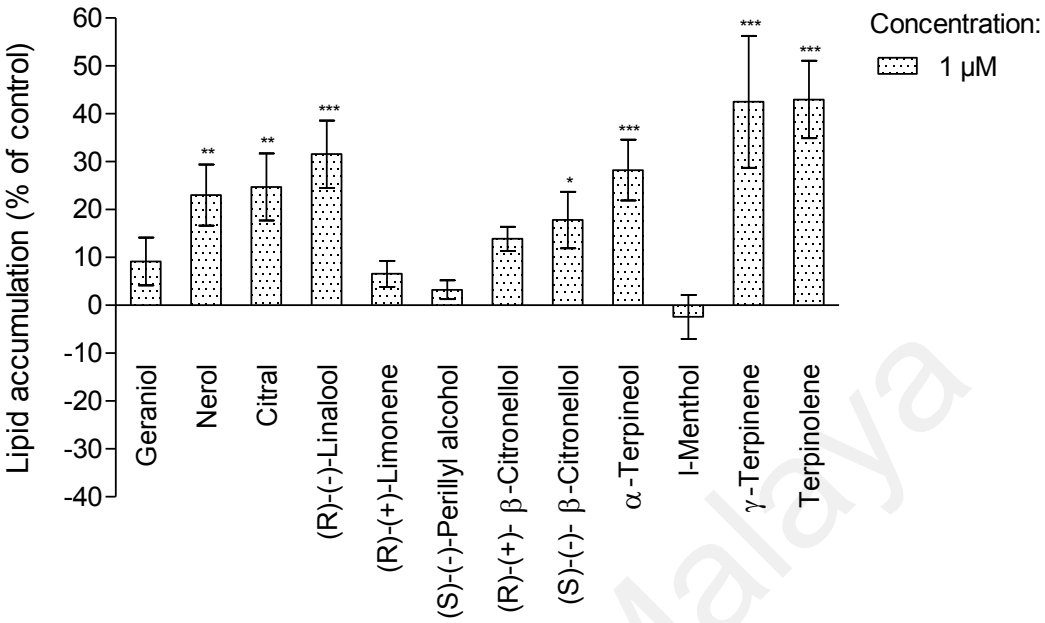


Figure 4.6A, continued: Effect of monoterpenes on total lipid accumulation in 3T3-L1 adipocytes. 3T3-L1 adipocytes were exposed to various monoterpenes at concentration of 1 µM for 48 h on (i) 2DPD, (ii) day 0 and (iii) day 2. Mature adipocytes were subjected to ORO assay to quantify the total lipid accumulation. Results expressed are means ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to negative controls. $n = 3$ for each group.

(iii) Monoterpenes incubated on Day 2

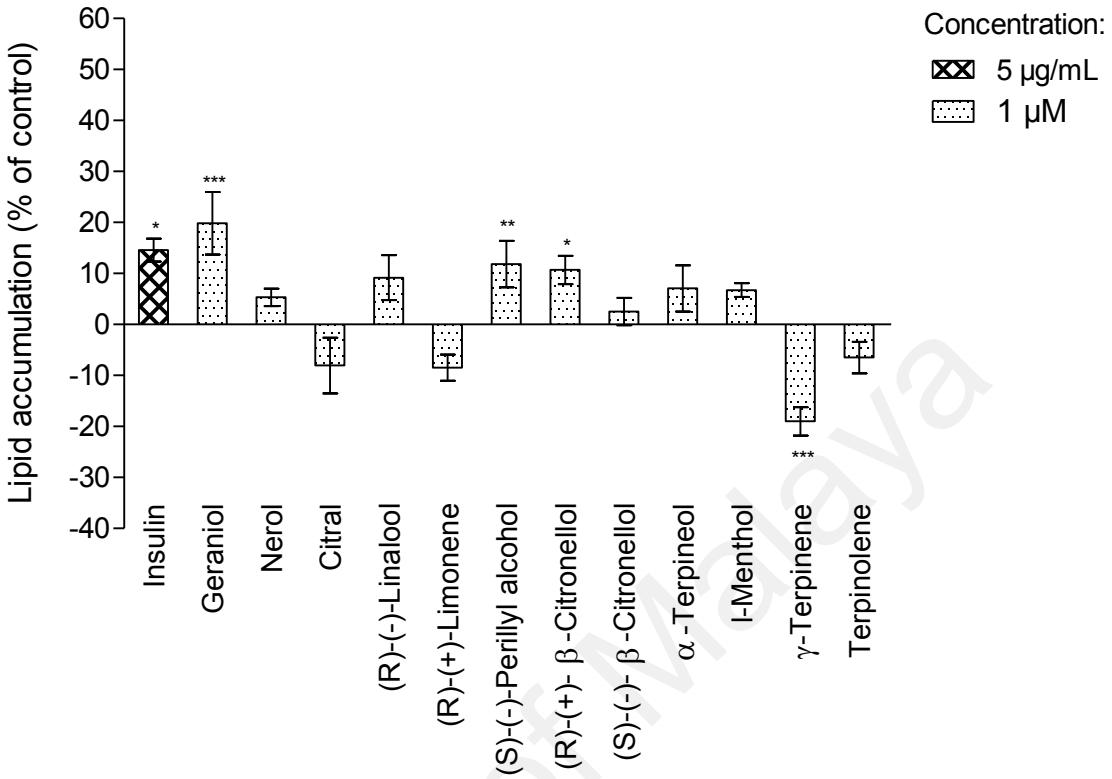


Figure 4.6A, continued: Effect of monoterpenes on total lipid accumulation in 3T3-L1 adipocytes. 3T3-L1 adipocytes were exposed to various monoterpenes at concentration of 1 µM for 48 h on (i) 2DPD, (ii) day 0 and (iii) day 2. Mature adipocytes were subjected to ORO assay to quantify the total lipid accumulation. Results expressed are means ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to negative controls. $n = 3$ for each group.

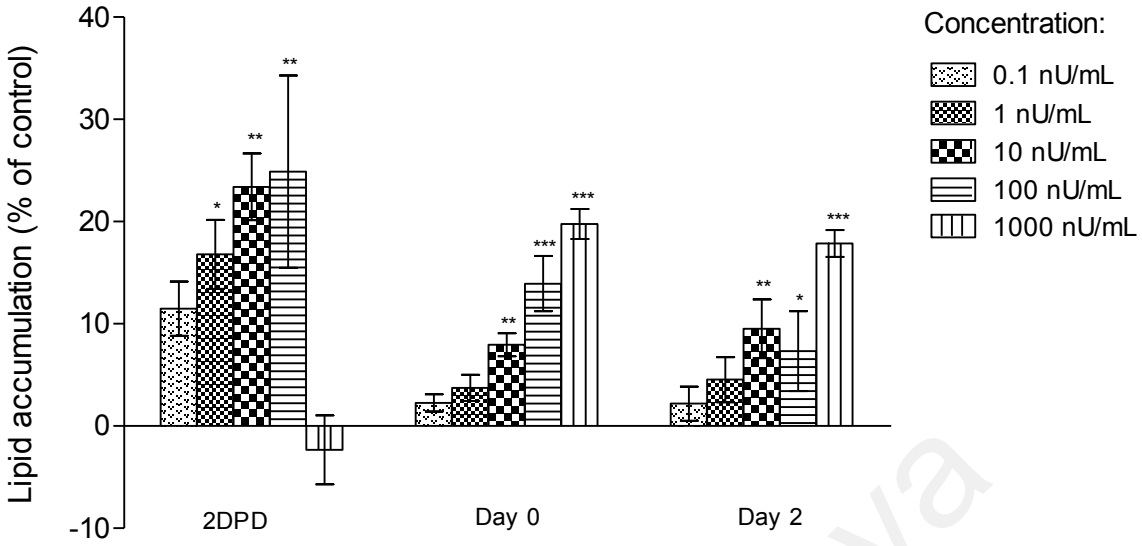


Figure 4.6B: Effect of glucose oxidase on total lipid accumulation in 3T3-L1 adipocytes. 3T3-L1 adipocytes were exposed to various concentrations of glucose oxidase for 48 h on 2DPD, day 0 and day 2. Mature adipocytes were subjected to ORO assay to quantify the total lipid accumulation. Results expressed are means ± SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to negative controls. *n* = 3 for each group.

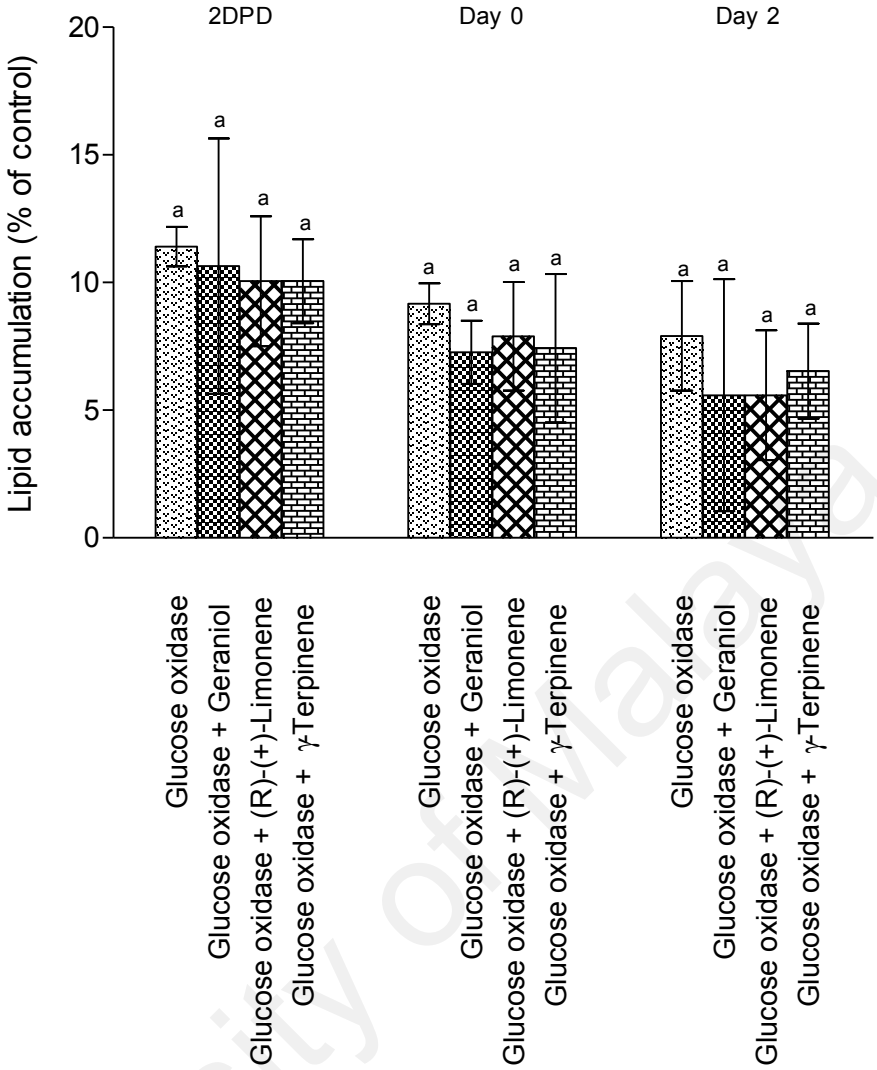


Figure 4.6C: Effect of monoterpenes on total lipid accumulation in 3T3-L1 adipocytes in the presence of glucose oxidase. 3T3-L1 adipocytes were co-incubated with 1 µM monoterpene and 100 nU/ml glucose oxidase for 48 h on 2DPD, day 0 and day 2. Mature adipocytes were subjected to ORO assay to quantify the total lipid accumulation. Results expressed are means ± SD. Data with the same superscript are not significantly different ($P < 0.05$).

4.8 EFFECT OF MONOTERPENES ON GLUCOSE UPTAKE IN MATURE 3T3-L1 ADIPOCYTES

In this study, results showed that only certain monoterpenes exerted stimulatory effect on the glucose uptake. Geraniol stimulated the highest increase, 21.1%, in the glucose uptake (Fig. 4.7). Citral, (R)-(+)-limonene and (R)-(+)-β-citronellol also enhanced glucose uptake by 17.6%, 17.4% and 16.1%, respectively. Nerol, (S)-(-)-perillyl alcohol, γ-terpinene and α-terpineol relatively showed a weaker stimulatory effect on glucose uptake, by 10.9% to 14.6%. On the other hand, (S)-(-)-β-citronellol, terpinolene and (R)-(-)-linalool did not affect the glucose uptake in 3T3-L1 adipocytes.

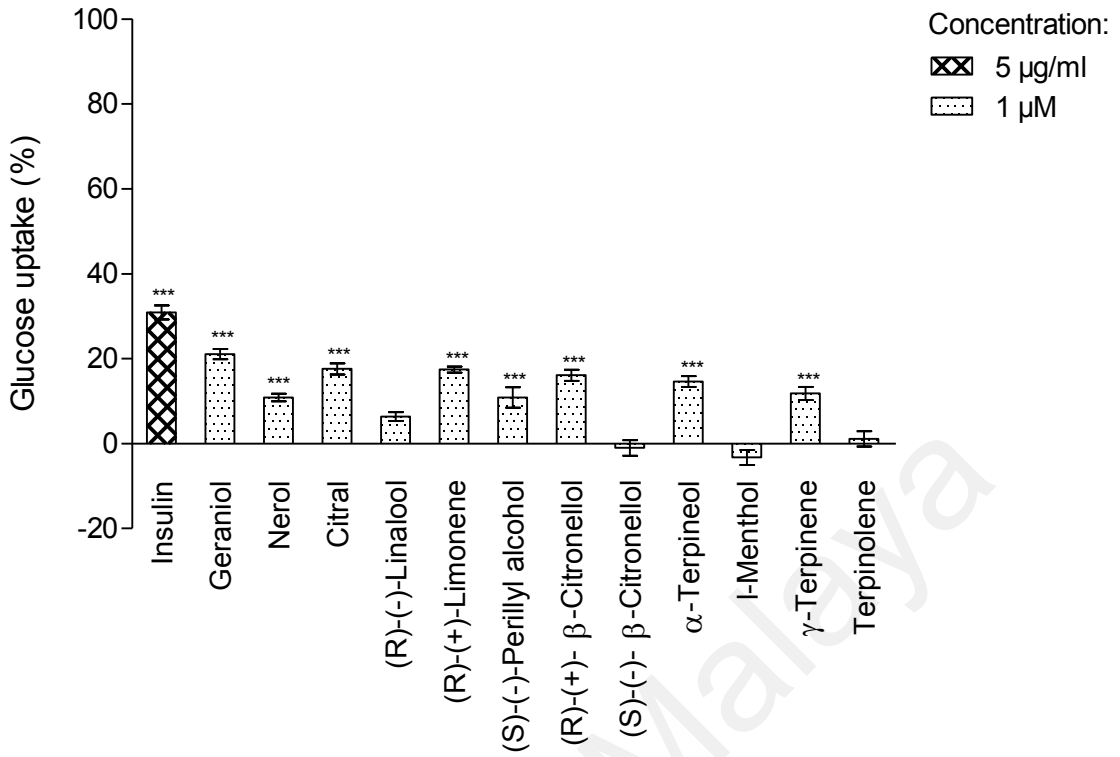


Figure 4.7: Effect of monoterpenes on total glucose uptake. Mature 3T3-L1 adipocytes were exposed to various monoterpenes at a concentration of 1 µM. After 5 h, total glucose in the medium was quantitated. Total reduction of glucose content in the medium in comparison to the blank indicated the total glucose uptake by the adipocytes. Results expressed are means ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to negative controls. *n* = 3 for each group.

4.9 EFFECT OF MONOTERPENES ON LIPOLYSIS IN MATURE 3T3-L1 ADIPOCYTES

(R)-(-)-Linalool that did not exert any effect on glucose uptake, exhibited 11.8% decrease in the lipolysis (Fig. 4.8). Interestingly, (R)-(+)-limonene, (S)-(-)-perillyl alcohol, (R)-(+)-β-citronellol and geraniol which enhanced glucose uptake, also stimulated lipolysis, by 17.7%, 17.3%, 12.0% and 11.1%, respectively.

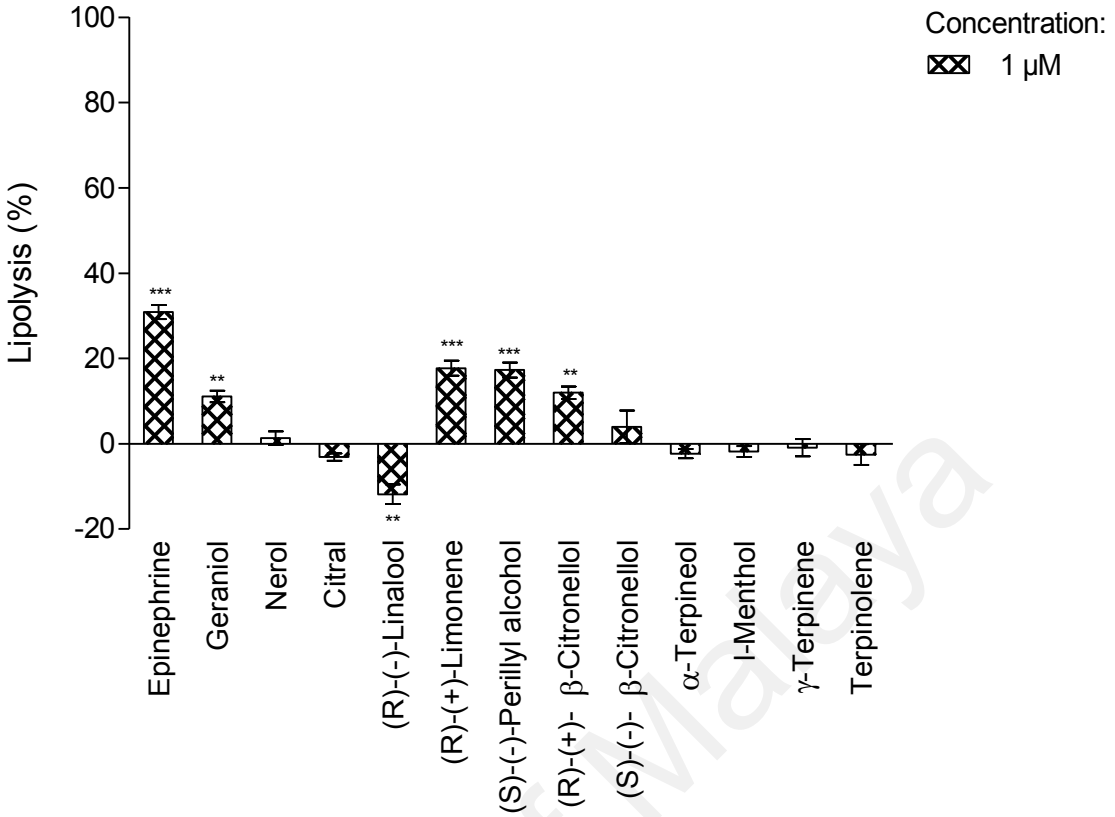


Figure 4.8: Effect of monoterpenes on lipolysis. Mature 3T3-L1 adipocytes were exposed to various monoterpenes at concentration of 1 µM. After 5 h, measurement of free glycerol released into the medium was performed. Total glycerol content in the medium indicated the lipolytic effect of monoterpenes in 3T3-L1 adipocytes. Results expressed are means ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to negative controls. $n = 3$ for each group.

4.10 EFFECT OF MONOTERPENES AND GLUCOSE OXIDASE ON THE REGULATION OF GENE EXPRESSION

4.10.1 TREATMENT WITH MONOTERPENES

(R)-(+)-Limonene was selected to study its effect on the regulation of gene expression based on its stimulatory effect on glucose uptake (section 4.8) and lipolysis (section 4.9); and insignificant effect on total lipid accumulation (section 4.7.1). After 48-hr exposure of 1 μM (R)-(+)-limonene to the cells during cell-inhibition stage (2DPD) and differentiation of 3T3-L1 adipocytes (day 0), mRNA expression of peroxisome proliferator activated-receptor-gamma (PPAR-γ) was measured. In the current study, (R)-(+)-limonene treatment did not alter the mRNA expression of PPAR-γ in 3T3-L1 adipocytes [Fig. 4.9A(i, ii)].

Subsequently, after 48-hr exposure to 1 μM (R)-(+)-limonene,the expression of several genes which participated in the early maturation stage of 3T3-L1 adipocytes (day 2) were investigated. Presence of 1 μM (R)-(+)-limonene in 3T3-L1 adipocyte culture significantly increased the mRNA expression of GLUT1 by 1.2 fold whereas the mRNA expression of GLUT4 remained unchanged [Fig. 4.9A(iii)].

With regards to lipolysis, the gene expression study focused primarily on ATGL and HSL mRNA expression. This study demonstrated that treatment with 1 μM (R)-(+)-limonene in mature 3T3-L1 adipocytes down-regulated the mRNA expression of ATGL but did not alter mRNA expression of HSL [Fig. 4.9A(iv)].

4.10.2 TREATMENT WITH GLUCOSE OXIDASE

Glucose oxidase at a concentration of 100 nU/ml was selected for gene expression studies as it stimulated highest lipid accumulation when introduced into the system on 2DPD, day 0 and day 2 as discussed in Section 4.7.3.

After 48-hr exposure to 100 nU/ml glucose oxidase on 2DPD and day 0, PPAR-γ mRNA expression in 3T3-L1 adipocytes was shown to be unaffected [Fig. 4.9B(i, ii)]. However, after 48-hr exposure to 100 nU/ml glucose oxidase on day 2, mRNA expression of GLUT1 was down-regulated whereas the mRNA expression of GLUT4 remained unchanged [Fig. 4.9B(iii)]. Exposure to 100 nU/ml glucose oxidase also resulted in the down-regulation of ATGL mRNA expression but not HSL mRNA expression, which remained unaffected [Fig. 4.9B(iv)].

To investigate the effect of oxidative stress on GPx mRNA expression in 3T3-L1 adipocytes, cells were exposed to 100 nU/ml glucose oxidase on 2DPD, day 0 and day 2 for 48 h. Interestingly, such exposure in these three stages did not alter the mRNA expression of GPx [Fig. 4.9B(v)].

(i) (ii)

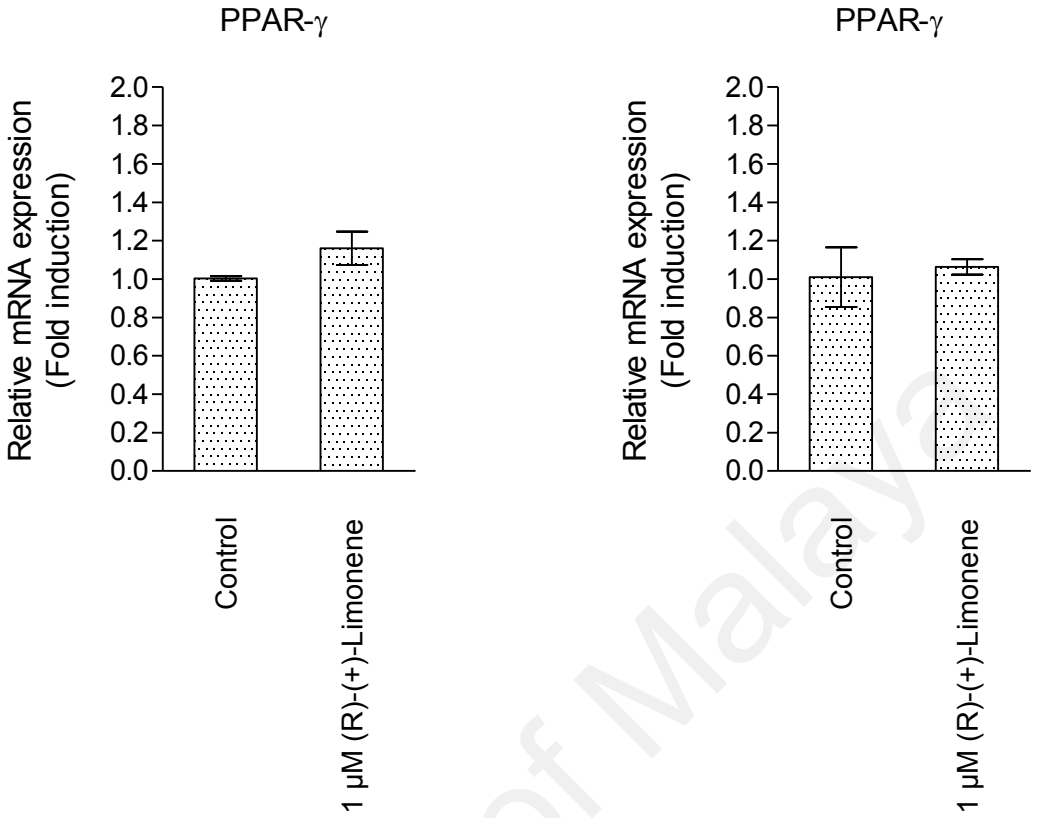


Figure 4.9A: The effect of (R)-(+)-limonene on the expression of genes associated with differentiation and lipid metabolism in 3T3-L1 adipocytes. Cells were exposed to 1 µM (R)-(+)-limonene on (i) 2DPD, (ii) day 0 and (iii, iv) day 2 for 48 h. Total mRNA was isolated on day 2 for the evaluation of the expression of PPAR-γ. Isolation of mRNA from the cells treated on day 2 was performed on day 4 to study the expression of downstream genes involved in glucose uptake and lipolysis. Results expressed are means ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to negative controls. $n = 3$ for each group. Control indicates untreated cells.

(iii) (iv)

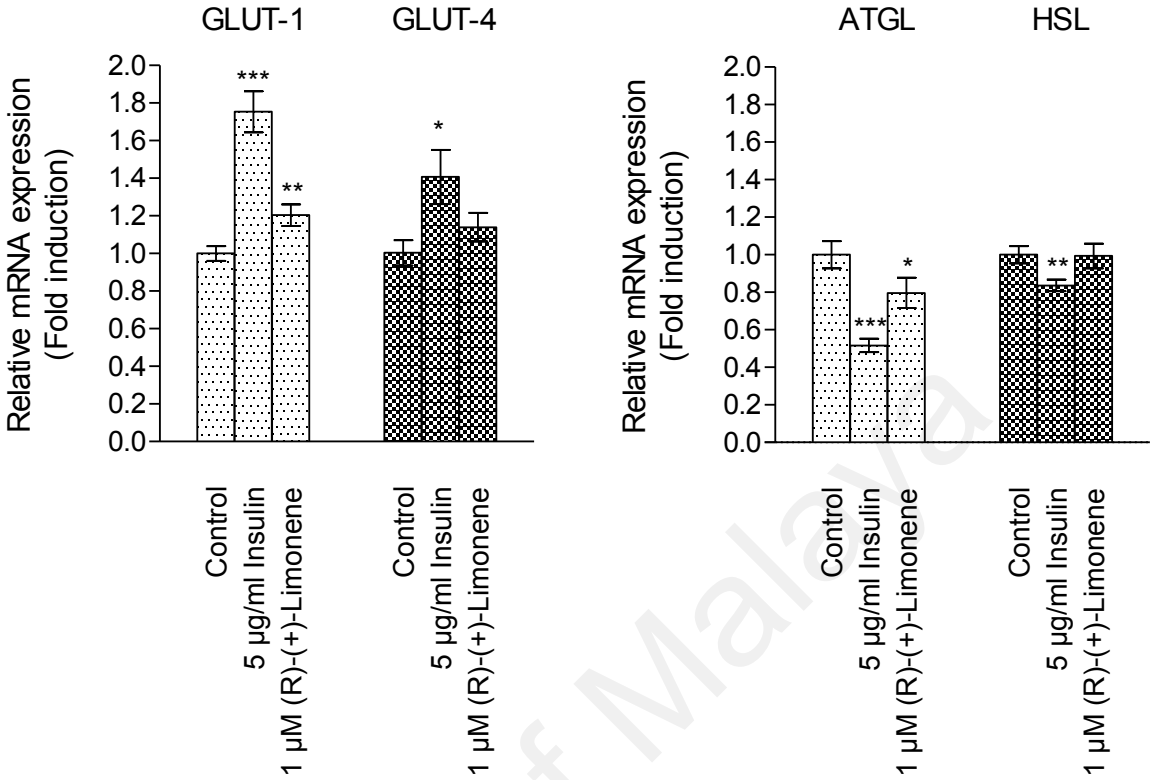


Figure 4.9A, continued: The effect of (R)-(+)-limonene on the expression of genes associated with differentiation and lipid metabolism in 3T3-L1 adipocytes. Cells were exposed to 1 µM (R)-(+)-limonene on (i) 2DPD, (ii) day 0 and (iii, iv) day 2 for 48 h. Total mRNA was isolated on day 2 for the evaluation of the expression of PPAR-γ. Isolation of mRNA from the cells treated on day 2 was performed on day 4 to study the expression of downstream genes involved in glucose uptake and lipolysis. Results expressed are means ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to negative controls. $n = 3$ for each group. Control indicates untreated cells.

(i) (ii)

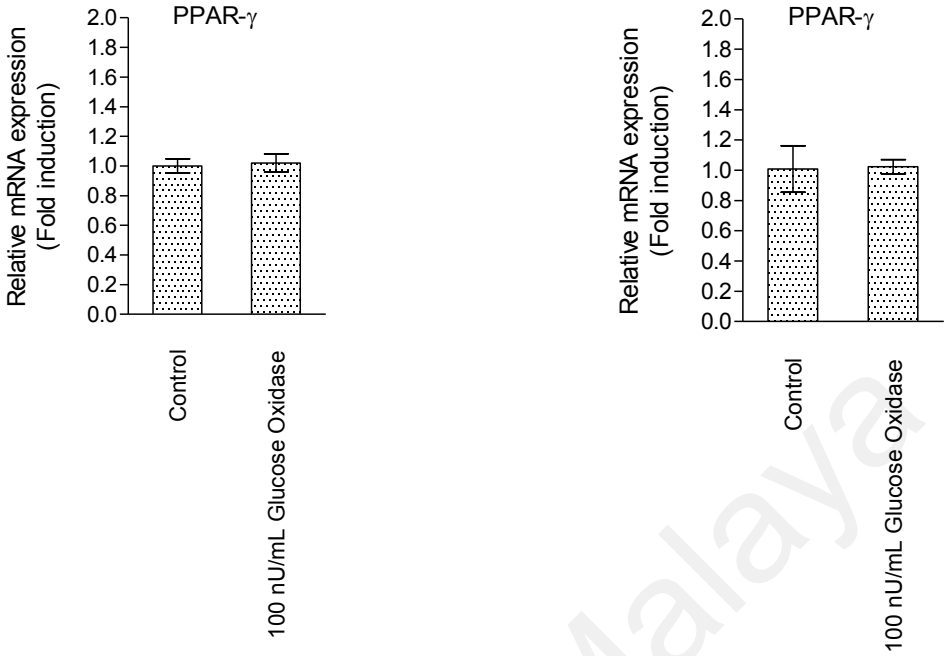


Figure 4.9B: The effect of glucose oxidase on the expression of genes associated with differentiation and lipid metabolism and GPx expression in 3T3-L1 adipocytes. Cells were exposed to 100 nU/ml glucose oxidase on (i) 2DPD, (ii) day 0 and (iii, iv) day 2 for 48 h. Total mRNA was isolated on day 2 for the evaluation of the expression of PPAR-γ. Isolation of mRNA from the cells treated on day 2 was performed on day 4 to study the expression of downstream genes involved in glucose uptake and lipolysis. (v) mRNA expression of GPx was quantified after 48-h incubation with 100 nU/ml glucose oxidase on 2DPD, day 0 and day 2. Results expressed are means ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to negative controls. $n = 3$ for each group. Control indicates untreated cells.

(iii) (iv)

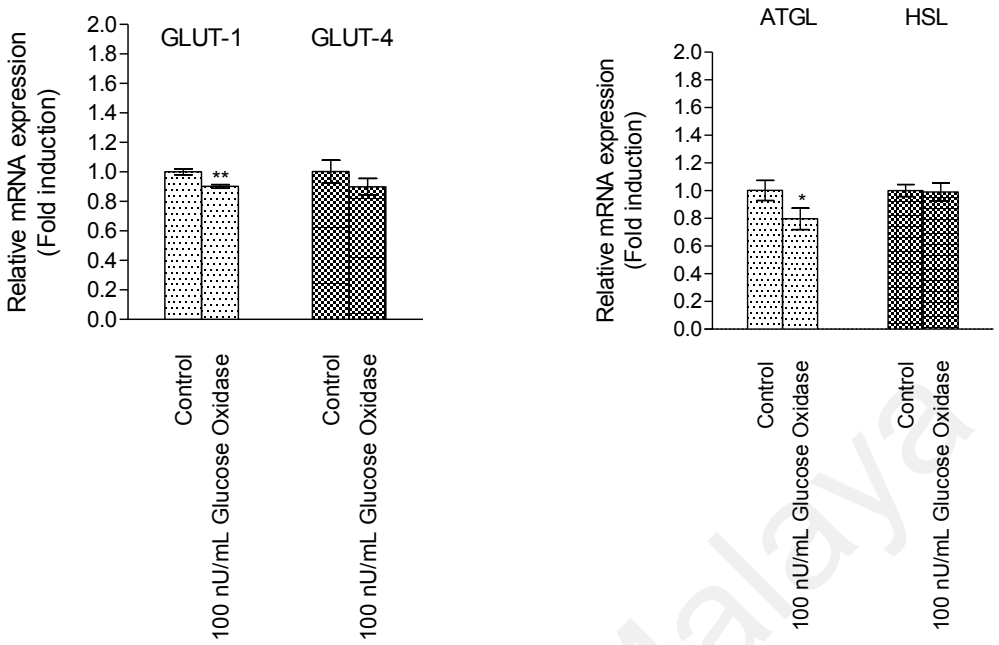


Figure 4.9B, continued: The effect of glucose oxidase on the expression of genes associated with differentiation and lipid metabolism and GPx expression in 3T3-L1 adipocytes. Cells were exposed to 100 nU/ml glucose oxidase on (i) 2DPD, (ii) day 0 and (iii, iv) day 2 for 48 h. Total mRNA was isolated on day 2 for the evaluation of the expression of PPAR-γ. Isolation of mRNA from the cells treated on day 2 was performed on day 4 to study the expression of downstream genes involved in glucose uptake and lipolysis. (v) mRNA expression of GPx was quantified after 48-h incubation with 100 nU/ml glucose oxidase on 2DPD, day 0 and day 2. Results expressed are means ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to negative controls. $n = 3$ for each group. Control indicates untreated cells.

(v)

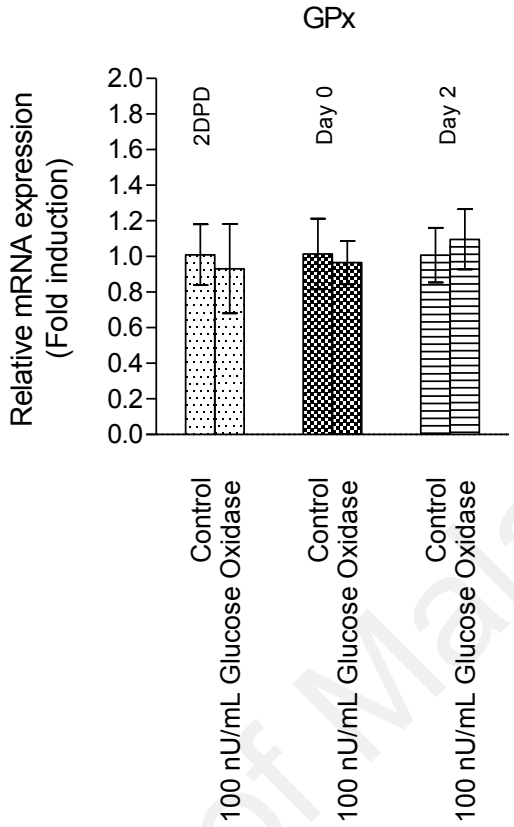


Figure 4.9B, continued: The effect of glucose oxidase on the expression of genes associated with differentiation and lipid metabolism and GPx expression in 3T3-L1 adipocytes. Cells were exposed to 100 nU/ml glucose oxidase on (i) 2DPD, (ii) day 0 and (iii, iv) day 2 for 48 h. Total mRNA was isolated on day 2 for the evaluation of the expression of PPAR-γ. Isolation of mRNA from the cells treated on day 2 was performed on day 4 to study the expression of downstream genes involved in glucose uptake and lipolysis. (v) mRNA expression of GPx was quantified after 48-h incubation with 100 nU/ml glucose oxidase on 2DPD, day 0 and day 2. Results expressed are means ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to negative controls. $n = 3$ for each group. Control indicates untreated cells.

CHAPTER FIVE: DISCUSSION

5.1 RADICAL SCAVENGING EFFECT AND REDUCING POWER OF MONOTERPENES

The current finding showed that DPPH radical scavenging activity of monoterpenes fell within milimolar range (Fig. 4.1) which was in concordance with several other reports (Bicas, Neri-Numa, Ruiz, De Carvalho, & Pastore, 2011; Di Sotto, Durazzi, Sarpietro, & Mazzanti, 2013; Junior et al., 2009). In a previous study, Bicas et al. (2011) showed that the IC_{50} of DPPH radical scavenging for α-terpineol and perillyl alcohol were IC_{50} = 332.8 g L^{-1} (approximately 2 M) and IC_{50} = 738.3 g L^{-1} (approximately 5 M), respectively. Junior et al. (2009) also showed that limonene (0.030%, v/v, approximately 2 mM) inhibited less than 5% of DPPH radicals. These findings contradicted the report by Choi et al. (2000) which showed that some of the monoterpenes such as γ-terpinene, terpinolene and citral exerted greater DPPH radical scavenging activity than Trolox. Several factors could affect the efficacy of antioxidants, including emulsion system, test system, methods and oxidation time. For instance, lipophilic antioxidant such as α-tocopherol is more efficient in oil-in-water emulsion system than a bulk oil system whereas hydrophilic antioxidant such as Trolox acts in the opposite trend (Shahidi & Zhong, 2011). Other than that, the possibility that synthesis process of monoterpenes and storage conditions like temperature, exposure to light and auto-oxidation could also alter the efficacy of monoterpenes resulting in the discrepancy between studies (Laguerre et al., 2015). Nevertheless, the monoterpenes used in this study were handled appropriately (stored in the dark and at the recommended temperature) to minimize or avoid auto-oxidation.

Similar to DPPH radical scavenging activity, most of the tested monoterpenes showed ABTS radical scavenging activity at milimolar range, except geraniol, l-menthol, nerol, (R)-(-)-linalool and (S)-(-)-β-citronellol which did not exert any

significant ABTS radical scavenging activity (Fig. 4.2). Both terpinolene and γ-terpinene respectively scavenged 59.5% and 32.7% ABTS radicals at a lower concentration (1 mM) (Fig. 4.2) compared to DPPH radical scavenging (10 mM) (Fig. 4.1). The different potencies observed for both assays can be explained by the mechanisms involved. ABTS radicals and DPPH radicals involve electron transfer and hydrogen donation, respectively, in which the former takes place in a much faster rate than the latter (Bendaoud, Bouajila, Rhouma, Savagnac, & Romdhane, 2009).

Compounds having high ferric reducing power (FRAP) value may serve as good antioxidants by donating their electrons to eliminate the free radicals (Benzie & Strain, 1996). However, monoterpenes that showed radical scavenging activities in DPPH and ABTS radical scavenging assays (Fig. 4.1 & Fig. 4.2) did not show any significant reducing power in FRAP assay (Table 4.1). Taken together, it is possible to conclude that synthetic monoterpenes used in the current study exhibited a weak antioxidant capacity with a weak radical scavenging activity and did not have any ferric reducing power.

Oxidative stress is closely associated with obesity and T2DM. Antioxidant treatment has been reported to reduce ROS and attenuate complications caused by diabetes (Huynh et al., 2014). Thus, dietary monoterpenes with antioxidant capacity can be beneficial to human health by alleviating complications caused by T2DM. Nevertheless, as antioxidant capacity of monoterpenes remains a controversy, the other potential aspects of monoterpenes that can possibly aid in the control of T2DM were investigated in this study.

5.2 α-AMYLASE AND α-GLUCOSIDASE INHIBITORY EFFECT OF MONOTERPENES

One of the current treatments of T2DM is the use of oral hypoglycemic agents which inhibit activity of carbohydrate-hydrolyzing enzymes, namely, α-amylase and α-glucosidase. Pancreatic α-amylase hydrolyzes starch into oligosaccharides which are further degraded by intestinal α-glucosidase into glucose that is readily absorbed into systemic circulation (Jo et al., 2013). In T2DM patients, due to impaired glucose tolerance, sudden rise in the plasma glucose level can lead to complications. Inhibition of these enzymes delays overall carbohydrate digestion and glucose absorption rate consequently blunting the increase of postprandial plasma glucose level. Thus, carbohydrate-hydrolyzing enzyme inhibitors such as acarbose, miglitol and voglibose have been clinically used as oral anti-hyperglycemic agents in the effective control of T2DM (Jo et al., 2013; Sugihara et al., 2014).

In the current study, the potential inhibitory effect of monoterpenes on both α-amylase and α-glucosidase were assessed. Citral was the only monoterpene that showed α-amylase inhibitory effect of 45.7%, at a concentration of 10 mM as compared to the positive control, acarbose which inhibited 48.2% of α-amylase activity at a concentration of 10 µM suggesting that citral was a weak α-amylase inhibitor (Fig. 4.3).

On the other hand, most of the tested monoterpenes (10 mM) showed inhibitory effect on α-glucosidase activity, ranging from 4.2% to 21.3%. In comparison, the positive control, acarbose exerted α-glucosidase inhibitory effect with a much greater potency, which was 49.6% at a concentration of 5 µM (Fig. 4.4). These findings suggest that monoterpenes are weak α-glucosidase inhibitors.

Clinical use of acarbose as oral hypoglycemic agent has been well established. Nonetheless, the main drawbacks of acarbose are gastrointestinal effects including abdominal distention, flatulence, meteorism and possibly diarrhea, due to abnormal

fermentation of undigested carbohydrates by the bacteria in the colon as a result of over inhibition of α-amylase (Jo et al., 2013). A more effective strategy in the control of T2DM involves a moderate α-amylase inhibition with a stronger inhibition on α-glucosidase. Therefore, with respect to diet, dietary intervention with high consumption of monoterpenes with a mild α-glucosidase inhibitory property, could offer a better control of glycemic index of food products together with the therapeutic approach.

5.3 EFFECT OF MONOTERPENES AND GLUCOSE OXIDASE ON VIABILITY OF 3T3-L1 PRE-ADIPOCYTES

5.3.1 TREATMENT WITH MONOTERPENES

Viability of pre-confluent 3T3-L1 pre-adipocytes after 48-hr exposure to various monoterpenes was determined using MTT assay. At a concentration of 1 μM, monoterpenes such as citral, geraniol and α-terpineol exhibited inhibitory effect on 3T3-L1 pre-adipocyte proliferation and thus, higher concentrations of monoterpenes were not investigated in the cell culture system in this study due to their potential cytotoxic effect on 3T3-L1 adipocytes. On the other hand, most other monoterpenes tested did not show any negative effect on proliferation of 3T3-L1 pre-adipocytes (Fig. 4.5A). These findings suggest that the suppressive effect of monoterpenes on 3T3-L1 pre-adipocyte proliferation was selective but the mechanism is unclear. One of the possible mechanisms involved in the suppression of 3T3-L1 pre-adipocyte growth is discussed below.

Monoterpenes are one of the major constituents of essential oils apart from sesquiterpene, diterpene, triterpene, tetraterpene and polyterpene. Due to their strong lipophilicity, essential oils can permeate and disrupt cytoplasmic membrane phospholipid layers leading to membrane damage (Bakkali et al., 2008). Essential oils can also lower the membrane potential and lead to depolarization of mitochondrial membranes, which in turn leads to the leakage of radicals, cytochrome C, calcium ions and proteins. Permeabilizations of outer and inner mitochondrial membranes result in apoptosis and necrosis (Armstrong, 2006; Bakkali et al., 2008). The speculations seem to be convincing, however, more in depth studies are necessary to understand the mechanism involved.

5.3.2 TREATMENT WITH GLUCOSE OXIDASE

To investigate the effect of low grade oxidative stress on the viability of pre-confluent 3T3-L1 pre-adipocytes, glucose oxidase was introduced to the system to constantly generate hydrogen peroxide at low concentrations, mimicking physiological environment. Glucose oxidase is capable of generating hydrogen peroxides from oxygen in the process of oxidizing glucose into glucolactone, resulting in oxidative stress (Milton, Giroud, Thumser, Minteer, & Slade, 2013). In the current study, growth inhibition of 3T3-L1 pre-adipocytes in response to the treatment of glucose oxidase (100 nU/ml to 1000 nU/ml) was observed (Fig. 4.5B). The inhibition of growth of 3T3-L1 pre-adipocytes by glucose oxidase can be explained by the deleterious effect of hydrogen peroxide, a ROS. ROS can lead to the damage of all macromolecules, including nucleic acids, carbohydrates, proteins and lipids. If the damages transcend the repair mechanism, death signaling pathways would be activated. Depending on the severity of the damage, different modes of cell death would be induced, namely, apoptosis and necrosis (Fulda, Gorman, Hori, & Samali., 2010). Therefore, in the present study, as the concentration of glucose oxidase increased, more hydrogen peroxide molecules were generated which then resulted in more cell damage and cell death, and thus, stronger growth inhibition was observed.

5.4 EFFECT OF MONOTERPENES AND GLUCOSE OXIDASE ON ADIPOGENESIS IN 3T3-L1 ADIPOCYTES

Treatments of T2DM, in part, target on the improvement of insulin sensitivity and glucose uptake. For instance, one of the commonly used drugs, TZD, a PPARγ ligand, is known to improve insulin sensitivity by increasing differentiation of new adipocytes with higher insulin sensitivity (Choi et al., 2011). Another treatment used to treat T2DM targets on the improvement of glucose uptake by the cells. Insulin is one of the most common drugs used in this type of treatment (Olokoba et al., 2012). Insulin treatment stimulates glucose uptake by skeletal muscle and adipocytes via glucose transporters. In both rodents and humans, up to 40% of whole-body *de novo* fatty acid synthesis from glucose occurs in the white adipocytes, resulting in the increased lipid accumulation (Leto & Saltiel, 2012; Masoodi et al., 2015). Therefore, screening the effect of monoterpenes on 3T3-L1 adipocyte differentiation and lipid accumulation may reveal the potential in the control of T2DM. It is also important to know if the tested monoterpenes cause an excessive lipid accumulation as adipocytes with excessive lipid accumulation and enlarged lipid droplets will experience a decrease in insulin sensitivity (Löfgren, Hoffstedt, Näslund, Wiren, & Arner, 2005).

5.4.1 TREATMENT WITH MONOTERPENES

To evaluate the effect of monoterpenes on adipocyte differentiation and lipid accumulation, 3T3-L1 adipocytes were treated with 1 µM of monoterpenes for 48 h at three distinct stages, which were during the cell-contact inhibition stage of post-confluent pre-adipocytes (2DPD), mitotic clonal expansion and terminal differentiation of adipocytes (day 0) and maturation of adipocytes (day 2) (Guo et al., 2009; Ntambi & Young-Cheul, 2000). Changes that occur during any of these stages could directly impact on downstream activities such as lipid accumulation. Therefore, by treating the

cells with monoterpenes during the different stages, alteration of total lipid accumulation could provide an insight into the stages affected by the treatment. In the present study, Oil Red O quantitative assay was used to quantify the total lipid accumulation in mature adipocytes.

When monoterpenes were introduced during 2DPD, only α-terpineol, geraniol and terpinolene showed stimulatory effect on total lipid accumulation in 3T3-L1 adipocytes [(Fig. 4.6A(i)]. The increase of lipid accumulation indicates their effect on the cell-contact inhibition stage of post-confluent pre-adipocytes, a stage that involves epigenetic modifications (DNA methylation and histone modification) (Guo et al., 2009).

Overall, more significant stimulatory effect of monoterpenes on total lipid accumulation in 3T3-L1 adipocytes was observed when they were introduced into the cell culture during day 0, a stage at which mitotic clonal expansion and terminal differentiation of adipocytes take place (Guo et al., 2009; Ntambi & Young-Cheul, 2000). Monoterpenes that showed stimulatory effect on total lipid accumulation was deduced to be due to their stimulatory effect on the differentiation of adipocytes. Terpinolene and γ-terpinene showed the strongest stimulatory effect on the differentiation of 3T3-L1 adipocytes, approximately 43%. Most but not all the tested monoterpenes showed stimulatory effect on the differentiation of 3T3-L1 adipocytes [(Fig. 4.6A(ii)]. Monoterpenes that stimulated the differentiation of 3T3-L1 adipocytes might have the potential in the control of T2DM by producing newly differentiated small adipocytes with higher insulin sensitivity (Choi et al., 2011).

To investigate the effect of monoterpenes on the maturation of adipocytes, 3T3-L1 adipocytes were exposed to monoterpenes on day 2 for 48 h, a stage at which glucose uptake and lipolysis occur (Guo et al., 2009; Ntambi & Young-Cheul, 2000).

The Oil Red O quantitative assay results indicate the direct effect of monoterpenes on lipid accumulation. As increase in lipid accumulation is closely associated with increase in glucose uptake (Masoodi et al., 2015), monoterpenes such as geraniol, (S)-(-)-perillyl alcohol and (R)-(+)-β-citronellol (1 µM) that exhibited a stimulatory effect on total lipid accumulation comparable to insulin (5 µg/ml), are likely to have the potential in the control of T2DM. On the contrary, amongst all the tested monoterpenes, only γ-terpinene showed inhibitory effect on the total lipid accumulation [(Fig. 4.6A(iii)].

Interestingly, the present findings showed that treatment of 1 µM (R)-(+)-limonene during these three distinctive stages had similar degree of total lipid accumulation in mature adipocytes as compared to negative control [Fig. 4.6A(i-iii)]. Our results concur with animal study by Murali and Saravanan (2012), which showed that there was no significant change in body weight in normal rats fed with (R)-(+)-limonene. These findings also suggest that (R)-(+)-limonene did not affect cell-contact inhibition, mitotic clonal expansion and terminal differentiation of adipocytes. Nevertheless, maturation of adipocytes involves both glucose uptake and lipolysis, which can result in the shift in the total lipid accumulation. Therefore, the study was proceeded with glucose uptake assay and lipolysis assay, which are discussed in Section 5.5 and Section 5.6.

5.4.2 TREATMENT WITH GLUCOSE OXIDASE

The effect of oxidative stress on adipocyte differentiation and total lipid accumulation in 3T3-L1 adipocytes were investigated by treating the cells with glucose oxidase which can gradually generate and accumulate hydrogen peroxide in the system mimicking physiological environment. Studies of oxidative stress in 3T3-L1 adipocytes are complex whereby concentrations and incubation period of ROS in adipocytes can directly affect the outcomes. For instance, prolonged incubation with high concentration of glucose oxidase can impair GLUT4 translocation (Rudich et al, 1998). Another report showed that treatment of antioxidant attenuated 3T3-L1 adipocyte differentiation (Imhoff & Hansen, 2010). Therefore, further investigations on the effect of oxidative stress on 3T3-L1 adipocyte differentiation and lipid accumulation are necessary.

In this study, the exposure to low concentrations of glucose oxidase in 3T3-L1 adipocytes on 2DPD, day 0 and day 2 resulted in increases of lipid accumulation (Fig. 4.6B), indicating oxidative stress showed a stimulatory effect on the events that occurred during these three stages: cell-inhibition stage (2DPD), mitotic clonal expansion and adipocyte differentiation (day 0) and lipid accumulation in adipocytes (day 2).

To date, the only events that are known to take place during cell-inhibition stage are epigenetic modifications which include DNA methylation and histone modifications (Guo et al., 2009). Several reports had shown that increased intracellular ROS can result in long-lasting epigenetic modifications (Giacco & Brownlee, 2010). Therefore, it is feasible to postulate that the stimulatory effect of glucose oxidase when introduced into the system during cell-inhibition (2DPD) (Fig. 4.6B) was closely associated with the epigenetic modifications.

Arising evidences showed that oxidative stress plays important roles in the differentiation of adipocytes. Adipogenesis was found to be increased under oxidizing condition and decreased under reducing conditions (Imhoff & Hansen, 2010). In relation to this, intracellular ROS derived from mitochondria stress, endoplasmic reticulum stress and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases were also found to enhance adipocyte differentiation (Liu, Chan, Higuchi, Dusting, & Jiang, 2012). Conversely, during adipogenesis, treatment of NADPH oxidase inhibitors or antioxidants such as genistein, resveratrol or N-acetylcysteine repressed ROS levels which in turn resulted in the inhibition of adipocyte differentiation and lipid accumulation in 3T3-L1 adipocytes (Calzadilla et al., 2011; Lee, Lee, Choi, Ko, & Kim, 2009). The downregulation of NADPH oxidase 4 (NOX4), one of the multiple isoforms of NADPH oxidase, serving as the major source of ROS in pre-adipocytes, resulted in the repression of ROS production and adipogenesis. The effect was reversed when NOX4 was over-expressed (Ye, Zhang, Townsend, & Tew, 2015). In concordance with the previous studies, the present study which used glucose oxidase to generate hydrogen peroxide to induce oxidative stress also showed stimulatory effect on adipogenesis (Fig. 4.6B).

Oxidants such as hydrogen peroxide, produced upon the stimulation of insulin in the target cells, were shown to have insulin-mimetic properties in adipocytes, such as enhancement of glucose transport and lipogenesis, and inhibition of lipolysis - suggesting that ROS such as hydrogen peroxide may act as second signaling messengers in the insulin signaling cascade (Almoguera, Cubero, Moreno, & Medina., 1982; Liu et al., 2012; Mukherjee, 1980). The hypothesis of the involvement of an oxidation step and ROS as second signaling messengers in the insulin signaling cascade was proposed decades ago but was not tested until the scientists discovered the potential mechanisms recently. Recent identification of the family of NADPH oxidase that

generates ROS upon cellular stimulation by different growth factors has renewed the interest to investigate the role of ROS as second signaling messengers in response to insulin action. Several studies have shown that transient bursts of low levels of ROS upon the stimulation of growth factors, cytokines, and hormones, were observed. Signaling molecules that have reduced cysteine thiol side chains which are mandatory for their catalytic activity show the potential to be inhibited by biochemical oxidation, for instance, protein-tyrosine phosphatases (PTPs). In general, PTPs inhibits insulin action by dephosphorylating the insulin receptor and its tyrosine-phosphorylated cellular substrates. Nevertheless, the enzyme activity can be effectively halted upon the rapid oxidation of the catalytic cysteine of target PTPs by ROS, reversing their repression on insulin signaling (Goldstein et al., 2005). Recent studies suggest that NOX4 may be involved in the generation of cellular ROS upon the stimulation of insulin which is coupled to the insulin activity. Oxidation and inhibition of PTP1B (a major regulator of the insulin signaling cascade) were observed in the same study (Goldstein et al., 2005; Liu et al., 2012). Based on these findings, it is possible that low concentrations of glucose oxidase could lead to stimulation of lipid accumulation via similar mechanism. To unveil the potential mechanism involved, mRNA expression of selected genes were assessed and discussed in Section 5.7.2.

5.4.3 CO-TREATMENT WITH MONOTERPENES AND GLUCOSE OXIDASE

Recent studies showed that antioxidant therapies have the potential to improve T2DM related complications (Huynh et al., 2014; Kabel, 2014). Since the accumulation of lipid in adipocytes may reflect insulin-sensitivity, the effect of monoterpenes on total lipid accumulation in 3T3-L1 adipocytes under oxidative stress was further investigated in the current study. Co-incubation of 100 nU/mL of glucose oxidase with 1 μM geraniol, (R)-(+)-limonene and γ-terpinene on 2DPD, day 0 and day 2 did not show any significant difference compared to the negative control (100 nU/mL of glucose oxidase) (Fig. 4.6C). The results suggest that during the co-incubation of glucose oxidase and monoterpenes, the effect of glucose oxidase on differentiation and lipid accumulation in 3T3-L1 adipocytes was predominant compared to geraniol (that stimulated both adipocyte differentiation and lipid accumulation) and γ-terpinene (that stimulated adipocyte differentiation and inhibited lipid accumulation) (Fig. 4.6A). Although several studies had shown that antioxidants suppressed ROS levels in 3T3-L1 adipocytes and in turn, inhibited adipocyte differentiation and lipid accumulation (Ye et al., 2015), the lack of adipogenesis inhibition in the current study was speculated to be due to the mild antioxidant property of monoterpenes that was not sufficient to counteract the effect of ROS in adipogenesis (Fig. 4.1, Fig. 4.2 and Table 4.1).

Since co-treatment of glucose oxidase and monoterpenes did not result in any significant results compared to the negative control (Fig. 4.6C), gene expression study for the combination effect of monoterpenes and glucose oxidase in adipocyte differentiation and total lipid accumulation was not carried out.

5.5 EFFECT OF MONOTERPENES ON GLUCOSE UPTAKE IN MATURE 3T3-L1 ADIPOCYTES

Glucose uptake plays an important role in the control of plasma glucose level, thus directly influencing glucose tolerance. The effect of monoterpenes on glucose uptake was examined by exposing mature 3T3-L1 adipocytes to 1 µM of various monoterpenes. Results showed that only certain but not all monoterpenes exerted stimulatory effect on the glucose uptake (Fig. 4.7). Interestingly, treatment of 1 µM(R)-(+)-limonene stimulated glucose uptake in 3T3-L1 adipocytes by 17.4%, which is in concordance with the previous report by Murali and Saravanan (2012), which showed that streptozotocin-induced diabetic rats fed with (R)-(+)-limonene experienced a repression in plasma glucose level, suggesting an improvement of glucose uptake. Another study by Jing et al. (2013) also showed that limonene reduced fasting plasma glucose. Further, More, Kulkarni, Nalawade, & Arvindekar (2014) and Santiago, Jayachitra, Shenbagam, & Nalini (2012) also reported that limonene improved glucose uptake in streptozotocin-induced diabetic rat and high-fat-diet-fed rat supplemented with limonene respectively with no significant change in the body weight of the untreated groups.

Glucose uptake in cells is mediated by glucose transporters (GLUTs), GLUT1 to GLUT14, a family of integral membrane proteins that mediate sodium-independent, facilitated-diffusion of hexoses across plasma membrane. Two main glucose transporter isoforms, namely, GLUT1 and GLUT4 are responsible for the glucose uptake in insulin-responsive tissues such as skeletal muscles and adipocytes. Predominantly, GLUT1 is expressed and found on the plasma membrane and intracellular membranes of both pre-adipocytes and adipocytes. On the other hand, GLUT4 is expressed during the course of adipocyte differentiation and sequestered in intracellular vesicles and translocated to the plasma membrane upon insulin stimulation. As a major insulin-

responsive glucose transporter, GLUT4 serves as the rate-controlling step in insulin-mediated glucose disposal which directly affects the insulin sensitivity (Herman & Kahn, 2006; Mueckler & Thorens, 2013). Extensive studies have been targeted exclusively on the expression of GLUT4 in glucose uptake in the control of T2DM (Jiang et al., 2014). Nonetheless, there is increasing evidence which shows that the enhancement of glucose uptake can commence via GLUT1 (Choi et al., 2011). Therefore, in the current study, the mRNA expression of both GLUT1 and GLUT4 were further investigated and the results are discussed in Section 5.7.

5.6 EFFECT OF MONOTERPENES ON LIPOLYSIS IN MATURE 3T3-L1 ADIPOCYTES

Lipolysis is a catabolic process that hydrolyzes TGs stored in lipid droplets liberating glycerols and fatty acids which serve as energy substrates during energy deprivation (Zechner et al., 2012). In the present study, the lipolytic effect of monoterpenes in 3T3-L1 adipocytes was assessed. Interestingly, monoterpenes such as (R)-(+)-limonene, (S)-(-)-perillyl alcohol, (R)-(+)-β-citronellol and geraniol which enhanced glucose uptake (Fig. 4.7), also stimulated lipolysis (Fig. 4.8). However, (S)-(-)-perillyl alcohol, (R)-(+)-β-citronellol and geraniol that stimulated both glucose and lipolysis also increased total lipid accumulation in 3T3-L1 adipocytes [(Fig. 4.6A(iii)]. This scenario was speculated to be due to the faster rate of glucose uptake than lipolysis. On the other hand, in concordance with earlier findings in this report, the insignificant change in total lipid accumulation in 3T3-L1 adipocytes treated with (R)-(+)-limonene during day 2 [(Fig. 4.6A(iii)] was possibly due to the concurrent elevation of glucose uptake and lipolysis.

In general, excessive circulating fatty acids caused by increased lipolysis is closely associated with the development of insulin resistance (Ahmadian et al., 2010). However, recent findings have demonstrated that increased lipolysis is not necessary to induce elevated serum fatty acids, suggesting enhancement of insulin sensitivity via lipolysis is possible. Adipose-specific phospholipase A_2 (AdPLA), an intracellular calcium-dependent PLA, has been shown to elevate prostaglandin E2 (PGE2) level that exhibited anti-lipolytic effect at low concentrations via cAMP suppression. Ablation of AdPLA and overexpression of AGTL, respectively, exhibited elevated lipolysis in murine adipocytes without causing an increase in serum fatty acid level, attributed to the concomitant increase of fatty acid oxidation within adipocytes (Ahmadian et al., 2010). This fatty acid oxidation within adipocytes might also explain the inhibitory effect of γ-

terpinene on total lipid accumulation in 3T3-L1 adipocytes [(Fig. 4.6A(iii)] though γ-terpinene stimulated glucose uptake and did not seem to stimulate lipolysis (Fig. 4.8) in the current study. Hence, it is imperative that further in depth investigation on compounds that can stimulate lipolysis, especially those with stimulatory effect on glucose uptake is carried out.

5.7 EFFECT OF MONOTERPENES AND GLUCOSE OXIDASE ON THE REGULATION OF GENE EXPRESSION

5.7.1 TREATMENT WITH (R)-(+)-LIMONENE

Effects of monoterpenes on glucose uptake and lipid metabolism in 3T3-L1 adipocytes are rarely studied and the underlying mechanisms remain unclear. Therefore, in the current study, all monoterpenes that showed significant changes in the total glucose uptake or/and lipolysis deserve further investigation. However, only one representative monoterpene was selected for the gene expression study.

The monoterpene, (R)-(+)-limonene, was selected for the gene expression study since it showed the positive stimulatory effect on glucose uptake (Fig. 4.7) and lipolysis (Fig. 4.8) with no increase in the total lipid accumulation in 3T3-L1 adipocytes [(Fig. 4.6A(iii)]. In addition, previous studies conducted by More, Kulkarni, Nalawade, & Arvindekar (2014) and Murali and Saravanan (2012) showed that streptozotocin-induced diabetic rats fed with (R)-(+)-limonene experienced a repression in plasma glucose level and insignificant change of body weight, suggesting an improvement of glucose uptake with improved insulin sensitivity but the underlying mechanism was not revealed. Therefore, the underlying mechanism of (R)-(+)-limonene on glucose uptake and lipid metabolism was investigated in this study.

After 48-hr exposure of 1 μM (R)-(+)-limonene to the cells during cell-inhibition stage (2DPD) and differentiation of 3T3-L1 adipocytes (day 0), mRNA expression of PPARγ was quantified. PPARγ, a ligand-activated transcriptional factor, is expressed predominantly in adipocytes and serves as the primary regulator of adipocyte differentiation. Activation of PPARγ regulates downstream genes involved in glucose and lipid metabolism through insulin-signaling cascade, giving rise to improved insulin sensitivity. Consistent decrease in the plasma glucose level in T2DM patients under the treatment of TZD, a PPARγ ligand, was concomitantly accompanied by the

remodeling of adipose tissues, with a marked replacement of large adipocytes by small insulin-sensitive adipocytes showing the importance of PPARγ activation (Choi et al., 2011). However, in the current study, exposure to 1 µM (R)-(+)-limonene [Fig. 4.9A(i-ii)] did not affect the mRNA expression of PPARγ in 3T3-L1 adipocytes. The insignificant change in the total lipid accumulation (Fig. 4.6A) suggests that 1 µM (R)-(+)-limonene did not exert significant effect on the differentiation of 3T3-L1 adipocytes.

The expression of several genes participating in the earlier maturation stage of 3T3-L1 adipocytes (day 2) after 48-hr exposure to 1 µM (R)-(+)-limonene was studied. Glucose uptake in adipocytes is attributed to the increased constitutive expression of GLUT1 proteins, or/and increase of the expression and translocation of GLUT4 from the cytosol to plasma membrane (Ku et al., 2014). Yamamoto et al. (2000) and Kohn, Summers, Birnbaum, & Roth (1996) demonstrated that active mitogen-activated protein kinase kinase (MAPKK) and Akt Ser/Thr kinase increased glucose uptake in 3T3-L1 adipocytes and mRNA expression of GLUT1 was augmented, but not mRNA expression of GLUT4. Further investigation revealed that the elevation of glucose uptake was attributed to not only to the increased expression of GLUT1, but also the translocation of GLUT4 from cytosol to plasma membrane. In this regard, cellular mRNA level alone can only serve as a reference but is not adequate to act as the absolute indicator of GLUT4 activity. The present results showed that treatment of 1 µM (R)-(+)-limonene in 3T3-L1 adipocytes significantly increased the mRNA expression of GLUT1 by 1.2 fold whereas the mRNA expression of GLUT4 remained unchanged [(Fig. 4.9A(iii)]. Therefore, the stimulatory effect of (R)-(+)-limonene on glucose uptake was likely, in part, rendered by its ability to upregulate the mRNA expression of GLUT1, possibly via MAPK signaling that is predominantly involved in the expression of GLUT1, but not GLUT4 (Yamamoto et al., 2000). Induction of glucose uptake by (R)-(+)-limonene via insulin signaling pathway was less likely as

insulin signaling is inversely correlated with increased lipolysis (Fig. 4B) (Zechner et al., 2012). Insulin downregulated both ATGL and HSL mRNA expression [Fig. 4.9A(iv)] whereas (R)-(+)-limonene decreased only ATGL mRNA expression but not HSL, suggesting (R)-(+)-limonene mediated activity possibly involved a pathway distinct from insulin. Stimulatory effect of (R)-(+)-limonene on glucose uptake via 5' adenosine monophosphate-activated protein kinase (AMPK) signaling was also less convincing as activation of this pathway not only markedly upregulated the expression of GLUT1, but also GLUT4 (Holmes & Dohm, 2004). Taken together, the current findings suggest that increased glucose uptake in 3T3-L1 adipocytes by (R)-(+)-limonene might possibly be via MAPK signaling.

Since ATGL and HSL are principal and rate limiting enzymes responsible for over 90% of TG hydrolase activity in murine white adipocytes (Zechner et al., 2012), the present gene expression study therefore focused primarily on ATGL and HSL mRNA expression. This study demonstrated that treatment with 1 μM (R)-(+)-limonene in mature 3T3-L1 adipocytes downregulated the mRNA expression of ATGL but mRNA expression of HSL remained unaffected, as compared to the negative control [Fig. 4.9A(iv)]. While gene expression results appeared to be counterintuitive with the elevated lipolysis by (R)-(+)-limonene treatment (Fig. 4.8), previous studies have also reported that isoproterenol and tumor necrosis factor-α exerted elevated lipolytic activity, even though with an attenuation of ATGL mRNA expression. The increase of lipolysis can be, however, explained by the increase of lipase activity via the activation of cAMP signaling. Perillipin-1, a lipid droplet-associated protein, interacts with comparative gene identification-58 (CGI-58), a coactivator protein of ATGL, preventing its binding to and, hence, activation of ATGL. Upon β-adrenergic stimulation, phosphorylation of perilipin-1 at multiple sites by PKA releasing CGI-58 results in the activation of ATGL activity and thus, lipolysis increases. Conversely, HSL

activity is directly activated by PKA phosphorylation (Holmes & Dohm, 2004; Zechner et al., 2012). Taken together, it is suggested that (R)-(+)-limonene-induced lipolysis could at least be, in part, via the same mechanism and downregulation of ATGL mRNA level by (R)-(+)-limonene could possibly be target-specific, rather than via the inhibition of cAMP signaling or PPAR signaling that regulates adipocyte differentiation. In order to determine the signaling pathways involved in both glucose uptake and lipolysis, further gene expression studies are necessary. In addition, proteomics assays and *in vivo* studies can further be conducted to confirm the protein expressions and anti-obese and anti-diabetic effects of monoterpenes in animal model.

5.7.2 TREATMENT WITH GLUCOSE OXIDASE

Since oxidative stress plays important roles in the pathogenesis of T2DM (Kabel, 2014), effect of glucose oxidase on the expression of genes associated with differentiation and lipid metabolism and GPx expression in 3T3-L1 adipocytes were studied. Several studies showed that ROS stimulated adipogenesis by activating redox-sensitive signaling pathways and transcription factors, including PPARγ (Ye et al., 2015). However, results of the present study showed that 48-h incubation of 100 nU/ml glucose oxidase in 3T3-L1 adipocytes on 2DPD and day 0 did not alter the mRNA expression of PPARγ [Fig. 4.9B(i-ii)] though increased adipogenesis was observed (Fig. 4.6B) suggesting the increased adipogenesis was via another mechanism.

Previous studies also showed that after the exposure to hydrogen peroxide in 3T3-L1 adipocytes, expression of GLUT1 increased whereas expression of GLUT4 decreased (Rains & Jain, 2011). Interestingly, in the current study, glucose oxidase that stimulated lipid accumulation (Fig. 4.6B) downregulated mRNA expression of GLUT1 whereas the mRNA expression of GLUT4 remained unaffected [Fig. 4.9B(iii)]. However, the stimulatory effect of glucose oxidase on lipid accumulation in 3T3-L1

adipocytes (Fig 4.6B) can be explained by the downregulation of ATGL [Fig. 4.9B(iv)], a rate limiting enzyme in lipolysis. The potential of anti-lipolytic effect of glucose oxidase in the present study can be supported by the insulin-mimetic properties of hydrogen peroxide including inhibition of lipolysis as described by Almoguera et al. (1982) and Mukherjee (1980).

In general, upon the exposure to hydrogen peroxide, GPx expression would be upregulated in order to catalyze the reduction of excessive hydrogen peroxide (ROS) to protect the cell from oxidative damage (Pendergrass, Rafferty, & Davis, 2011). However, in the present findings, mRNA expression of GPx was not altered after the exposure to 100 nU/ml glucose oxidase suggesting that the constant generation of hydrogen peroxide at low dose was not sufficient to stimulate the mRNA expression of GPx.

Collectively, these controversies discussed above have suggested that constant low dose of hydrogen peroxide generated by glucose oxidase which mimics physiological environment, might stimulate adipogenesis and lipid accumulation in 3T3-L1 adipocytes were via another mechanism.

Paradoxically, chronic oxidative stress has been closely related to reduced insulin sensitivity, impaired β-cell functions and pathophysiology of T2DM (Goldstein et al., 2005). However, taken together the positive response of glucose oxidase in adipocyte differentiation and lipid accumulation, suggests that it is important to understand the role of oxidative stress in adipocytes to help in the discovery of a variety of possible and effective treatments for obesity and T2DM.

CHAPTER SIX: CONCLUSION

The current comprehensive study supports the underlying mechanism of some novel biological effects of monoterpenes, including a mild antioxidant capacity, inhibition of α-amylase and α-glucosidase activity, stimulation of glucose uptake and lipolysis. It is hoped that these novel biological findings will be useful in increasing the awareness on monoterpenes not only as food additives but also potential as a cost-effective strategy in the control of obesity and T2DM.

The current study also showed that low levels of constant oxidative stress does not necessarily upregulate the GPx mRNA expression, however, it is adequate to initiate the stimulation of adipogenesis and lipid accumulation in 3T3-L1 adipocytes. Remarkably, this novel finding serves to provide some insights into the role of low oxidative stress in adipogenesis and lipid accumulation opening new avenues of research concerning pathophysiology of obesity and T2DM, with the hope of discovering better strategies in the control of obesity and T2DM.

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LIST OF PUBLICATION AND PROCEEDINGS

LIST OF PUBLICATION

Tan, X. C., Chua, K. H., Ram, M. R., & Kuppusamy, U. R. (2016). Monoterpenes: Novel insights into their biological effects and roles on glucose uptake and lipid metabolism in 3T3-L1 adipocytes. *Food chemistry, 196*, 242-250.

LIST PROCEEDINGS

Tan, X. C., Chua, K. H., & Kuppusamy, U. R. (2012, December). *Prolonged oxidative stress stimulates differentiation and lipogenesis in 3T3-L1 adipocytes.* Poster presentation at 17th Biological Science Graduate Congress, Bangkok, Thailand.

Tan, X. C., Chua, K. H., & Kuppusamy, U. R. (2013, November). *d-Limonene promotes glucose uptake and lipolysis in 3T3-L1 adipocytes.* Poster presentation at 3rd Asia-Korea Conference on Science and Technologies 2013, Singapore.

APPENDICES

APPENDIX A: List of chemicals and assay kits used in the current study.

Description	Brand
2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) radical scavenging assay	
DPPH	Sigma-Aldrich, USA
Ascorbic acid	Sigma-Aldrich, USA
2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity	
ABTS	Boehringer Mannheim, Germany
Potassium persulphate ($K_2O_8S_2$)	Merck, Germany
Trolox	Sigma-Aldrich, USA
Ferric reducing antioxidant power (FRAP) assay	
Sodium acetate trihydrate	Merck, Germany
Glacial acetic acid	Merck, Germany
2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ)	Sigma-Aldrich, USA
Hydrochloric acid	Merck, Germany
Ferric chloride ($FeCl_3$•$6H_2O$)	Merck, Germany
Ferrous sulphate ($FeSo_4$•H_2O)	Sigma-Aldrich, USA
α-Glucosidase inhibitory assay	
α-Glucosidase (from *S. cerevisiae*)	Sigma-Aldrich, USA
Bovine serum albumin (BSA)	Sigma-Aldrich, USA
4-nitrophenyl-α-D-glucopyranoside (PNPG)	Sigma-Aldrich, USA
Sodium carbonate	Fisher Scientific Limited, UK
α-Amylase inhibitory assay	
Porcine pancreatic α-amylase	Sigma-Aldrich, USA
Starch	Sigma-Aldrich, USA
3,5-Dinitrosalicylic_acid (DNS)	Sigma-Aldrich, USA
Sodium potassium tartrate dehydrate	Fisher Scientific Limited, UK
Sodium hydroxide (NaOH)	Fisher Scientific Limited, UK
Cell culture	
Dulbecco's Modification of Eagle's Medium (DMEM)	Sigma-Aldrich, USA
Minimum Essential Medium (MEM)	Sigma-Aldrich, USA
Fetal bovine serum (FBS)	Sigma-Aldrich, USA
L-glutamine	Sigma-Aldrich, USA
Penicillin-streptomycin	Invitrogen, California
Insulin	Invitrogen, California
1-methyl-3-isobuylxanthine (IBMX)	ICN,Ohio
Dexamethasone (DEX)	ICN, Ohio
0.25% trypsin-EDTA	Sigma-Aldrich, USA
Phosphate-buffered saline tablets (PBS)	Flowlab, Australia

APPENDIX A, continued: List of chemicals and assay kits used in the current study.

Description	Brand
Cell culture	
Trypan blue dye	Sigma-Aldrich, USA
Cell proliferation assay (MTT assay)	
3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)	Merck, Germany
Oil Red O quantification assay	
Oil Red O dye	Sigma-Aldrich, USA
Formaldehyde	Sigma-Aldrich, USA
Isopropanol	Fisher Scientific Limited, UK
Glucose uptake assay	
Glucose GOD kit	DiaSys Diagnostic Systems GmbH, Germany
Glucose	Sigma-Aldrich, USA
Lipolysis assay	
EnzyChromTM Adipolysis Assay Kit	BioAssay Systems, USA
RNA extraction	
Ambion-RNAqueous Micro® kit	Applied Biosystems, USA
RNA integrity check	
RNA 6000 Nano Assay	Applied Biosystems, USA
RNAseZAP	Applied Biosystems, USA
RNase-free water	Applied Biosystems, USA
cDNA synthesis	
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems, USA
Quantitative real time PCR	
TaqMan® Fast Advanced Master Mix	Applied Biosystems, USA
TaqMan® probe mix	Applied Biosystems, USA
Optical reaction plate (96-well)	Applied Biosystems, USA
Optical film	Applied Biosystems, USA
Common reagent/chemical	
Acarbose	Sigma-Aldrich, USA
Monosodium phosphate (sodium dihydrogen phosphate)	Fisher Scientific Limited, UK
Disodium phosphate (sodium hydrogen phosphate)	Fisher Scientific Limited, UK
Dimethyl sulphoxide (DMSO)	Fisher Scientific Limited, UK
Ethanol (EtOH)	Fisher Scientific Limited, UK

APPENDIX B: List of consumables used in the current study.

Description	Brand
75 cm^3 tissue culture flasks	Nunc, Denmark
25 cm^3 tissue culture flasks	Nunc, Denmark
96-well microplate, sterile	Nunc, Denmark
24-well microplate, sterile	Nunc, Denmark
96-well microplate, non-sterile	ISS, UK
Minisart®syringe filter (0.2 µm, sterile)	Sartorius, Germany

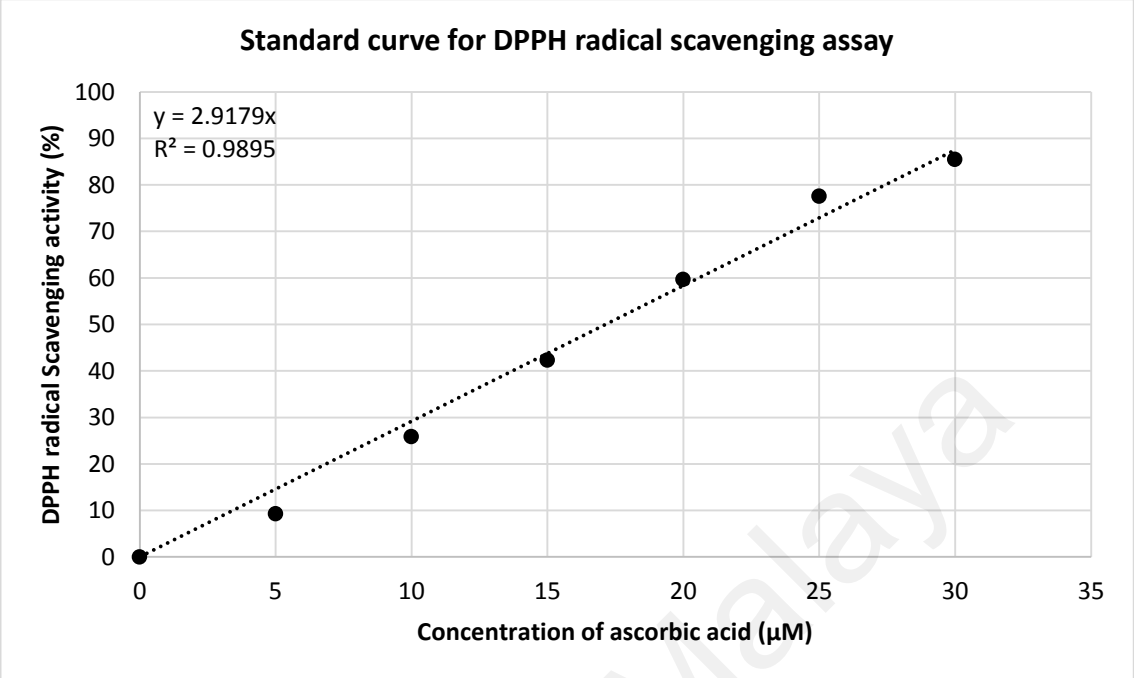
APPENDIX C: List of instruments used in this study.

Description	Instrument/Software	Brand
DPPH assay	Eon™ Microplate Spectrophotometer **Software:** Gen5 v2.00.18	Bio-Tek Instruments Inc, USA
ABTS assay		
FRAP assay		
α-Glucosidase inhibitory activity		
α-Amylase inhibition activity		
Cell proliferation assay (MTT assay)		
Oil Red O quantification assay		
Total glucose uptake assay		
Lipolysis assay		
Cell culture work	Autoclave machine (HVE-50 HiClave)	Hirayama, USA
	Class II biohazard safety cabinet (NU 425-400E)	Nuaire™, USA
	Water Jacket CO_2 Incubator (AutoFlow NU-4750)	Nuaire™, USA
	Inverted lab microscope system (Olympus CK40-SL)	Olympus Optical Co., Japan
	Haemocytometer set	Hirschmann Laborgerate, Germany
	Tally counter (RS-4)	Milky Way, Taiwan
RNA Integrity Check	Agilent® 2100 Bioanalyzer **Software:** 2100 Expert Software	Applied Biosystems, USA

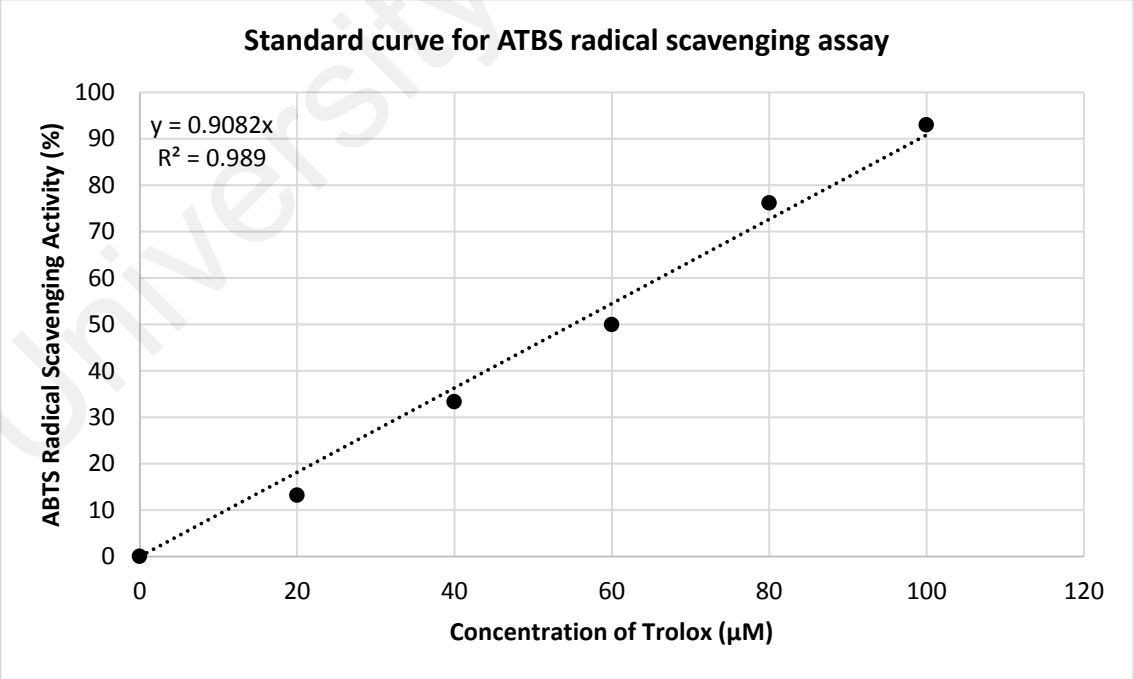
APPENDIX C, continued: List of instruments used in this study.

Description	Instrument/Software	Brand
cDNA synthesis	Thermal cycler (MyCycler™)	Bio-RAD Laboratories Inc., USA
Quantitative real time PCR	Applied Biosystems 7500 Fast Real-Time PCR System **Software:** 7500 Software v2.0.4	Applied Biosystems, USA
	Vortex mixer (MS2-S8/MS2-S9)	Applied Biosystems, USA
Common equipment	Laboratory safety hood	IRYAS, Malaysia
	Ultrapure water purification system (MiliQ® system)	Milipore Co., USA
	Refrigerator (NR-B35TA/37T/41FB)	Matsushita National Electronic Industrial Co., Japan
	Freezer (-80 °C Forma Scientific/ULT)	Thermo Electron Corporation, USA
	Water bath system	Memmert GmbH, Germanay
	Centrifuge System (C312)	Jouan, France
	Microcentrifuge system (BIOFUGE *pico*)	Heraeus, Germany
	Autovortex mixer (Stuart® SA2)	Bibby Scientific Ltd., UK
	Minishaker (MSI)	IKA® Werke GmbH, Germany
	Pipette: 0.5–10 µl, 10–100 µl, 20–200 µl, 100–1,000 µl (Eppendorf® Research® plus)	Eppendorf AG, Germany
	12-channel pipettes: 50–1200 µl (Sartorius eLINE®)	Sartorius, Germany
	Electronic clock timer (CT-20)	Canon Inc., Japan

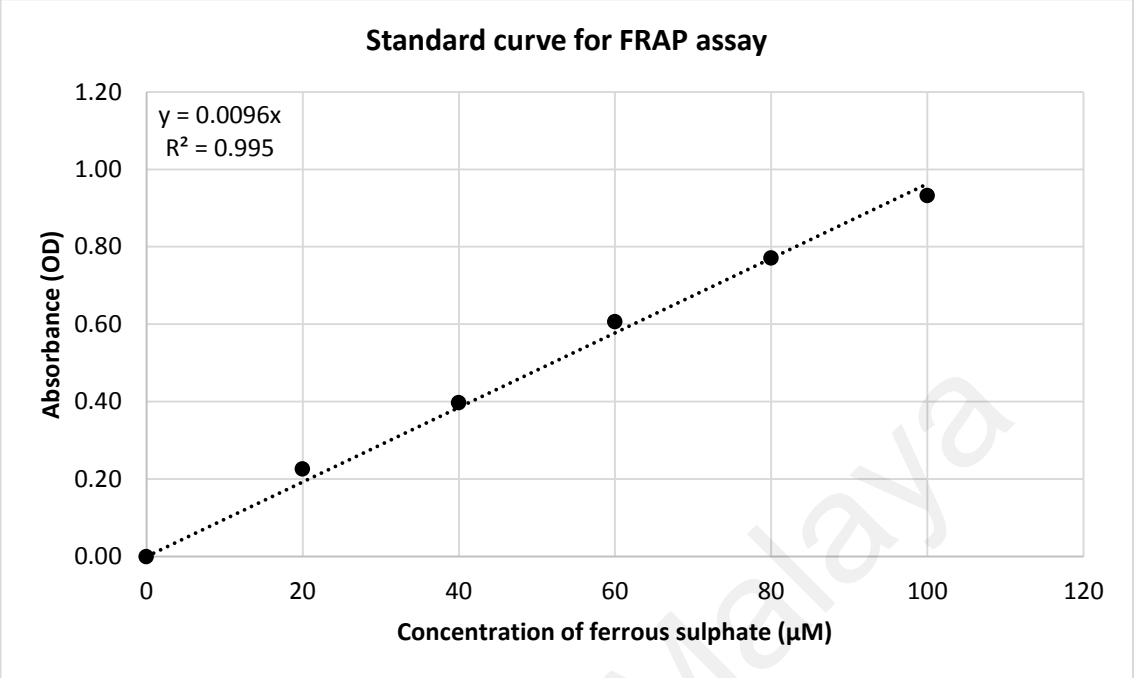
APPENDIX D: Standard curve for DPPH radical scavenging assay.



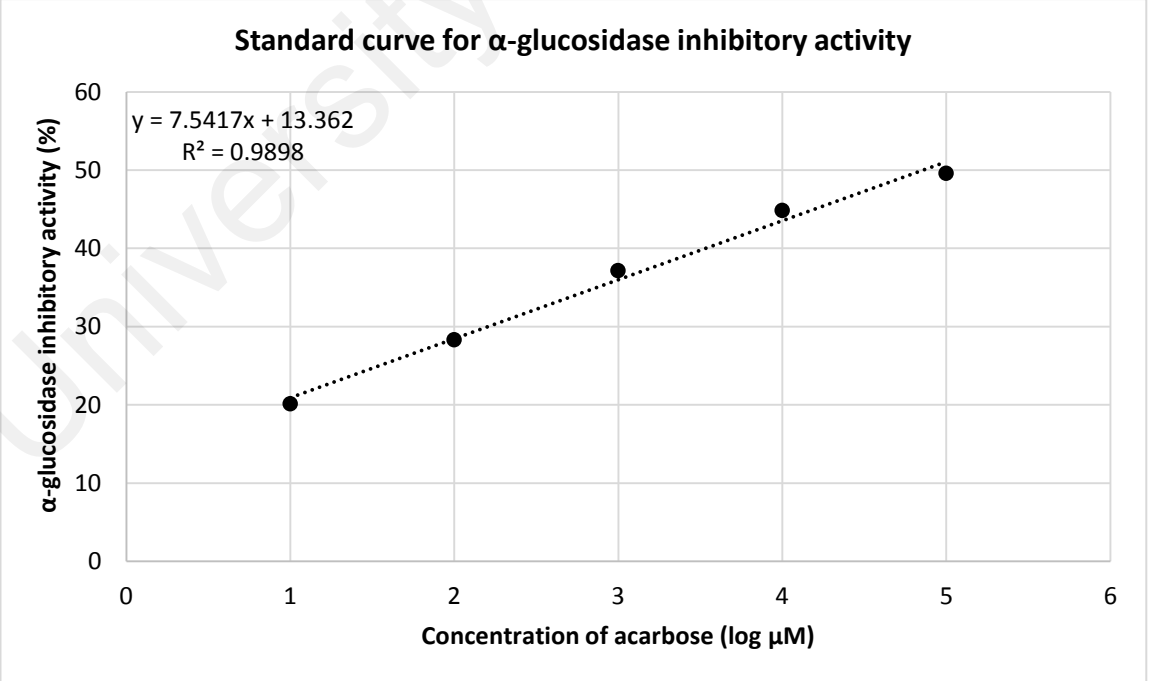
APPENDIX E: Standard curve for ABTS radical scavenging assay.



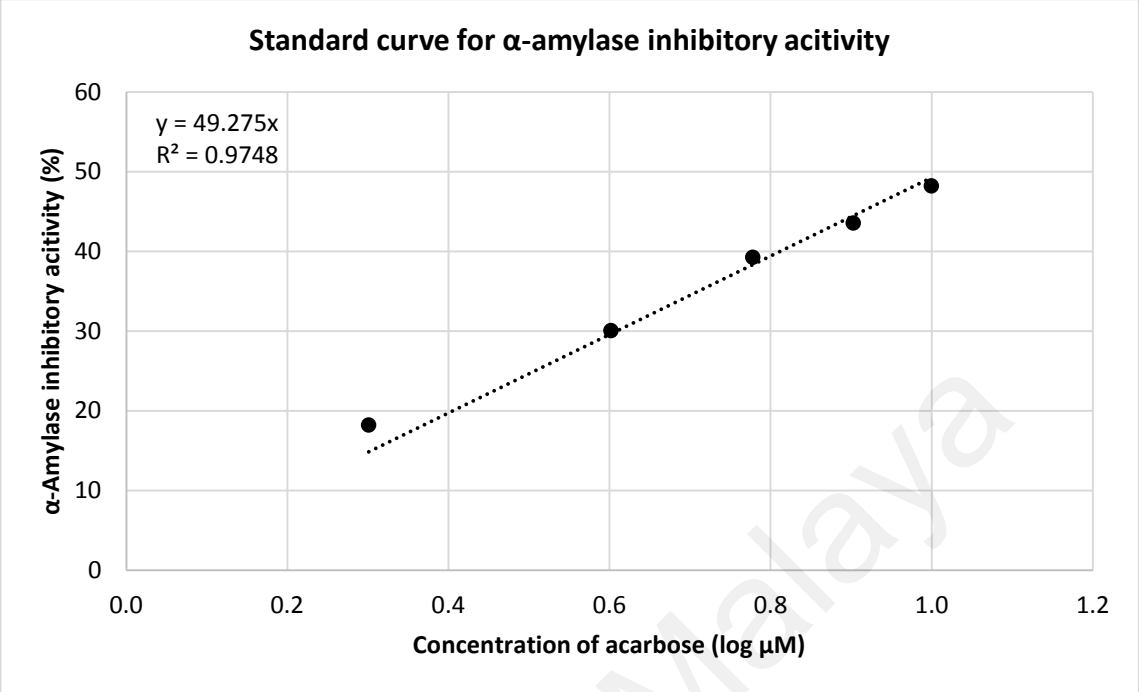
APPENDIX F: Standard curve for FRAP assay.



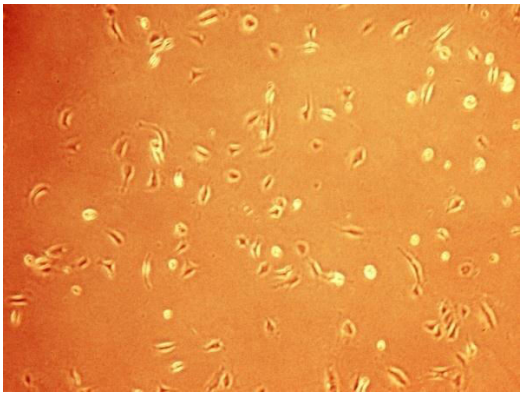
APPENDIX G: Standard curve for α-glucosidase inhibitory activity.



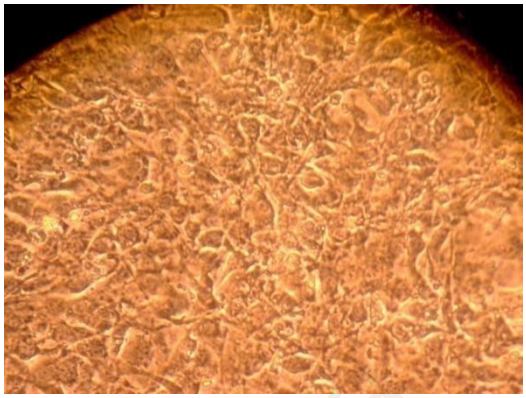
APPENDIX H: Standard curve for α-amylase inhibitory activity.



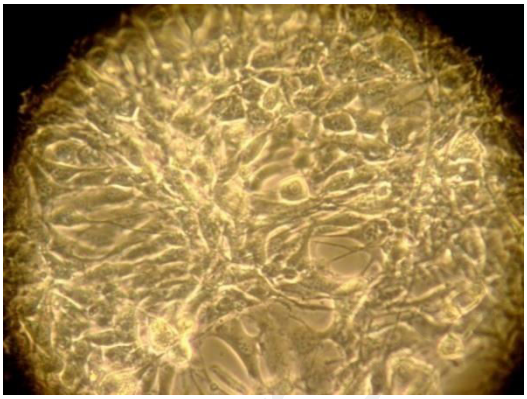
APPENDIX I: Morphology of 3T3-L1 adipocytes when cells were (A) pre-confluent, (B), confluent, (C) differentiating and (D) maturing.



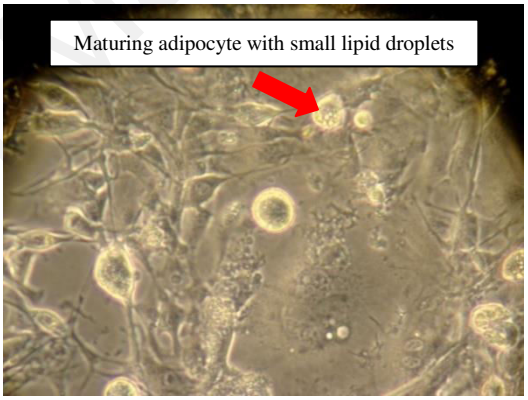
(A) Pre-confluent pre-adipocyte (100x magnification)



(B) Confluent pre-adipocyte (100x magnification)

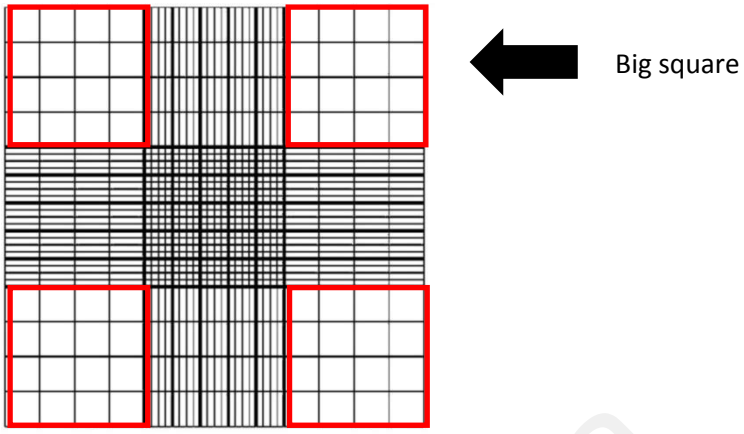


(C) Differentiating adipocyte (200x magnification)

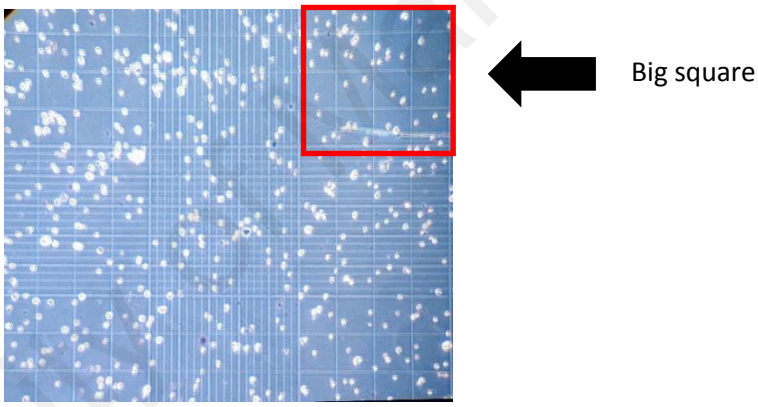


(D) Maturing adipocyte (200x magnification)

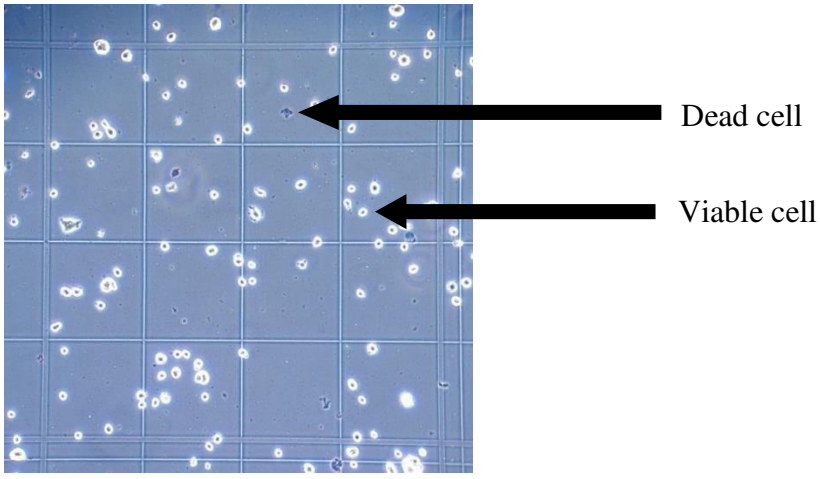
APPENDIX J: Illustration of haemocytometer's squares.



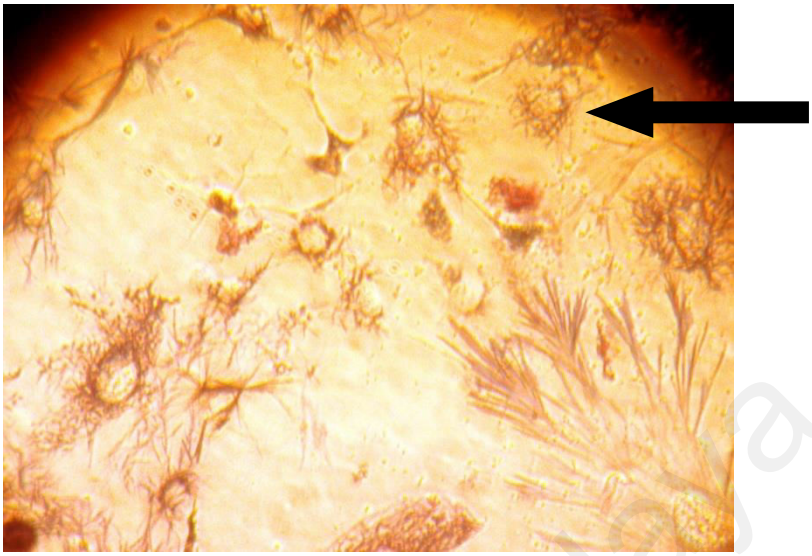
APPENDIX K: 3T3-L1 pre-adipocytes stained with trypan blue viewed under an inverted microscope (50x magnification).



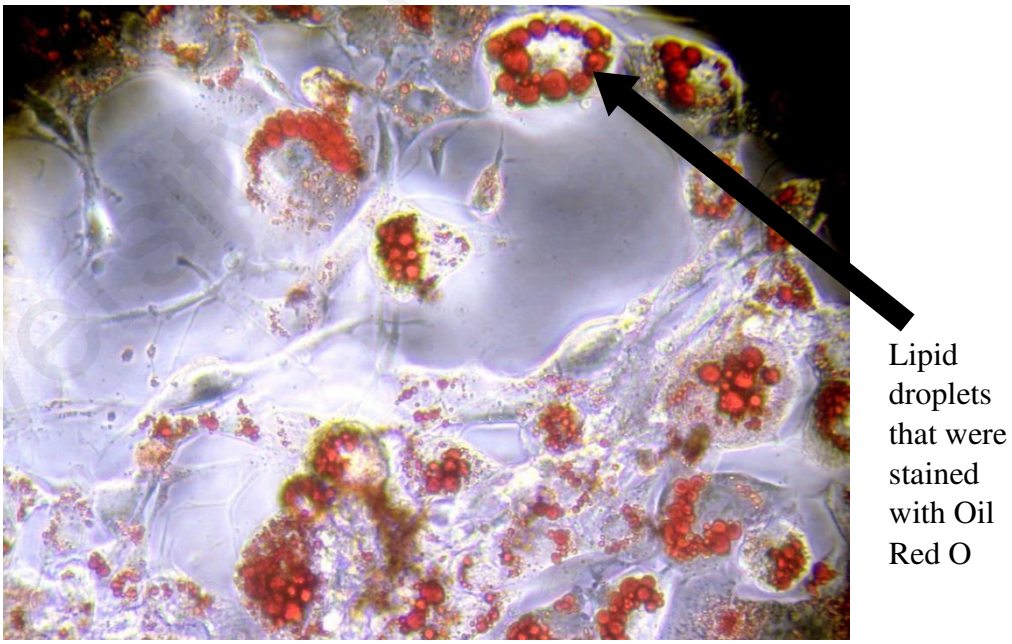
APPENDIX L: 3T3-L1 pre-adipocytes stained with trypan blue viewed under an inverted microscope (100x magnification).



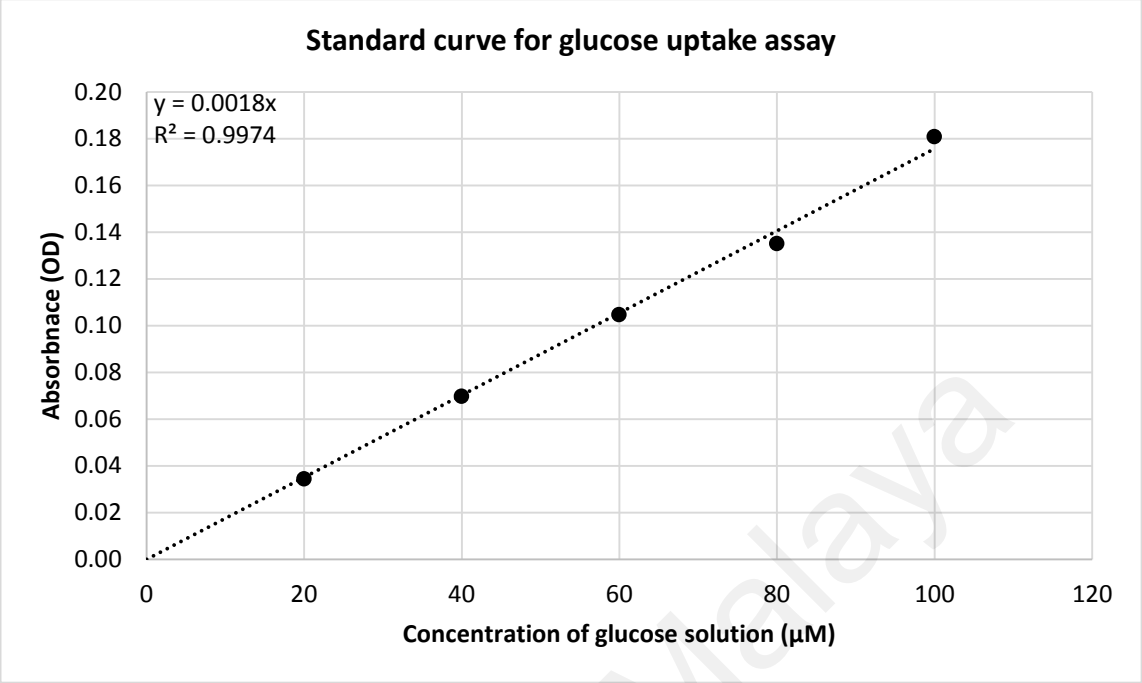
APPENDIX M: Purple formazan crystals in 3T3-L1 pre-adipocytes after 4-hour incubation with MTT in dark (200x magnification).



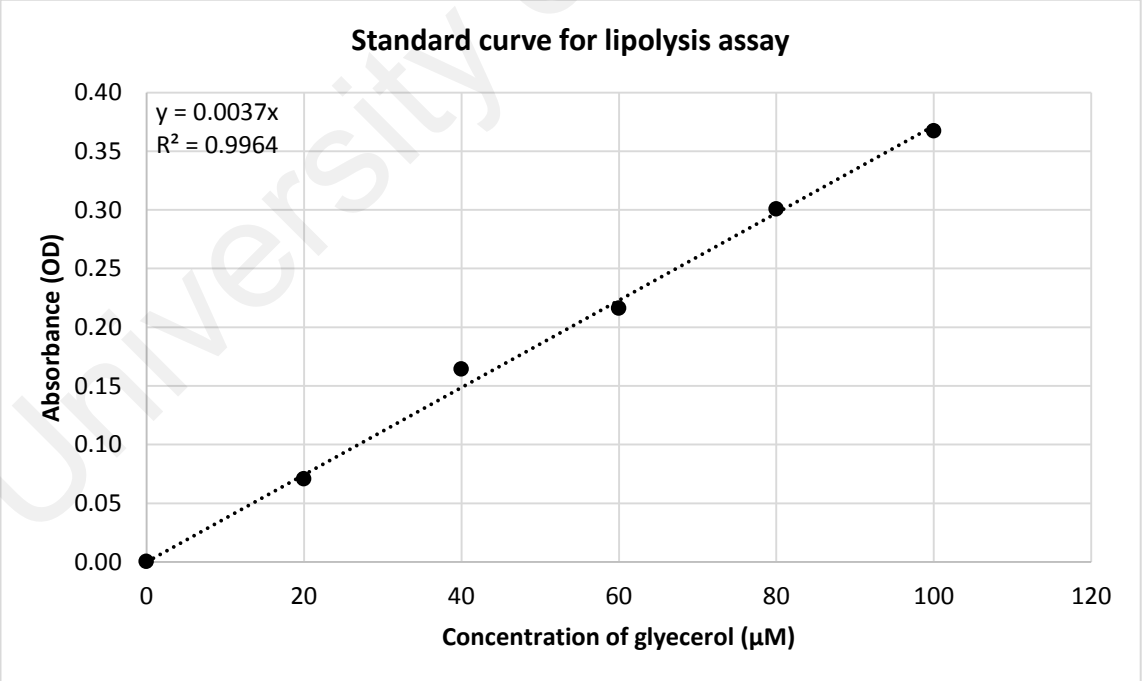
APPENDIX N: Mature adipocytes with lipid droplets that were stained with Oil Red O (200x magnification).



APPENDIX O: Standard curve for glucose uptake assay.



APPENDIX P: Standard curve for lipolysis assay.



APPENDIX Q: Set up for RNA integrity check.

Isolated RNA was subjected to purity and integrity check using RNA 6000 Nano Assay according to manufacturer's protocol. Firstly, the chip priming station was set up. A new syringe was inserted into the clip followed by the insertion into the hole of the luer lock adapter and screwed tightly to the chip priming station. Secondly, the electrode of the Agilent 2100 Bioanalyzer was decontaminated as described by the following process. One of the wells of an electrode cleaner was slowly filled with 350 µl RNAseZAP. The electrode cleaner was then placed in the Agilent 2100 Bioanalyzer. The lid of the bioanalyzer was closed and incubated for 1 min before removing it. After that, one of the wells of another electrode cleaner was slowly filled with 350 µl RNase-free water and placed into the same Agilent 2100 Bioanalyzer. The lid of the bioanalyzer was closed and incubated for about 10 sec. Subsequently, the electrode cleaner was removed and the lid of bioanalyzer was let open for another 10 sec to allow the water to evaporate. The bioanalyzer was then ready for use.

All reagents of RNA 6000 Nano Assay were acclimatized to room temperature 30 min before use. RNA 6000 Nano dye concentrate was light sensitive and kept in the dark. After 30 min, 550 µl of Agilent RNA 6000 Nano gel matrix was pipetted into a spin filter and centrifuged for 10 min at 1500 x *g* unit. The filtered gel was then aliquoted into several microfuge tubes with 65 µl each. The aliquots were stored at 4 °C and expired within a month. Next, Agilent RNA 6000 dye concentrate was vortex-mixed and centrifuged. After that, 1 µl of RNA 6000 Nana dye concentrate was added to a 65 µl of filtered gel and vortex-mixed thoroughly. This gel-dye mix was then centrifuged for 10 min at 13,000 x *g* unit.

After the gel-dye mix was ready, a new RNA Nano chip was placed on the chip priming station and 9 µl of the gel-dye mix was pipetted at the bottom of the well marked "G". The plunger of the syringe was ensured to be positioned at 1 ml and the

chip priming station was closed. The plunger of the syringe was pressed down until it was held by the clip. After 30 sec, the plunger was released until it at least moved back to the 0.3 ml mark. After another 5 sec, the plunger was slowly pulled back to the 1 ml position. The chip priming station was opened and 9 µl of gel-dye mix was pipetted into each of the indicated well. The RNA Nano chip was now ready for sample loading and subsequent steps.

APPENDIX R: RNA integrity and purity.

Treatment day	Treatment	RIN	RNA purity (A260/A280 ratio)
2DPD	Negative control (Growth medium/1% EtOH)	9.9	2.064
	1 µM of (R)-(+)-Limonene	10.0	2.054
	Negative control (Growth medium)	10.0	2.042
	100 nU/ml of Glucose oxidase	10.0	2.051
Day 0	Negative control (Growth medium/1% EtOH)	9.9	2.058
	1 µM of (R)-(+)-Limonene	10.0	2.070
	Negative control (Growth medium)	10.0	2.019
	100 nU/ml of Glucose oxidase	10.0	2.055
Day 2	Negative control (Growth medium/1% EtOH)	9.9	2.046
	5 µg/ml of Insulin	10.0	1.994
	1 µM of (R)-(+)-Limonene	9.7	2.042
	Negative control (Growth medium)	10.0	2.020
	100 nU/ml of Glucose oxidase	10.0	2.021

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Monoterpenes: Novel insights into their biological effects and roles on glucose uptake and lipid metabolism in 3T3-L1 adipocytes

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ABSTRACT

Various strategies have been adopted to combat complications caused by Type 2 diabetes mellitus and controlled diet is one of them. Monoterpenes, major constituents of essential oils, are synthesized and widely used as artificial food flavors. A series of twelve monoterpenes were assessed in the present study. Monoterpenes, exhibited low 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) and 2,2′-azinobis-(3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity even at high concentrations. Some monoterpenes inhibited α-amylase and α-glucosidase activity and stimulated glucose uptake and lipolysis. Monoterpenes such as (R)-(+)-limonene stimulated both glucose uptake (17.4%) and lipolysis (17.7%); the mRNA expression of glucose transporter 1 (GLUT1) was upregulated but glucose transporter 4 (GLUT4) was unaffected, and adipose triglyceride lipase (ATGL) was suppressed. Taken together, the selected monoterpenes may not confer strong protection against free radicals but nevertheless, their positive influence on lipid and glucose metabolism may have potential in the control of obesity and Type 2 diabetes mellitus.

1. Introduction

Monoterpenes are amongst the major constituents of essential oils and abundantly found in citrus fruits, vegetables, spice and herbs. Monoterpenes are a class of terpenes, consisting of two isoprene units in the structures, which can either be in the form of acyclic (linear) or cyclic (ring). Monoterpenes are volatile compounds and characterized by strong odors responsible for the fragrance in many plants and fruits. Monoterpenes which are well known for their fragrance have been commercially used as artificial flavors in food, sanitary, cosmetic and perfume industries. In the past decades, increasing evidence showing biological effects of monoterpenes, such as antioxidant, anti-phlogistic, anti-tumor, antiviral and anti-nociceptive properties have intensified the usage and consumption of monoterpenes (Bakkali, Averbeck, Averbeck, & Idaomar, 2008; Choi, Song, Ukeda, & Sawamura, 2000; Kamatou, Vermaak, Viljoen, & Lawrence, 2013). Dietary intake of monoterpenes in human is fairly high. For instance, concentration of d-limonene, a fragrance additive with a lemon-like odor in candy, ice cream, orange juice and chewing gum is 49 ppm, 68 ppm, 100 ppm, and 2300 ppm, respectively. The U.S. per capita dietary consumption of both naturally occurring and artificial d-limonene is 0.27 mg/kg body weight/day. By this large scale consumption of monoterpenes, significant beneficial biological effects could possibly be reflected on human health in the long run. Evidence from the phase I clinical trials had showed that oral consumption of d-limonene in advanced cancer patients helped in the control of breast cancer and colorectal carcinoma which further strengthens the benefits of dietary monoterpenes in human health (Sun, 2007).

There have been numerous studies focused primarily on anti-cancer and anxiolytic properties of monoterpenes but not much is known with regards to their effects on obesity and Type 2 diabetes mellitus (T2DM). T2DM is one of the metabolic disorders characterized by chronic hyperglycemia due to impaired insulin production, insulin function, or both. T2DM is associated with complications such as retinopathy, nephropathy, peripheral neuropathy, cardiovascular complications and sexual dysfunction. Generally, obesity is the main predisposing factor to T2DM due to the increased insulin resistance in obese people (American Diabetes Association, 2014). Several effective interventions have been implemented over the past decades for the effective control of diabetes, such as changes in diet and exercise. In recent years, there is emerging evidence which indicates that quality and type of food are highly associated with the risk factors of T2DM. For instance, whole-grain diets with high fiber and polyunsaturated fat are associated with a lower risk of T2DM whereas diets with

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high *trans* fat increase the risk of T2DM (Hu, 2011). More interestingly, diet can induce changes in the composition of gut microbiota which then alters the metabolism of short-chain fatty acids (SCFA). SCFA such as butyrate have been shown to improve insulin resistance in dietary-obese mice (Shen, Obin, & Zhao, 2013). Collectively, these findings suggest that components in diet play important roles in the development of T2DM. This necessitates further studies and better understanding on food components.

Therefore, in the current study, the biological effects of monoterpenes were studied and their roles on glucose uptake and lipid metabolism in 3T3-L1 adipocytes were investigated. 3T3-L1 adipocytes were used as they are one of the most common cell culture models used in the study of obesity and T2DM due to their roles on glucose uptake, lipogenesis and lipolysis which contribute directly to the control of energy balance (Arsenijevic, Gregoire, Delforge, Delporte, & Perret, 2012). By understanding the biological effects of food components including additives such as monoterpenes, diet can be more effectively planned for a better control of obesity and T2DM.

2. Materials and methods

2.1. Materials

Geraniol (PubChem CID: 637566), nerol (PubChem CID: 643820), citral (PubChem CID: 638011), (R)-(−)-linalool (PubChem CID: 443158), (R)-(+)-limonene (PubChem CID: 440917), (S)-(−)-perillyl alcohol (PubChem CID: 369312), (R)-(+)-β-citronellol (PubChem CID: 101977), (S)-(−)-β-citronellol (PubChem CID: 7793), α-terpineol (PubChem CID: 443162), l-menthol (PubChem CID: 16666), γ-terpinene (PubChem CID: 7461) and terpinolene (PubChem CID: 11463) were purchased from Sigma–Aldrich (USA).

2.2. 2,2-Diphenyl-2-picrylhydrazyl hydrate (DPPH) radical scavenging activity

DPPH radical scavenging activity of monoterpenes was assessed as described by Tan et al. (2015) with slight modifications. To examine the DPPH radical scavenging activity of monoterpenes, 10 μl of test sample was mixed with 290 μl of 0.1 mM of DPPH ethanol solution and incubated for 3 h in the dark at room temperature prior to the measurement of absorbance at 517 nm. Ascorbic acid was used as a positive control. The radical-scavenging activity of test sample was expressed in percentage of inhibition, based on the following equation, DPPH radical scavenging activity (%) = (OD of blank − OD of test compound)/OD of blank × 100.

2.3. 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity

ABTS radical scavenging activity of monoterpenes was assessed as described by Tan et al. (2015) with slight modifications. ABTS radical monocations were generated by mixing 5 ml of 7 mM ABTS solution and 89 μl of 2.45 mM potassium persulphate ($K_2O_8S_2$). The solution was incubated for 12–16 h at room temperature in the dark before use. The working solution was prepared by diluting the previous solution with ethanol to obtain an absorbance of 0.7 ± 0.02 at a wavelength of 734 nm. The absorbance was measured using a spectrophotometer (Bio-Tech Instruments Inc, USA). Next, 10 μl of the test sample was added to 90 μl of the working solution in a 96-well plate and incubated for 2 h before the absorbance was measured. Trolox was used as the positive control. The radical-scavenging activity of the test sample was expressed in percentage of inhibition, calculated according to the following equation, ABTS radical scavenging activity (%) = (OD of blank − OD of test compound)/OD of blank × 100.

2.4. Ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power of monoterpenes was evaluated as described by Tan et al. (2015) with slight modifications. FRAP reagent was prepared by mixing 300 mM acetate buffer (pH of 3.6), 10 mM 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) in 40 mM hydrochloric acid and 20 mM ferric chloride ($FeCl_3{\cdot}6H_2O$) in a ratio of 10:1:1. The resulting orange-coloured reagent was incubated at 37 °C before use. To evaluate the reducing power of test sample, 10 μl of test sample was added to 290 μl of FRAP reagent for 3 h. The absorbance of the mixture was measured at 593 nm. Ferrous sulphate ($FeSO_4{\cdot}7H_2O$) was used as a positive control. The results were expressed as FRAP value (mol $FeSO_4{\cdot}7H_2O$/mol test compound).

2.5. α-Glucosidase inhibitory activity

α-Glucosidase solution was prepared by dissolving α-glucosidase from Saccharomyces *cerevisiae* in 0.1 M sodium phosphate buffer pH 6.8 supplemented with 0.2% bovine serum albumin (BSA) to give a concentration of 0.4 U/mL. Then, equal volumes (20 μl) of test sample, 0.4 U/mL α-glucosidase solution and 6 mM 4-nitrophenyl-α-d-glucopyranoside (PNPG) were mixed and allowed to stand at 37 °C for 15 min. To terminate the reaction, 80 μl of 0.2 M sodium carbonate was added to the reaction mixture and the absorbance was measured at 400 nm. Acarbose was used as a positive control. The α-glucosidase inhibitory activity of monoterpenes was expressed in percentage of inhibition, calculated as follows, α-Glucosidase inhibitory activity (%) = (OD of control − OD of test sample)/OD of control × 100 (Manaharan et al., 2011).

2.6. α-Amylase inhibitory activity

α-Amylase solution (2 U/ml) was prepared by dissolving porcine pancreatic α-amylase (Sigma Type IV-B) in ice-cold distilled water. Next, potato soluble starch solution (1%) was prepared by boiling starch in 20 mM phosphate buffer pH 6.9 until the solution became fully transparent. Then, 80 μl of the test sample was added to 40 μl of α-amylase solution. After 10 min incubation at room temperature, 40 μl of starch solution was added to the reaction mixture and allowed to stand for 10 min at 37 °C. To terminate the reaction, 80 μl of 3,5-dinitrosalicylic acid (DNS) solution consisting of 1 g DNS and 30 g sodium potassium tartrate dehydrate (dissolved in 100 ml of 2 M sodium hydroxide) was added and incubated at 95 °C for 10 min. The absorbance was measured at 540 nm. Acarbose was used as a positive control. The α-amylase inhibitory activity of monoterpenes was expressed in percentage of inhibition, calculated as follows, α-Amylase inhibitory activity (%) = (OD of control − OD of test sample)/OD of control × 100 (Manaharan et al., 2011).

2.7. 3T3-L1 cell culture and differentiation

Murine 3T3-L1 pre-adipocytes were grown and induced to differentiate into mature adipocytes as described by Kanagasabapathy, Chua, Malek, Vikineswary, and Kuppusamy (2014) with slight modifications. 3T3-L1 pre-adipocytes (American Type Culture Collection, Rockville, MD, USA) were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin–streptomycin (10,000 U/mL penicillin and 10,000 μg/mL streptomycin) and 4 mM L-glutamine at 37 °C in 95% air and 5% CO_2. To induce adipocyte

differentiation, 2-day post-confluent 3T3-L1 pre-adipocytes (day 0) were treated with DMEM/10% FBS supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 μM dexamethasone and 5 μg/ml insulin. After two days (day 2), the medium was replaced with DMEM/10% FBS and was changed at a 2-day interval thereafter until analysis.

2.8. Proliferation assay

Effect of monoterpenes on cell proliferation was evaluated using *in vitro* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Kanagasabapathy et al. (2014) with slight modifications. Pre-confluent 3T3-L1 pre-adipocytes were seeded in the 96-well plates at a density of 1000 cells/well and incubated for 24 h to allow the cells to adhere to the plate prior to the treatment with monoterpenes at a concentration of 1 μM. After 48 h incubation, 0.5 mg/mL MTT was added to the cells and incubated in the dark for 4 h at 37 °C. The medium was aspirated and the formazan crystals were solubilized in dimethyl sulphoxide (DMSO). The absorbance of the solution was measured at 550 nm using a spectrophotometer. Cell viability was expressed in percentage of control, calculated using the following equation, Cell viability (% of control) = (OD of treated cells/OD of negative control) × 100.

2.9. Total glucose uptake assay

Effect of monoterpenes on total glucose uptake in 3T3-L1 adipocytes were assessed as described by Cheng, Huang, Chang, Tsai, and Chou (2008) with slight modifications. 3T3-L1 pre-adipocytes were seeded in 96-well plates at a density of 20,000 cells/well and induced to differentiate into mature adipocytes. Mature 3T3-L1 adipocytes with lipid droplets were starved in serum-free DMEM. After 2 h, the medium was replaced with Eagle's Minimum Essential Medium (MEM). Monoterpenes (1 μM) was added to the medium and incubated for 5 h. Incubations were carried out at 37 °C in 95% air and 5% CO_2. After 5 h of glucose uptake by adipocytes, remaining glucose in the medium was measured by adding 5 μl of the medium to 500 μl of Glucose GOD kit (DiaSys Diagnostic Systems GmbH, Germany) in a 24-well plate and incubated at room temperature for 20 min prior to the absorbance reading at 500 nm. Insulin was used as a positive control. The glucose uptake by the cells was expressed in percentage of difference, based on the following equation, Glucose uptake (% of difference) = (OD of negative control − OD of treated cells)/OD of negative control × 100.

2.10. Lipolysis assay

Effect of monoterpenes on lipolysis in 3T3-L1 adipocytes was evaluated as described by Murosaki et al. (2007) with modifications. 3T3-L1 pre-adipocytes were seeded into 96-well plates at the density of 20,000 cells/well and induced to differentiate into mature adipocytes with lipid droplets. Mature adipocytes were starved in serum-free DMEM for 2 h before the medium was replaced with MEM. Monoterpenes (1 μM) was added to the medium at this point and further incubated for 5 h. Incubations were carried out at 37 °C in 95% air and 5% CO_2. Subsequently, free glycerol released into the medium was assayed using EnzyChrom™ Adipolysis Assay Kit (Bio Assay Systems, USA) following the manufacturer's protocol. Epinephrine was used as a positive control. The free glycerol released by the cells was expressed in percentage of difference, calculated using the following equation, Glycerol released by adipocytes (% of difference) = (OD of treated cells − OD of negative control)/OD of negative control × 100.

2.11. Oil Red O quantitative assay

(R)-(+)-Limonene was selected to study its effect on 3T3-L1 adipocyte differentiation and lipid accumulation using Oil Red O quantitative assay as described by Chai, Lim, Kanthimathi, and Kuppusamy (2011) with slight modifications. (R)-(+)-Limonene (1 μM) was added to the culture medium and incubated for 48 h at three distinct time points in three separated experiments, which were two days prior to differentiation (2DPD), day 0 and day 2. The lipid content in the mature adipocytes was quantified when adipocytes were mature with large lipid droplets. The mature adipocytes were washed with phosphate-buffered saline (PBS) followed by 10% formalin fixation for 1 h at room temperature. After that, the cells were washed with PBS and rinsed with 60% isopropanol and allowed to air dry. Subsequently, fixed cells were incubated with filtered 0.3% Oil Red O working solution (60% isopropanol and 40% water) in the dark for 1 h. After staining, the cells were washed with PBS and allowed to air dry. Isopropanol (100% v/v) was added to the stained cells to solubilize the Oil Red O. The absorbance of solution was measured spectrophotometrically at 510 nm. The total lipid accumulation by the cells was expressed in percentage of difference, calculated using the following equation, Total lipid accumulation (% of difference) = (OD of treated cells − OD of negative control)/OD of negative control × 100.

2.12. Gene expression study using quantitative real time PCR

To study the effect of the selected monoterpene, d-limonene, on expression of genes pertinent to the properties mentioned above, 3T3-L1 pre-adipocytes were grown and induced to differentiate. Cells treated on 2DPD and day 0, were harvested on day 2 whereas cells treated on day 2 were harvested on day 4. Gene expression studies carried out on day 2 and day 4 were to investigate the regulation of different sets of genes involved in the differentiation of adipocytes and lipid metabolism, respectively. The total RNA from the adipocytes was isolated using an Ambion-RNAqueous Micro® kit from Applied Biosystems and stored at −80 °C until use. Extracted RNA was subjected to purity and integrity check using an Agilent® 2100 Bio analyzer (Applied Biosystems, USA). Single stranded complementary DNA (cDNA) was synthesized from 1 μg of purified RNA [RNA Integrity Number (RIN) of 8–10] using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Quantification of gene expression was conducted using an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, USA). In brief, 10 ng of sample was mixed with TaqMan® Fast Advanced Master Mix (Applied Biosystems, USA) together with the TagMan® Gene Expression Assay resulting in a final volume of 20 μl. The reaction mixtures were incubated following manufacturer's protocol. The following genes were investigated in this study: peroxisome proliferator activated-receptor-gamma (PPAR-γ) (assay ID: Mm 01184322_m1), glucose transporter 1 (GLUT1) (assay ID: Mm 00441480_1), glucose transporter 4 (GLUT4) (assay ID: Mm 00436615_m1), hormone sensitive lipase (HSL) (assay ID: Mm 00495359_m1) and adipose triglyceride lipase (ATGL) (assay ID: Mm 00503040_m1). Assay ID refers to the proprietary primer and TaqMan® probe mix of the Applied Biosystems Gene Expression Assays kits. Gene expression was normalized using β-actin (assay ID: Mm 00607939_s1) as a reference gene. Relative mRNA expression was calculated based on the comparative CT method, which is also known as $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

2.13. Statistical analysis

Statistical analysis was performed using Graph Pad Prism version 5. Data shown are expressed in mean ± SEM unless indicated otherwise. The significance of differences between

means at a confidence limit of 95%, set at $P < 0.05$, was assessed using one-way analysis of variance (ANOVA), followed by Tukey–Kramer test for multiple variable comparison.

3. Results and discussion

3.1. Radical scavenging effect and reducing power of monoterpenes

At a concentration of 100 mM, γ-terpinene exhibited the strongest DPPH radical scavenging activity (83.0%) among the tested monoterpenes, followed by citral (77.9%) and terpinolene (75.3%) (Fig. 1A). (R)-(+)-β-Citronellol exerted a weaker DPPH radical scavenging (44.0%). On the other hand, the radical scavenging effect of (R)-(+)-limonene, (R)-(−)-linalool, (S)-(−)-β-citronellol, nerol, geraniol, (S)-(−)-perillyl alcohol and α-terpineol were shown to be weak, ranging from 5.3% to 18.4%. L-Menthol was shown to have no significant DPPH radical scavenging activity. Conversely, in comparison, ascorbic acid (positive control) scavenged 77.6% of DPPH radicals at 25 μM, which was significantly more potent than monoterpenes. Our current finding showed that DPPH radical scavenging activity of monoterpenes fell within milimolar range which was in concordance with several other reports (Bicas, Neri-Numa, Ruiz, De Carvalho, & Pastore, 2011; Di Sotto, Durazzi, Sarpietro, & Mazzanti, 2013; Junior et al., 2009). In a recent study, Bicas et al. (2011) showed that the IC_{50} of DPPH radical scavenging for α-terpineol and perillyl alcohol were $IC_{50} = 332.8\ g\ L^{-1}$ (approximately 2 M) and $IC_{50} = 738.3\ g\ L^{-1}$ (approximately 5 M), respectively. These findings contradicted the report by Choi et al. (2000) which showed that some of the monoterpenes such as γ-terpinene, terpinolene and citral exerted greater DPPH radical scavenging activity than Trolox. Several factors could affect the efficacy of antioxidants, including emulsion system, test system, methods and oxidation time. For instance, lipophilic antioxidant such as α-tocopherol is more efficient in oil-in-water emulsion system than a bulk oil system whereas hydrophilic antioxidant such as Trolox acts in the opposite trend (Shahidi & Zhong, 2011). We do not rule out the possibility that synthesis process of monoterpenes and storage condition like temperature, exposure to light and auto-oxidation could also alter the efficacy of monoterpenes resulting in the discrepancy between studies. Nevertheless, the monoterpenes used in this study were handled appropriately (stored in the dark and at the recommended temperature) to avoid or minimize auto-oxidation.

Similar to DPPH radical scavenging activity, γ-terpinene (93.7%), terpinolene (89.5%) and citral (45.7%) exhibited the strongest ABTS radical scavenging activity at a concentration of 100 mM (Fig. 1B). Both terpinolene (59.5%) and γ-terpinene (32.7%) showed ABTS radical scavenging capacity at a lower concentration (1 mM) compared to DPPH radical scavenging (10 mM). ABTS radical scavenging activity at 100 mM were followed by (S)-(−)-perillyl alcohol (34.9%), (R)-(+)-β-citronellol (16.8%), α-terpineol (11.2%) and (R)-(+)-limonene (10.4%). Geraniol, l-menthol, nerol, (R)-(−)-linalool and (S)-(−)-β-citronellol did not exert any significant ABTS radical scavenging activity. The different potencies observed for both assays can be explained by the mechanism involved. ABTS radicals and DPPH radicals involve electron transfer and hydrogen donation, respectively, in which the former takes place in a much faster rate than the latter (Bendaoud, Bouajila, Rhouma, Savagnac, & Romdhane, 2009).

In the ferric reducing assay, monoterpenes were shown to have no significant reducing power in comparison to the positive control, ferric sulphate (data not shown). Overall, synthetic monoterpenes used in the current study exhibited a weak radical scavenging activity and did not have any ferric reducing power.

Oxidative stress is closely associated with obesity and T2DM. Antioxidant treatment has been reported to reduce reactive oxygen species (ROS) and lead to attenuation of complications caused by diabetes (Huynh, Bernardo, McMullen, & Ritchie, 2014). Thus, dietary monoterpenes with antioxidant capacity can be beneficial to human health by alleviating complications cause by T2DM. Nevertheless, as antioxidant capacity of monoterpenes remains a controversy, we further examined the other potential aspects of monoterpenes that can possibly aid in the control of T2DM.

3.2. α-Amylase and α-glucosidase inhibition by monoterpenes

One of the current treatments of T2DM is the use of oral hypoglycemic agents which inhibit activity of carbohydrate-hydrolyzing enzymes, namely, α-amylase and α-glucosidase. Pancreatic α-amylase hydrolyzes starch into oligosaccharides which are further degraded by intestinal α-glucosidase into glucose that is readily absorbed into systemic circulation. In T2DM patients, due to impaired glucose tolerance, sudden rise in the plasma glucose level can lead to complications. Inhibition of these enzymes delays overall carbohydrate digestion and glucose absorption rate consequently blunting the increase of postprandial plasma glucose level. Thus, carbohydrate-hydrolyzing enzyme inhibitors such as acarbose, miglitol and voglibose have been clinically used as oral anti-hyperglycemic agents in the effective control of T2DM (Jo et al., 2013; Sugihara et al., 2014).

In the current study, we examined the potential inhibitory effect of monoterpenes on both α-amylase and α-glucosidase.

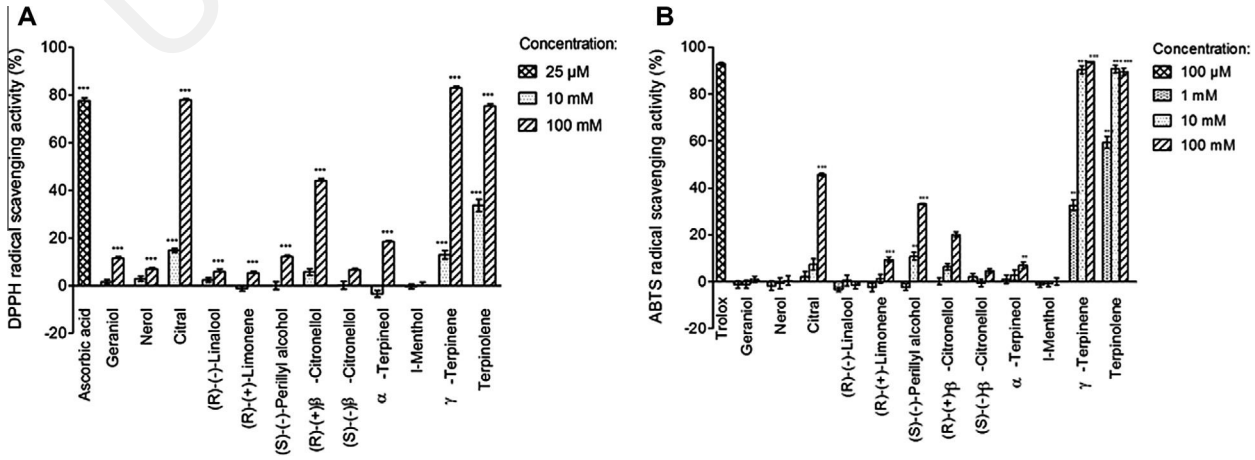

Fig. 1. Scavenging effects of monoterpenes on (A), DPPH and (B), ABTS radicals. Results are expressed as mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 3$ for each group.

Citral was the only monoterpene which showed α-amylase inhibitory effect of 45.7%, at a concentration of 10 mM (Fig. 2A) as compared to the positive control, acarbose which inhibited 48.2% of α-amylase activity at a concentration of 10 μM suggesting that citral was a weak α-amylase inhibitor.

On the other hand, at 10 mM, (R)-(+)-limonene and (S)-(−)-perillyl alcohol exhibited the strongest inhibitory effect on α-glucosidase activity, 21.3% and 21.1%, respectively, followed by α-terpineol, 14.0% (Fig. 2B). (R)-(+)-β-citronellol, terpinolene, citral, (R)-(−)-linalool, nerol, geraniol and (S)-(−)-β-citronellol exerted relatively weaker α-glucosidase inhibitory effect, ranging from 4.2% to 8.1%. L-menthol and γ-terpinene did not show any significant α-glucosidase inhibitory activity. In comparison, the positive control, acarbose exerted α-glucosidase inhibitory effect with a much greater potency, which was 49.6% at a concentration of 5 μM.

Clinical use of acarbose as an oral hypoglycemic agent has been well established. Nonetheless, the main drawbacks of acarbose are gastrointestinal effects including abdominal distention, flatulence, meteorism and possibly diarrhea, due to abnormal fermentation of undigested carbohydrates by colonial bacteria as a result of over inhibition of α-amylase (Jo et al., 2013). A more effective strategy in the control of T2DM involves a moderate α-amylase inhibition with a stronger inhibition on α-glucosidase. With respect to diet, dietary intervention with high consumption of monoterpenes with a mild α-glucosidase inhibitory property, could offer a better control of glycemic index of food products together with the therapeutic approach.

3.3. Effect of monoterpenes on proliferation of 3T3-L1 pre-adipocytes

Proliferation of pre-confluent 3T3-L1 pre-adipocytes after 48-h exposure to various monoterpenes was determined using MTT assay. At a concentration of 1 μM, citral, geraniol and α-terpineol were found to suppress the proliferation of 3T3-L1 pre-adipocytes by 29.2%, 19.9% and 13.6%, respectively (Fig. 3). Nerol, (R)-(−)-linalool, (R)-(+)-limonene, (S)-(−)-perillyl alcohol, (R)-(+)-β-citronellol, (S)-(−)-β-citronellol, l-menthol, γ-terpinene and terpinolene were found to have no negative proliferative effect on 3T3-L1 pre-adipocytes. These results indicate that the suppressive effect of monoterpenes on 3T3-L1 pre-adipocyte proliferation was selective. However, more in depth studies are necessary to understand the mechanism involved.

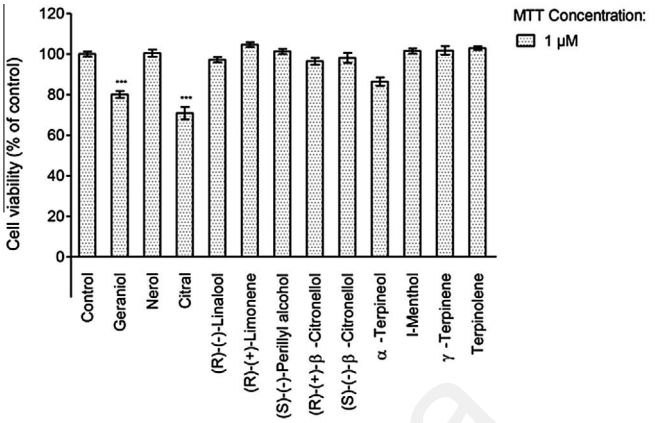

Fig. 3. Effect of monoterpenes on cell viability in 3T3-L1 pre-adipocytes. 3T3-L1 pre-adipocytes were exposed to various monoterpenes at a concentration of 1 μM for 48 h prior to MTT assay. Results are expressed as mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 3$ for each group.

3.4. Effect of monoterpenes on glucose uptake in mature 3T3-L1 adipocytes

Glucose uptake plays an important role in the control of plasma glucose level, thus directly influencing glucose tolerance. Here, we examined the effect of monoterpenes on glucose uptake by exposing mature 3T3-L1 adipocytes to 1 μM of various monoterpenes. Results showed that only certain but not all monoterpenes exerted stimulatory effect on the glucose uptake. Geraniol stimulated the highest increase, 21.1%, in the glucose uptake (Fig. 4A). Citral, (R)-(+)-limonene and (R)-(+)-β-citronellol also enhanced glucose uptake by 17.6%, 17.4% and 16.1%, respectively. Nerol, (S)-(−)-perillyl alcohol, γ-terpinene and α-terpineol relatively showed a weaker stimulatory effect on glucose uptake, by 10.9% to 14.6%. On the other hand, (S)-(−)-β-citronellol, terpinolene and (R)-(−)-linalool did not affect the glucose uptake in 3T3-L1 adipocytes.

Glucose uptake in cells is mediated by glucose transporters (GLUTs), GLUT1 to GLUT14, a family of integral membrane proteins that mediate sodium-independent, facilitated-diffusion of hexoses across plasma membrane. Two main glucose transporter isoforms, namely, GLUT1 and GLUT4 are responsible for the glucose uptake in insulin-responsive tissues such as skeletal muscles and adipocytes. Predominantly, GLUT1 is expressed and found on the plasma

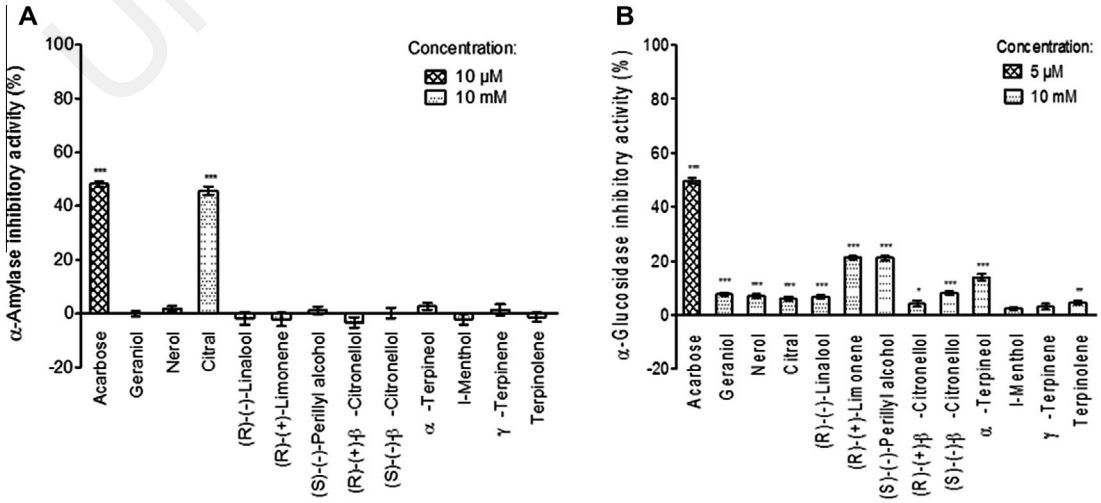

Fig. 2. (A) α-Amylase and (B) α-glucosidase inhibitory activity of monoterpenes. Results are expressed as mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 3$ for each group.

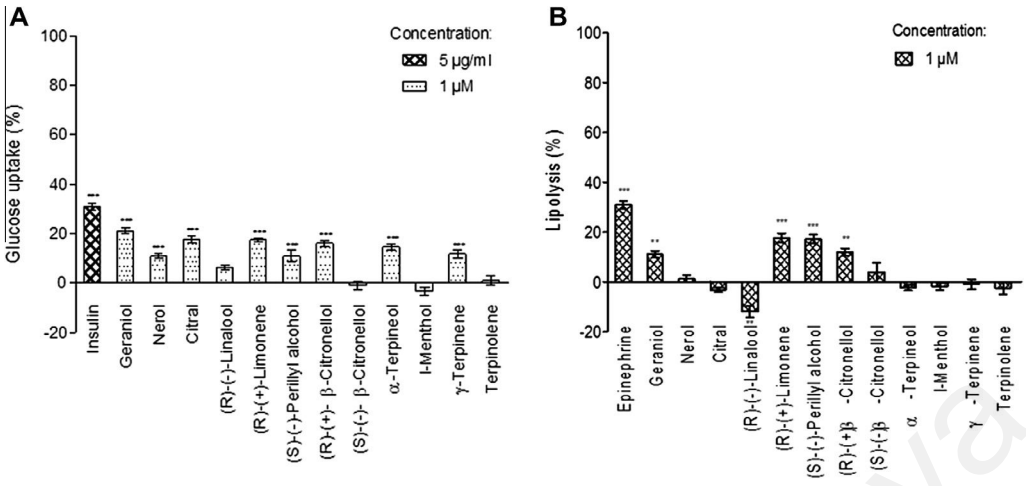

Fig. 4. Effect of monoterpenes on glucose uptake and lipid metabolism. Mature 3T3-L1 adipocytes were exposed to various monoterpenes at a concentration of 1 μM. After 5 h, measurements of (A) total glucose uptake and (B) free glycerol released into the medium were performed. Total glycerol content in the medium indicates the lipolytic effect of monoterpenes in 3T3-L1 adipocytes. Results are expressed as mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 3$ for each group.

membrane and intracellular membranes of both pre-adipocytes and adipocytes. On the other hand, GLUT4 is expressed during the course of adipocyte differentiation and sequestered in intracellular vesicles and translocated to the plasma membrane upon insulin stimulation. As a major insulin-responsive glucose transporter, GLUT4 serves as the rate-controlling step in insulin-mediated glucose disposal which directly affects the insulin sensitivity (Herman & Kahn, 2006; Mueckler & Thorens, 2013). Extensive studies have been targeted exclusively on the expression of GLUT4 in glucose uptake in the control of T2DM (Jiang et al., 2014). Nonetheless, increasing evidence shows that the enhancement of glucose uptake can commence via GLUT1 (Choi et al., 2011). Thereby, in the current study, we further investigated the mRNA expression of both GLUT1 and GLUT4.

3.5. Effect of monoterpenes on lipolysis in mature 3T3-L1 adipocytes

Lipolysis is a catabolic process that hydrolyzes triacylglycerols (TGs) stored in lipid droplets liberating glycerols and fatty acids which serve as energy substrates during energy deprivation. Neutral hydrolysis of TGs involves three distinct enzymes: adipose triglyceride lipase (ATGL) initiates lipolysis by hydrolyzing TGs to diacyglycerols (DGs); hormone sensitive lipase (HSL) converts DGs into monoacylglycerols (MGs), which in turn, acted by monoacylglycerol lipase (MGL) releasing glycerol and fatty acids (Zechner et al., 2012). In the present study, we conducted a quick screening on the lipolytic effect of monoterpenes in 3T3-L1 adipocytes. (R)-(−)-Linalool that did not exert any effect on glucose uptake, exhibited 11.8% decrease in the lipolysis (Fig. 4B). Interestingly, (R)-(+)-limonene, (S)-(−)-perillyl alcohol, (R)-(+)-β-citronellol and geraniol which enhanced glucose uptake, also stimulated lipolysis, by 17.7%, 17.3%, 12.0% and 11.1%, respectively.

In general, high level of circulating fatty acids due to excessive lipolysis is closely associated with the development of insulin resistance. However, recent findings have demonstrated that increased lipolysis does not necessarily lead to elevated serum fatty acids, suggesting enhancement of insulin sensitivity via lipolysis is possible. Adipose-specific phospholipase A_2 (AdPLA), an intracellular calcium-dependent PLA, has been shown to elevate prostaglandin E2 (PGE2) level that exhibited anti-lipolytic effect at low concentrations via cyclic adenosine monophosphate (cAMP) suppression. Ablation of AdPLA and overexpression of AGTL, respectively, exhibited elevated lipolysis in murine adipocytes without causing an increase in serum fatty acid level, attributed to the concomitant increase of fatty acid oxidation within adipocytes (Ahmadian, Wang, & Sul, 2010). Hence, it is imperative that further in depth investigation on compounds that can stimulate lipolysis, especially those with stimulatory effect on glucose uptake is carried out.

3.6. Effect of (R)-(+)-limonene on total lipid accumulation in 3T3-L1 adipocytes

For a better understanding of the effect of monoterpenes on glucose uptake, lipid metabolism and gene expression, we selected one of the monoterpenes for further investigations. Based on the previous report (Murali & Saravanan, 2012), streptozotocin-induced diabetic rats fed with (R)-(+)-limonene experienced a repression in plasma glucose level suggesting an improvement of glucose uptake. On the other hand, administration of (R)-(+)-limonene in normal rats did not result in significant difference in body weight as compared to negative controls. These findings give rise to the interesting prospects to investigate the effect of (R)-(+)-limonene on glucose uptake and the maintenance of body weights. Taken together with the positive stimulatory effect in glucose uptake and lipolysis (Fig. 4A and B), (R)-(+)-limonene was selected for the following experiments.

To examine the effect of (R)-(+)-limonene on total lipid accumulation, 3T3-L1 adipocytes were treated with 1 μM (R)-(+)-limonene for 48 h at three distinctive stages, which were during the cell-contact inhibition stage of post-confluent pre-adipocytes (2DPD), mitotic clonal expansion and terminal differentiation of adipocytes (day 0) and maturation of adipocytes (day 2). Changes in the occurrence during any of these stages could directly impact on downstream activities such as lipid accumulation. Therefore, by treating the cells with (R)-(+)-limonene during different stages, alteration of total lipid accumulation could provide an insight into the stages affected by the treatment. In the present study, Oil Red O quantitative assay was used to quantify the total lipid accumulation in mature adipocytes.

Interestingly, results obtained showed that treatment of 1 μM (R)-(+)-limonene during these three distinctive stages had similar degree of total lipid accumulation in mature adipocytes as compared to negative control (data not shown). Our results concur with animal study by Murali and Saravanan (2012), which showed that there was no significant change in body weight in normal rats

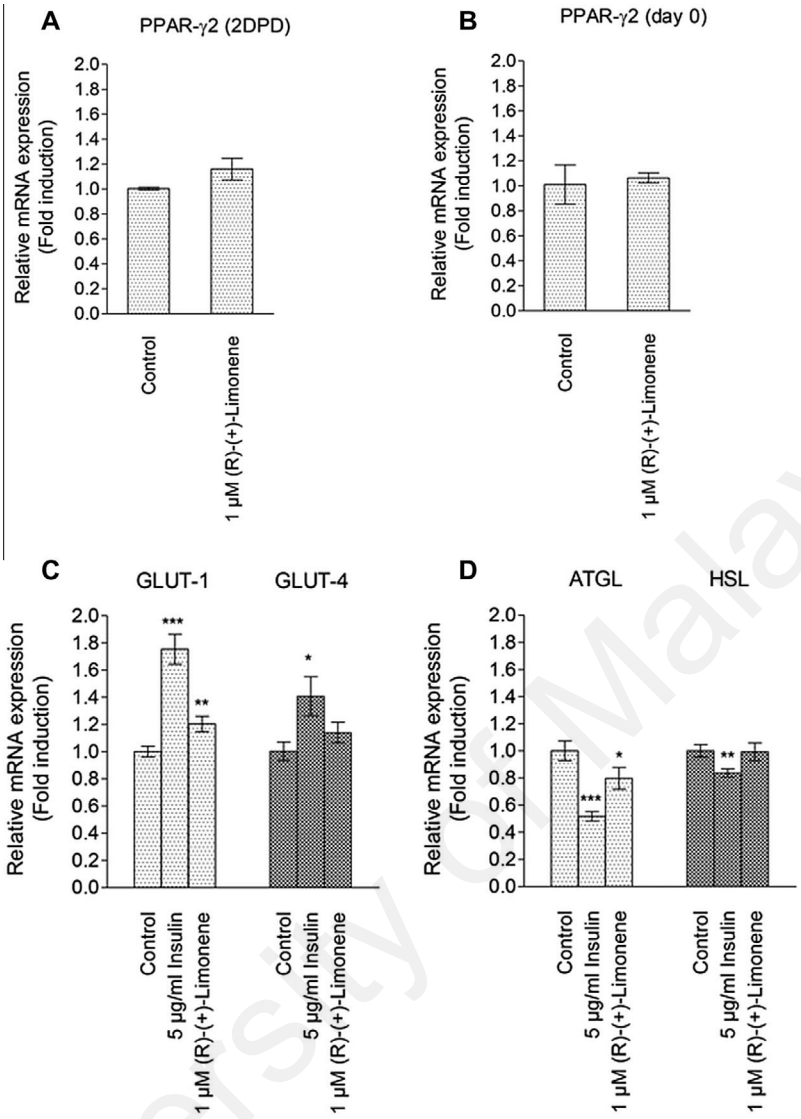

Fig. 5. The effect of (R)-(+)-limonene on the expression of genes associated with differentiation, glucose uptake and lipid metabolism in 3T3-L1 adipocytes. Cells were exposed to 1 μM (R)-(+)-limonene for 48 h on (A) 2DPD (two days prior to differentiation), (B) day 0 and (C, D) day 2. Isolation of mRNA from the cells treated on 2DPD and day 0 was performed on day 2 for the evaluation of the expression of PPAR-γ. Isolation of mRNA from the cells treated on day 2 was performed on day 4 to study the expression of downstream genes involved in glucose uptake (GLUT1 and GLUT4) and lipolysis (ATGL and HSL). Results expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 3$ for each group.

fed with (R)-(+)-limonene. These findings also suggest that (R)-(+)-limonene did not affect cell-contact inhibition, mitotic clonal expansion and terminal differentiation of adipocytes. Nevertheless, maturation of adipocytes involves both glucose uptake and lipolysis, which can result in the alteration of total lipid accumulation. Considering earlier findings in this report, we speculate that insignificant change in total lipid accumulation in 3T3-L1 adipocytes treated with (R)-(+)-limonene during day 2 was possibly due to the concurrent elevation of glucose uptake and lipolysis.

3.7. Effect (R)-(+)-limonene on the regulation of gene expression

After 48-h exposure of 1 μM (R)-(+)-limonene to the cells during cell-inhibition stage (2DPD) and differentiation of 3T3-L1 adipocytes (day 0), mRNA expression of peroxisome proliferator activated-receptor-gamma (PPAR-γ) was quantified. PPAR-γ, a ligand-activated transcriptional factor, is expressed predominantly in adipocytes and serves as the primary regulator of adipocyte differentiation. Activation of PPAR-γ regulates downstream genes involved in glucose uptake and lipid metabolism through insulin-signaling cascade, giving rise to improved insulin sensitivity. Consistent decrease in the plasma glucose level in T2DM patients under the treatment of thiazolidinedione (TZD), a PPAR-γ ligand, was concomitantly accompanied by the remodeling of adipose tissues, with a marked replacement of large adipocytes by small insulin-sensitive adipocytes showing the importance of PPAR-γ (Choi et al., 2011). However, in the current study, (R)-(+)-limonene treatment did not affect the mRNA expression of PPAR-γ in 3T3-L1 adipocytes (Fig. 5A and B). Corresponding to insignificant change in the total lipid accumulation in Oil Red O quantitative assay (data not shown), these findings suggest that 1 μM (R)-(+)-limonene did not exert significant effect on the differentiation of 3T3-L1 adipocytes.

Subsequently, the expression of several genes which participated in the early maturation stage of 3T3-L1 adipocytes (day 2) after 48-h exposure to 1 μM (R)-(+)-limonene were investigated. Glucose uptake in adipocytes is attributed to the increased constitutive expression of GLUT1 proteins, or/and increase of the expression and translocation of GLUT4 from the cytosol to plasma membrane (Ku et al., 2014). Yamamoto et al. (2000) and Kohn,

Summers, Birnbaum, and Roth (1996) demonstrated that constitutively active mitogen-activated protein kinase kinase (MAPKK) and Akt Ser/Thr kinase increased glucose uptake in 3T3-L1 adipocytes and mRNA expression of GLUT1 was augmented in parallel, but not mRNA expression of GLUT4. Further investigation revealed that the elevation of glucose uptake was attributed not only to the increased expression of GLUT1, but also the translocation of GLUT4 from cytosol to plasma membrane. In this regard, cellular mRNA level alone can only serve as a reference but is not adequate to act as the absolute indicator of GLUT4 activity. Our results showed that treatment of 1 μM (R)-(+)-limonene in 3T3-L1 adipocytes significantly increased the mRNA expression of GLUT1 by 1.2-fold whereas the mRNA expression of GLUT4 remained unchanged (Fig. 5C). Thereby, we hypothesize that the stimulatory effect of (R)-(+)-limonene on glucose uptake was likely, in part, rendered by its ability to upregulate the mRNA expression of GLUT1, via MAPK signaling that is predominantly involved in the expression of GLUT1, but not GLUT4 (Yamamoto et al., 2000). Induction of glucose uptake by (R)-(+)-limonene via insulin signaling pathway was less likely as insulin signaling is inversely correlated with increased lipolysis (Fig. 4B) (Zechner et al., 2012). In the meantime, insulin downregulated both ATGL and HSL mRNA expression (Fig. 5D) whereas (R)-(+)-limonene decreased only ATGL mRNA expression but not HSL, suggesting (R)-(+)-limonene possibly acted via a pathway distinct from insulin. Stimulatory effect of (R)-(+)-limonene on glucose uptake via 5′ adenosine monophosphate-activated protein kinase (AMPK) signaling was also less convincing as activation of this pathway not only markedly upregulated the expression of GLUT1, but also GLUT4 (Holmes & Dohm, 2004).

Since ATGL and HSL are principal and rate limiting enzymes responsible for over 90% of TG hydrolase activity in murine white adipocytes (Zechner et al., 2012), our gene expression study therefore focused primarily on ATGL and HSL mRNA expression. Our study demonstrated that treatment with 1 μM (R)-(+)-limonene in mature 3T3-L1 adipocytes downregulated the mRNA expression of ATGL but mRNA expression of HSL remained unaffected, as compared to the negative control (Fig. 5D). The gene expression results seem to be counterintuitive with the elevated lipolysis by (R)-(+)-limonene treatment (Fig. 4B). A previous study has also reported that isoproterenol and tumor necrosis factor-α exerted elevated lipolytic activity, despite the attenuation of ATGL mRNA expression. The increase of lipolysis can be explained by the increase of lipase activity but not necessarily via the activation of cAMP signaling. Perillipin-1, a lipid droplet-associated protein, interacts with comparative gene identification-58 (CGI-58), a coactivator protein of ATGL, hence, preventing its binding and activation of ATGL. Upon β-adrenergic stimulation, phosphorylation of perilipin-1 at multiple sites by protein kinase A (PKA) releases CGI-58 which results in the activation of ATGL and thus, lipolysis increases. Conversely, HSL activity is directly activated by PKA phosphorylation (Holmes & Dohm, 2004; Zechner et al., 2012). Taken together, it is suggested that (R)-(+)-limonene-induced lipolysis could possibly be target-specific, rather than via the inhibition of cAMP signaling or PPAR signaling that regulates adipocyte differentiation (Fig. 5A and B). More in depth studies are required in order to unravel the underlying mechanism involved in adipogenesis and lipid metabolism. In the last decade, studies have shown the potency of monoterpenes as strong radical scavengers, which further build up the confidence and interest in the application of monoterpenes in the food and cosmetic industries. However, present study, aligned with several other reports, collectively showed relatively weaker DPPH and ABTS radical scavenging activity of monoterpenes. These findings suggest that antioxidant capacities of monoterpenes are determined by multi factors, and therefore, controversies prevail.

4. Conclusion

The current comprehensive study supports the underlying mechanism of some novel biological effects of monoterpenes, including a mild antioxidant capacity, inhibition of α-amylase and α-glucosidase activity, as well as stimulation of glucose uptake and lipolysis. It is hoped that these novel biological findings will be useful in increasing the awareness on monoterpenes not only as food additives but also potentially a cost-effective strategy in the control of obesity and T2DM.

Acknowledgments

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