PTEROSTILBENE, A NATURAL PHYTOCHEMICAL, AMELIORATES FATTY LIVER DISEASE IN *IN VITRO* AND *IN VIVO* MODELS

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PhD

The Hong Kong Polytechnic University

2019

The Hong Kong Polytechnic University

Department of Applied Biology and Chemical Technology

Pterostilbene, a Natural Phytochemical, Ameliorates Fatty Liver Disease in *In Vitro* and *In Vivo* Models

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

June 2018

CERTIFICATE OF ORIGINALITY

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NG Yam Fung

Abstract

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease resulting from non-alcoholic causes of excessive deposition of fat in hepatocytes. It encompasses a spectrum of conditions ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), which can further progress to fibrosis, cirrhosis and hepatic carcinoma. Over the past few decades, the prevalence of NAFLD increases worldwide due to the spread of Western lifestyle in the developing countries. In recent years, there is increasing incidence of NAFLD in younger generation. The rising incidence of NAFLD has become a serious global public health issue.

Currently, there is no standard therapeutic medical treatment for NAFLD. Lifestyle modification is the mainstay of treatment, but it may be difficult for patients to maintain these long-term lifestyle changes. Medication to improve the efficacy of lifestyle measures is warranted.

Pterostilbene (3,5-dimethoxy-4-hydroxy-trans-stilbene), a methylated analogue of resveratrol, has been reported to possess various biological activities, including

antioxidative, anti-inflammatory, anti-diabetic, and anti-cancer effects. The properties of pterostilbene may be able to target the pathogenic processes in the progression of NAFLD.

The objectives of the present study include (1) to investigate the protective effect of pterostilbene against oxidative stress as well as its lipid reduction effect and the possible molecular mechanisms using steatotic hepatic cell model *in vitro*; (2) to evaluate the potential therapeutic effects of pterostilbene against NAFLD using high-fat diet-induced hepatic steatosis animal model *in vivo*; and (3) to investigate the possible mechanistic pathways of pterostilbene in treating NAFLD using molecular, lipidomic and genomic approaches.

Results of *in vitro* study demonstrated that pterostilbene could protect liver cells against oxidative stress via downregulation of MAPKs and upregulation of HO-1. Moreover, results of *in vitro* study showed that pterostilbene could reduce intracellular lipid accumulation in steatotic liver cells by inhibiting fatty acid synthesis as well as cholesterol synthesis and promoting fatty acid β-oxidation and lipophagy.

Results of *in vivo* study demonstrated that treatment with pterostilbene could help reduce liver lipid content and attenuate insulin resistance. The underlying mechanisms may include protection against high-fat diet-induced oxidative stress, inhibition of fatty acid synthesis as well as cholesterol synthesis, promoting fatty acid β-oxidation and lipophagy, restoration of the high-fat diet-induced changes in the serum metabolites of lysophospholipids as well as the high-fat diet-induced alteration of gut microbiota structure.

In conclusion, the present studies showed that pterostilbene could help ameliorate NAFLD *in vitro* and *in vivo* by multiple mechanisms. Further studies are required to determine the potential of using pterostilbene as therapeutic agents for NAFLD in clinical settings.

Acknowledgments

It is my great honour to express my sincere gratitude and deep appreciation to everyone providing continuous support to me throughout the journey in my Ph.D. study.

I would like to express my sincere gratefulness to my supervisor, Prof. Wong Wing-Tak, and co-supervisor, Dr. Chan Shun-Wan, for their patience, guidance and encouragement.

My sincere thanks go to Prof. Mok Kam-Wah Daniel, Dr. Chan Chi-On, Mr. Li Meng-Heng and Ms. Sham Tung-Ting for their help in lipidomic study. I would like to thank Mr. Lu Lu for his assistance in the *in vivo* study.

I am grateful to Dr. Zhang Huan and Ms. Cheng Huan-Le for their encouragement, advice and support during the project work. My special appreciation goes to staff of the Department of Applied Biology and Chemical Technology for their help and support.

My sincere thanks also go to Prof. Kwan Yiu-Wa, who provided me an opportunity to join his team and shared his research experience.

Last but not least, I would especially like to express my sincere appreciation to my parents for their immeasurable love and care as well as moral and emotional support. I would also like to thank my friends who have supported me along the way.

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List of Abbreviations

ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ACC	Acetyl-CoA carboxylase
ADP	Adenosine-5′-diphosphate
AGC	Automatic gain control
Akt	Protein kinase B
ALT	Alanine transaminase
AMP	Adenosine-5′-monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AP-1	Activator protein-1
AST	Aspartate transaminase
ATP	Adenosine-5′-triphosphate
BSA	Bovine serum albumin
COX	Cyclooxygenase
CV	Coefficient of variations
DCFH-DA	Dichloro-dihydro-fluorescein diacetate
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethyl sulfoxide
DPPH	2,2-Diphenyl-1-picryl-hydrazyl
ECL	Enhanced chemiluminescence
ERK	Extracellular signal-regulated kinase
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FFA	Free fatty acid
FXR	Farnesoid X receptor
GLUT4	Glucose transporter type 4
GPBAR1	G protein-coupled bile acid receptor 1
H&E	Haematoxylin and eosin
HDL-C	High-density lipoprotein cholesterol

H-ESI	Heated electrospray ion
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HMGCR	HMG-CoA reductase
HO-1	Heme oxygenase-1
HOMA-IR	Homeostasis model assessment of insulin resistance
HSD	Honestly significant difference
IKK	I kappa B kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRS	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
LC3	Light chain 3
LDL-C	Low-density lipoprotein cholesterol
LDLR	Low-density lipoprotein receptor
LPS	Lipopolysaccharide
LysoPC	Lysophosphatidylcholine
MAPK	Mitogen-activated protein kinase
MMP	Matrix metallopeptidase
MS	Mass spectrometry
MTBE	Methyl tert-butyl ether
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NF-κB	Nuclear factor-kappaB
NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2-related factor 2
OA	Oleic acid
OGTT	Oral glucose tolerance test
OPLS-DA	Orthogonal partial least squares-discriminant analysis
p38 MAPK	p38 mitogen-activated protein kinase

PA	Palmitic acid
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol 3-kinases
PLS-DA	Partial least squares-discriminant analysis
PMSF	Phenylmethylsulfonyl fluoride
PPAR	Peroxisome proliferator-activated receptors
PVDF	Polyvinylidene difluoride
QC	Quality control
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
SD rat	Sprague-Dawley rat
SREBP	Sterol regulatory element binding protein
STZ	Streptozotocin
TBST	Tris-buffered saline with Tween
TC	Total cholesterol
TG	Triglyceride
TNF-α	Tumor necrosis factor-alpha
UPLC	Ultra-performance liquid chromatography
VIP	Variable importance in the projection

Chapter 1 Introduction

1.1 Nonalcoholic fatty liver disease

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the world. Due to the spread of Western lifestyle in the developing countries, the prevalence of NAFLD keeps on rising throughout the world over the past few decades (Younossi et al., 2017). NAFLD affects 20 – 40% of the general population in Western countries and 5 – 40% of the general population across the Asia-Pacific region (Abd El-Kader and El-Den Ashmawy, 2015; Heidari and Gharebaghi, 2017; Sherif et al., 2016). According to the information released from the Centre for Health Protection, the prevalence of NAFLD is around 25 – 30% in Hong Kong and its annual incidence is 3 – 4% among Chinese adults (Centre for Health Protection, 2015). Generally, the prevalence of NAFLD increases with age. In recent years, the incidence of NAFLD in children and teenagers has been increasing (Singer et al., 2014; Sozio et al., 2010; Temple et al., 2016). With the increasing prevalence worldwide and rising incidence at younger age, NAFLD has become a serious global public health issue (Hamaguchi et al., 2005; Suzuki et al., 2005; Whalley et al., 2007). The growing clinical burden of NAFLD would finally lead to a negative impact on the health care system (Younossi et

al., 2016).

NAFLD is a hepatic manifestation of the metabolic syndrome with fat deposition in hepatocytes that is not due to alcohol consumption. It includes a spectrum of conditions associated with fat accumulation in hepatocytes ranging from nonalcoholic hepatic steatosis to nonalcoholic steatohepatitis (NASH) (Suyavaran et al., 2015). Nonalcoholic hepatic steatosis is the mildest form of NAFLD. It refers to the conditions that there is excessive fat accumulation in the liver. When there is a deposition of intracellular fat in more than 5% of hepatocytes, it would be considered as hepatic steatosis. NASH refers to an increased severity of steatosis in the liver with inflammation and injury of hepatocytes. In a more severe situation, liver fibrosis would occur. Long-term damage caused by NASH would lead to liver cirrhosis and even hepatocellular carcinoma (Sheedfar et al., 2013; Wilkins et al., 2013).

NAFLD is strongly associated with obesity and type II diabetes mellitus. Over 70% of patients with NASH are obese and up to 90% of obese individuals have NAFLD (Bellentani et al., 2010; Francque et al., 2016). It has been reported that about 70% of

patients with type II diabetes mellitus are found to have NAFLD and about 30% of them have NASH (Cusi, 2016; Hazlehurst et al., 2016). These findings indicated that obesity and type II diabetes mellitus may contribute to the pathogenesis of NAFLD or these metabolic syndromes share many pathogenic factors. In the following section, the pathogenesis of NAFLD will be discussed.

1.2 Pathogenesis of NAFLD

The pathogenesis of NAFLD is complicated. A variety of factors, such as lifestyles and genetic factors, are involved in the development and progression of NAFLD (Angulo 2002). Lifestyles including dietary habits and physical activity directly affect the amount of nutrient intake, energy consumption and utilization (Yasutake et al., 2014; Zelber-Sagi et al., 2016). Genetic factors affect the risk of having NAFLD by influencing the interaction between individuals and nutritional and environmental factors (Dongiovanni and Valenti, 2017; Ravi Kanth et al., 2016). Impaired lipid metabolism, hyperlipidemia, hyperinsulinemia, insulin resistance, oxidative stress and inflammation are believed to play a central role in the pathogenesis

of NAFLD (Caligiuri et al., 2016; Oliveira et al., 2006; Paschos and Paletas, 2009; Utzschneider and Kahn, 2006). The pathogenesis of NAFLD is still not fully understood due to its complexity. Initially, the "two-hit" hypothesis is generally accepted to be the prevailing theory to explain the pathogenesis of NAFLD (Dowman et al., 2010). However, it is considered to be too simplistic and inadequate to reflect the real situation that takes place in NAFLD. Consequently, the "two-hit" hypothesis is substituted by the "multiple-hit" hypothesis (Buzzetti et al., 2016).

1.2.1 The "two-hit" hypothesis

The "first hit" refers to an imbalance between uptake, production and usage of lipid, resulting in hepatic lipid accumulation. Excess amount of nutrient intake leads to increase in hepatic uptake of free fatty acids (FFAs). High levels of FFAs affect insulin signaling and induce insulin resistance. At the same time, insulin resistance affects glucose metabolism and leads to increase in the influx of FFAs to the liver. This in turn leads to lipid accumulation in the liver causing steatosis (Day and James, 1998; Dowman et al., 2010; Musso et al., 2010).

The “second hit” is caused by the consequences of high level of FFAs and excess hepatic lipid accumulation. Increased oxidation of FFAs generates reactive oxygen species (ROS) resulting in oxidative stress which triggers the release of inflammatory cytokines and adipokines causing liver inflammation. Oxidative stress also leads mitochondrion dysfunction resulting in apoptosis of hepatocytes. At this stage, NAFLD progress from simple steatosis to NASH (Day and James, 1998; Dowman et al., 2010; Musso et al., 2010).

1.2.2 The “multiple-hit” hypothesis

The “multiple-hit” hypothesis emphasizes that the pathogenesis of NAFLD is multifactorial and these factors work in parallel to cause NAFLD. Dietary habits, genetic factors and environmental factors contribute to the development and the progression of NAFLD (Takaki et al., 2013; Temple et al., 2016; Yu et al., 2016). They cause the development of insulin resistance, accumulation of hepatic lipid, dysfunction of adipose tissue, and alteration of gut flora.

1.2.3 Lipid accumulation

The accumulation of lipid in form of triglycerides (TGs) in hepatocytes is the major characteristic of NAFLD. The TGs stored in the liver are synthesized in the hepatocytes via esterification of FFAs and glycerol. The FFAs used for TGs synthesis are derived from few sources, including directly from dietary fat, *de novo* lipogenesis from dietary carbohydrate and lipolysis of lipid in adipose tissue. Liver removes FFAs by utilization of FFAs through β-oxidation or exportation as very low-density lipoprotein (Adams et al., 2005; Postic and Girard, 2008).

In normal condition, the influx and the clearance of FFAs in the liver are maintained at a state of equilibrium. However, when imbalance between the intake and the usage of FFAs occurs due to increased nutrient uptake or impaired lipid metabolism, excessive FFAs will be re-esterified to TGs and stored as lipid droplets in hepatocytes (Postic and Girard, 2008). Prolonged accumulation of lipid in hepatocytes results in hepatic steatosis. Hepatic lipid accumulation is associated with hepatic lipotoxicity and insulin resistance.

1.2.4 Insulin resistance

Same as the “two-hit” hypothesis, the development of insulin resistance is also considered to be one of the key factors in the pathogenesis of NAFLD in the “multiple-hit” hypothesis.

Insulin resistance develops along with the development and the progression of NAFLD. Intake of hypercaloric diet and sedentary lifestyle induce insulin resistance. In the situation of overnutrition, excess circulating fatty acids lead to fat accumulation in liver and adipose tissue causing insulin resistance (Postic and Girard, 2008). Due to insulin resistance, the body's cells lose their sensitivity to insulin. In order to compensate the resistance, pancreatic beta cells secrete more insulin resulting in an increased plasma insulin level. The raised plasma insulin level stimulates hepatic *de novo* lipogenesis which expedites accumulation of fat in the liver. On the other hand, the adipose tissue lipolysis suppression effect of insulin is impaired because of insulin resistance resulting in an increased flux of fatty acids from adipose tissue to the liver as well as driving *de novo* lipogenesis (Alam et al., 2016; Bugianesi et al., 2010).

As a result of increased circulating fatty acids, increased hepatic fat accumulation and dysregulated insulin secretion due to overload of pancreatic beta cells, insulin resistance also increased which leads to further fatty acid dysregulation, constituting a vicious cycle.

1.2.5 Adipose tissue dysfunction

Adipose tissue, also called fat tissue, is a loose connective tissue composed mainly of adipocytes. The main role of adipose tissue is for energy storage in form of fat, provides heat insulation and serves as mechanical cushion to protect abdominal organs. Recently, this view has been changed and adipose tissue was found to be able to synthesize and release a variety of hormones, cytokines, and proteins. Adipose tissue is now recognized as an endocrine organ and is involved in lots of physiological functions including maintenance of energy homeostasis, regulation of neuroendocrine and immune function (Coelho et al., 2013; Kershaw and Flier, 2004; Proença et al., 2014). Increasing evidence shows that adipose tissue dysfunction is associated with the pathogenesis of NAFLD.

In the situation of overnutrition, excess amount of nutrient intake causes accumulation of lipid in adipose tissue in form of TGs. This leads to adipocytes hypertrophy (enlargement of adipocytes). Progressive adipocytes hypertrophy causes increase in adipokines and pro-inflammatory cytokines production. Also, progressive adipocytes hypertrophy induces adipose tissue hypoxia resulting in adipocytes cell death. Besides, adipose tissue hypoxia initiates adipose tissue fibrosis. Progressive fibrosis of adipose tissue affects its ability of fat storage which in turn promotes the deposition of lipid in liver and muscle and increases insulin resistance (Cimini et al., 2017).

Leptin, an adipokine secreted by adipocytes, is found to play a role in the development and the progression of NAFLD, and is reported to have dual action on the pathogenesis of NAFLD, exerting anti-steatotic effect, but also pro-inflammatory and profibrogenic effects. In healthy individuals and individuals with NAFLD at initial stage, leptin helps prevent hepatic steatosis by suppressing hepatic glucose production, inhibiting hepatic *de novo* lipogenesis and promoting β-oxidation of fatty acids. Thus, leptin helps improve insulin sensitivity. However, when NAFLD progresses to a certain

point that leptin resistance is developed, the action of leptin is impaired. In order to compensate the resistance, more leptin will be produced and causes hyperleptinemia. At this stage, leptin exerts its pro-inflammatory and profibrogenic effects and promotes hepatic inflammation and fibrogenesis (Cimini et al., 2017; Jung and Choi, 2014; Makki et al., 2013; Polyzos et al., 2016).

Adiponectin is another major adipokine produced by adipocytes. It is a protein hormone serving as a regulator of energy homeostasis by regulating glucose and lipid metabolism. Adiponectin inhibits gluconeogenesis, suppressing fatty acids synthesis and promoting β-oxidation of fatty acids. These effects help prevent hepatic steatosis and improve insulin sensitivity. Apart from that, adiponectin exerts anti-inflammatory effect by reducing the expression of nuclear factor-kappaB (NF-κB), inhibiting NF-κB signaling pathway and antagonizing the actions of pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-α) and some of the interleukins (ILs) (Cimini et al., 2017; Jung and Choi, 2014; Makki et al., 2013; Polyzos et al., 2016).

1.2.6 Oxidative stress

Oxidative stress occurs when there is an imbalance between free radicals generation and antioxidant defenses. Over production of ROS or impairment of body system's ability to eliminate ROS result in accumulation of oxidative stress. Elevated intracellular ROS can lead to oxidative damage to DNA, proteins and phospholipids (Birben et al., 2012; Kurutas, 2016).

It has been well established that oxidative stress is a crucial factor in the pathogenesis of NAFLD and is associated with hepatocyte mitochondrial dysfunction and inflammation induction. Mitochondria are responsible for β-oxidation of fatty acids. When fatty acid load is high, β-oxidation will be overwhelmed. As a result, increased amount of ROS will be generated and oxidative stress will be induced. ROS not only causes oxidative damage to mitochondria resulting in mitochondrial dysfunction, but also attacks other cellular organelles and DNA resulting in apoptosis of hepatocytes. Apart from that, ROS also contributes in activation of inflammatory pathways and leads to liver inflammation (Gupte et al., 2013; Rodriguez-Ramiro et al.,

2016; Serviddio et al., 2013).

1.2.7 Gut microbiota

Gut microbiota is a complex and dynamic population of microorganisms that lives in the gastrointestinal tract and maintains a symbiotic relationship with the host. In healthy individuals, gut microbiota plays a lot of important roles. Gut microbiota perform fermentation of complex carbohydrates into short chain fatty acids. It also performs synthesis of vitamin K and several components of vitamin B. Good microbiota helps maintain the integrity of gut barrier and the structure of gastrointestinal tract, and protects against pathogens by fully colonizing the space and maintaining the immunity (Jandhyala et al., 2015).

Recent studies suggested that gut microbiota may play an important role in the pathogenesis of NAFLD. The configuration of gut microbiota is highly influenced by the nutrient intake of the host. Various studies found that high-fat diet causes alteration of the gut microbiota. These changes in the composition and the diversity of gut microbiota are associated with the development and the progression of NAFLD

(Bashiardes et al., 2016; Leung et al., 2016; J. Ma et al., 2017).

The liver receives 70% of its blood supply from the intestine via the portal vein, thus it is continually exposed to gut-derived factors including gut bacterial components, endotoxins (lipopolysaccharide, flagellin and lipoteichoic acid) and peptidoglycans (Son et al., 2010). Alteration of gut microbiota structure affects the integrity of the intestinal barrier, resulting in increased intestinal permeability which leads to translocation of microbiota-derived harmful substances into the portal circulation and directly to the liver (Nadeau and Conjeevaram, 2017). Besides, high-fat diet influences bacterial metabolism, resulting in alterations of the metabolic by-products of the gut microbiota, such as increased production of short-chain fatty acids and ethanol. These alterations are associated with the progression of obesity and NAFLD (Cummings et al., 2001; Zhu et al., 2013). Gut microbiota dysbiosis also affects bile acid metabolism and bile acid-related mechanistic pathways. Disrupted gut microbiota changes the composition of bile acid pool and involves in the activation of bile acid receptors, such as the Farnesoid X receptor (FXR) and the G-protein coupled bile acid receptor 1 (GPBAR1). Impaired bile acid metabolism not only affects glucose homeostasis,

cholesterol breakdown and excretion, but also initiates inflammatory response causing liver injury (de Aguiar Vallim et al., 2013; M. Li et al., 2017; Martoni et al., 2014; Ridlon et al., 2006).

1.2.8 Inflammation

Inflammation is a key process involved in the progression of NAFLD from simple steatosis to NASH. Chronic inflammation induces hepatic injury and is suggested to promote liver fibrosis and cirrhosis resulting in liver dysfunction. Various factors, including lipotoxicity due to elevated levels of FFAs and hepatic lipid accumulation, oxidative stress, adipose tissue dysfunction and endotoxins produced by gut microbiota, promote the production and the release of pro-inflammatory cytokines which activates inflammatory response in the liver (Buzzetti et al., 2016; Dowman et al., 2010).

I kappa B kinase (IKK)/NF-κB and c-Jun N-terminal kinase (JNK)/activator protein-1 (AP-1) signaling pathways are the two major inflammatory pathways involved in the hepatic chronic inflammation (Hotamisligil, 2006). Both of the two pathways can be directly activated by oxidative stress and FFAs. Activations of

IKK/NF-κB and JNK/AP-1 signaling pathways trigger the production of pro-inflammatory cytokines, such as TNF-α, IL-6 and AP-1, promoting inflammatory responses. It is suggested that both of the two pathways are involved in the development of insulin resistance (Cai et al., 2005).

1.3 Signaling pathways

1.3.1 AMPK

AMP-activated protein kinase (AMPK) is an enzyme that plays a central role in the regulation of cellular energy metabolism. AMPK expresses universally in all eukaryotes serving as cellular energy sensor to maintain metabolic homeostasis. It exists as a heterotrimeric protein complex consisting of a catalytic α subunit and regulatory β and γ subunits. These subunits are critical for maintaining the structure of AMPK and play specific roles on the activity of AMPK which enable AMPK to detect changes in the adenosine-5′-diphosphate (ADP):adenosine-5′-triphosphate (ATP) and adenosine-5′-monophosphate (AMP):ATP ratios. Elevation of ADP:ATP and AMP:ATP ratios leads to activation of AMPK which promotes the production of ATP

by increasing glucose uptake, glycolysis and fatty acid oxidation, and reduces the use of ATP by inhibiting the synthesis of glycogen, fat and cholesterol (Gowans and Hardie, 2014; Hardie, 2014; P. Sanz, 2008).

Sterol regulatory element binding proteins (SREBPs) are a family of transcription factors that regulate cellular lipid homeostasis (Eberlé et al., 2004; Horton, 2002). SREBP-1c is responsible for control of *de novo* lipogenesis and regulation of *de novo* lipogenesis related genes, such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS); while SREBP-2 is responsible for control of cholesterol metabolism and regulation of cholesterol metabolism related genes, such as HMG-CoA reductase (HMGCR) and low-density lipoprotein receptor (LDLR). Activation of AMPK downregulates the expression of both SREBP-1c and SREBP-2 (Y. Li et al., 2011), suppresses the expression of ACC, FAS, HMGCR and LDLR and inhibits fatty acids, TGs and cholesterol synthesis. This help alleviate TGs accumulation and prevent the development of hepatic steatosis.

1.3.2 MAPKs

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases that are responsible for transduction of extracellular stimuli into intracellular responses. They are involved in a variety of biological processes and cellular activities, including proliferation, differentiation, migration, stress response, survival and apoptosis. In mammalian cells, three sub-groups of MAPKs are well studied. They are extracellular signal-regulated kinase (ERK), JNK and p38 mitogen-activated protein kinase (p38 MAPK). ERK is majorly involved in the regulation of cell proliferation and survival; JNK is involved in the regulation of cell proliferation, apoptosis and inflammatory response; while p38 MAPK is mainly involved in the regulation of inflammatory response (Cargnello and Roux, 2011; E. K. Kim and Choi, 2010; W. Zhang and Liu, 2002).

MAPKs are involved in the pathogenesis of NAFLD. JNK has been recognized to be involved in the development of insulin resistance. Studies found that high-fat diet leads to activation of JNK resulting in insulin resistance (Kodama and Brenner, 2009;

Malhi et al., 2006; Tarantino and Caputi, 2011). High-fat diet also generates oxidative stress causing activation of p38 MAPK resulting in the release of inflammatory cytokines and the induction of inflammatory response. TNF-α, an inflammatory cytokine, is known as a potent activator of JNK. Activation of JNK is not only involved in the development of insulin resistance, but also promotes inflammatory response and cell apoptosis (Pearson et al., 2001). Several studies also observed that high-fat diet induces ERK activation in liver tissue (Bi et al., 2013; Jiao et al., 2013). Activation of hepatic ERK is suggested to be involved in inflammation as well as the increase in liver glycogen content and the decrease in energy expenditure. These cellular responses are highly related to the progression of NAFLD suggesting that MAPKs play an important role in the pathogenesis of NAFLD.

1.3.3 NF-κB

NF-κB is a family of transcription factors that functions as a sensor of cellular stressful changes and a key regulator of immune responses. It can be found in almost all types of cells. Activation of NF-κB promotes production of inflammatory cytokines,

such as TNF-α, IL-1, IL-6, resulting in the initiation of immune responses and inflammation. NF-κB can be activated by various stimuli, such as pathogen-derived molecules like lipopolysaccharide (LPS) and genetic materials of pathogens including viral and bacterial DNA and RNA, cellular stress like ROS induced oxidative stress, and inflammatory cytokines. NF-κB is critical in controlling immunity to protect cells against infection (Kaĭdashev, 2012). However, dysregulation of NF-κB causes multiple disorders including inflammatory disease, autoimmune disease and even cancer. NF-κB is known to be participated in the pathogenesis of NAFLD. High levels of FFAs and ROS activate NF-κB, leads to increase in expression of inflammatory cytokines (Duvnjak et al., 2007). These inflammatory cytokines can also activate NF-κB, which in turn increases the severity of inflammation. Apart from that, the inflammatory cytokines activate JNK. Both NF-κB and JNK are found to be involved in the development of hepatic insulin resistance (Tilg and Moschen, 2008). Chronic inflammation of the liver can cause liver fibrosis, cirrhosis and cancer (Anupam Bishayee, 2014).

1.3.4 PPARs

Peroxisome proliferator-activated receptors (PPARs) are a family of ligand-regulated nuclear receptor proteins consisting of three isotypes, PPAR-α, PPAR-β/δ, and PPAR-γ. PPARs serve as key regulators in lipid, lipoprotein and glucose metabolism. They also participate in cell proliferation, differentiation and apoptosis (Bensinger and Tontonoz, 2008; Berger et al., 2005; Stienstra et al., 2007). PPAR-α is highly expressed in metabolically active cell types including hepatocytes, skeletal muscle cells, enterocytes, the proximal tubule cells of kidney, cardiomyocytes and endothelial cells, and is mainly responsible to regulate lipid metabolism and modulate transportation and β-oxidation of fatty acids (Rakhshandehroo et al., 2010; Stienstra et al., 2007). PPAR-β/δ is highly and ubiquitously expressed and is suggested to be involved in the regulation of lipid metabolism, glucose homeostasis, cell differentiation, inflammation and angiogenesis (Müller et al., 2008). PPAR-γ is highly expressed in adipose tissue, the immune system and retina, and is mainly responsible in mediating adipocyte differentiation and controlling fatty acid uptake into adipocytes (Elangbam et al., 2001; Floyd and Stephens, 2012; Martin et al., 1998).

PPARs play an important role in the pathogenesis of NAFLD (Tailleux et al., 2012; Videla and Pettinelli, 2012). PPAR-α promotes fatty acid β-oxidation and inhibits lipogenesis and thus can help decrease hepatic lipid accumulation, alleviate insulin resistance of the liver and lower blood FFA level. Apart from that, PPAR-α suppresses NF-κB pathway and inhibits inflammation in the liver (N. Zhang et al., 2014). PPAR-γ promotes fatty acid uptake into adipocytes, this can reduce the amount of FFA delivery to the liver which in turn reduce hepatic lipid accumulation. Several studies showed activation of PPAR-γ help improve insulin sensitivity (S.-S. Choi et al., 2016; Leonardini et al., 2009; Olefsky, 2000). The role of PPAR-β/δ is still not well-defined, but evidence showed that activation of PPAR-β/δ also promotes fatty acid β-oxidation, improves insulin sensitivity and attenuates inflammatory responses (Luquet et al., 2005; Monsalve et al., 2013; Palomer et al., 2018). Overall, activation of PPARs shows to alleviate NAFLD.

1.3.5 PI3K/Akt

Phosphatidylinositol 3-kinases (PI3K) are a family of lipid kinases that regulates

diverse cellular activities including cell growth, cell survival and apoptosis. Protein kinase B (Akt), a serine/threonine kinase, is a major downstream target of PI3K (Akinleye et al., 2013; Cantley, 2002).

PI3K/Akt signaling pathway is insulin downstream molecular pathway, which plays an important role in regulating the glucose metabolic actions of insulin. Insulin activates the insulin receptor tyrosine kinase which phosphorylates insulin receptor substrate (IRS) resulting in the activation of PI3K/Akt signaling pathway (Metz and McGarry Houghton, 2011; Yoon, 2017). The activation of Akt activates glycogen synthase promoting glycogenesis in the liver. It has been observed that the activities of PI3K/Akt decreased in the liver of patients with NAFLD (Pang et al., 2018). Upregulation of PI3K/Akt is suggested to improve hepatic insulin sensitivity.

As discussed before, oxidative stress is one of the crucial factors involved in the pathogenesis of NAFLD. Studies found that restoration of PI3K/Akt signaling pathway induces the expression of the antioxidant enzyme heme oxygenase-1 (HO-1) in the liver, which protect hepatocytes against oxidative damage (Joung et al., 2007; W. Li et al.,

2016; A. Li et al., 2017).

PI3k/Akt signaling pathway also plays a role in the progression of inflammatory response. Activation of PI3K inhibits the production of inflammatory cytokines, such as TNF-α, and upregulate the anti-inflammatory cytokines, such as IL-10, to suppress inflammation (Cherla et al., 2009; Haidinger et al., 2010).

1.4 Therapeutic treatments

Currently, there is no standard therapeutic medical treatments for patients with NAFLD (Dajani and AbuHammour, 2016; Nascimbeni et al., 2013). Recommended treatments nowadays mainly focus on lifestyle modification and medications that target on the pathological mechanisms of NAFLD such as drugs that lower blood lipid level, insulin sensitizers, drugs that reduce body weight, bile acid sequestrants, antioxidant agents, and anti-inflammatory drugs, etc. These current recommended treatments for NAFLD will be reviewed as follows.

1.4.1 Lifestyle modification

Lifestyle modification is suggested to be the primary treatment strategy and the first-line therapy for patients with NAFLD (Eckard et al., 2013; Golabi et al., 2017; Harrison and Day, 2007). It is known that NAFLD is associated with obesity and metabolic syndromes including type II diabetes mellitus. Excessive nutrients intake and low level of energy utilization are major causes of NAFLD. Lifestyle modification, including dietary modification and increasing physical activity, has been demonstrated to alleviate NAFLD. However, lifestyle modification is sometimes difficult to implement because patients are not able to maintain long-term lifestyle changes.

1.4.1.1 Dietary modification

Diet plays a key role in the development of NAFLD (Mirmiran et al., 2017). Nutritional imbalance is considered to be one of the crucial factors of causing NAFLD. Western dietary pattern, characterized with high consumption of red meat, white bread, refined grains and processed sugar products including soft drinks and confectionary; and low consumption of fish, vegetables and fruits, is recognized to be positively associated with the risk of NAFLD (Oddy et al., 2013; Trovato et al., 2018). Western

diet, energy-rich and composed of high levels of saturated fats, trans-fats, cholesterol, sucrose and fructose, causes disorders in lipid and glucose metabolisms resulting in metabolic syndrome.

Modification of dietary patterns to restrict the intakes of saturated fats and simple carbohydrates reduces energy consumption and is investigated to be able to improve hepatic functions, including reduce hepatic lipid accumulation, increase liver enzyme levels and lower insulin resistance, and alleviate obesity and NAFLD (Ferolla et al., 2015). Increase intakes of fruits, vegetables and food with unsaturated fats are also shown to reduce risk of metabolic syndrome and NAFLD (Mirmiran et al., 2017; Yang et al., 2015).

1.4.1.2 Physical activity

It is widely accepted that physical activity and exercise provide benefits to our health. Regular exercise is known to reduce risk of obesity, cardiovascular diseases, type II diabetes mellitus and NAFLD (Bae et al., 2012; Keating et al., 2015). Improving physical activity behavior is considered as an important part of lifestyle

intervention of NAFLD. Increased intensity of physical activity promotes energy expenditure which in combination with dietary modification helps maintain better energy balance in NAFLD patients. It also helps reduce hepatic TG and serum FFA levels resulting in alleviation of NAFLD. Various studies demonstrated that exercise can achieve hepatic TGs reduction independently of weight loss (Alex et al., 2015; Golabi et al., 2016; Hallsworth et al., 2011). Moreover, exercise was demonstrated to improve insulin resistance and glucose metabolism (Venkatasamy et al., 2013). A few studies also found that exercise may help attenuate inflammation in NAFLD patients (Komine et al., 2017; Oh et al., 2017; Vilar-Gomez et al., 2015). During exercise, muscle contraction induces the production and release of myokines, such as IL-6. These myokines can mediate direct and indirect anti-inflammatory effects and may help attenuate hepatic inflammation (Oliveira et al., 2016). Regular physical activity is considered as a preventive measure and treatment of NAFLD.

1.4.2 Pharmacologic therapy

1.4.2.1 Insulin sensitizers

Metformin, a biguanide class antihyperglycemic drug, is a first-line treatment of

type II diabetes mellitus (Song, 2016). Metformin reduces blood glucose level by inhibiting gluconeogenesis in the liver and enhancing glycogen synthesis. Besides, it also facilitates glucose uptake in muscle by increasing the activity of glucose transporter type 4 (GLUT4). These help alleviate peripheral insulin resistance (Giannarelli et al., 2003). For lipid metabolism, metformin promotes fatty acid oxidation, enhance re-esterification of FFAs and inhibits lipolysis in adipose tissue (Viollet et al., 2012). These reduce hepatic lipotoxicity and may help improve hepatic insulin sensitivity. However, metformin causes adverse effect of gastrointestinal irritation (Bonnet and Scheen, 2017; Bouchoucha et al., 2011; McCreight et al., 2016). Long-term usage of metformin can cause malabsorption of vitamin B_{12} resulting in vitamin B_{12} deficiency (Aroda et al., 2016; K. W. Liu et al., 2006). Metformin is also contraindicated in patients with chronic kidney disease, liver cirrhosis, chronic obstructive pulmonary disease and heart failure because these complications increases the risk of metformin-associated lactic acidosis which can be fatal (Tahrani et al., 2007).

Thiazolidinediones, also known as glitazones, are a class of anti-diabetic drugs.

Rosiglitazone and pioglitazone are the two thiazolidinediones approved by the United States Food and Drug Administration for treating type II diabetes mellitus. Thiazolidinediones work by targeting PPARs, especially PPAR-γ (Hauner, 2002; Reginato and Lazar, 1999). The activation of PPAR-γ promotes storage of fatty acids in adipocytes and thus reduces circulation as well as hepatic uptake of fatty acids. As a result, the body turns to be more dependent on the oxidation of carbohydrates for yielding energy. These help reduce blood glucose level and improve insulin sensitivity. However, the promotion of fatty acid storage in adipocytes leads to side effect of weight gain (Mahady, 2013). Apart from that, several studies suggested that the use of thiazolidinediones may be associated with increased risk of cardiovascular disease, bladder cancer and bone fracture (Mahady, 2013; Stein et al., 2009; W.-h. Xiao et al., 2013).

1.4.2.2 Cholesterol-lowering drugs

Statins are a class of drugs that reduce the level of cholesterol in the blood working as HMGCR inhibitors (Istvan, 2002). Since the structures of statins and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) are similar, they compete the

active site of HMGCR with each other and thus reduce the rate of HMGCR in turning HMG-CoA to mevalonic acid resulting in inhibition of cholesterol synthesis in the liver. Statins also upregulate SREBP-2 in hepatocytes which stimulates the transcription of LDLR (Rashid et al., 2005). Increased LDLR expression promotes hepatic uptake of low-density lipoprotein cholesterol (LDL-C) from the circulation and thus reduces the LDL-C level in the blood. Statin treatment is generally safe and well tolerated. However, long-term high-intensity statin treatment may induce liver toxicity (Calderon et al., 2010; Pastori et al., 2015). Other commonly reported adverse effects of statins are statin-associated muscle-related adverse effects, including muscle pain, tenderness, or weakness. Statins can cause muscle inflammation and damage. In more severe conditions, statins can cause myopathy, myalgia, myositis or even rhabdomyolysis (Bitzur et al., 2013; Khayznikov et al., 2015). Breakdown of skeletal muscle leads to release of muscle fiber contents, such as myoglobin, into the bloodstream, which are harmful to the kidney and can cause kidney failure.

1.4.3 Treatments that promotes weight loss

1.4.3.1 Bariatric surgery

Bariatric surgery includes several types of surgery, such as gastric sleeve, gastric bypass, and gastric balloon, etc. These surgeries aim at achieving weight loss by reducing the size of the stomach, removing a portion of the stomach or rerouting of the intestines. Bariatric surgery helps reduce the amount of food intake and absorption resulting in weight loss. It is known that bariatric surgery is effective in alleviate nutritional diseases, including obesity, type II diabetes mellitus and NAFLD (Holterman et al., 2014; Koliaki et al., 2017). Like other treatments, bariatric surgery also carries risks and causes adverse side effects. Malnutrition is one of the most common complications of bariatric surgery (Malinowski, 2006). Patients undergoing bariatric surgery may experience chronic nausea and vomiting. Bariatric surgery can lead to dilation of oesophagus resulting in reflux of stomach acid. Besides, it can cause obstruction of stomach or intestine. Bariatric surgery can also cause internal hernia or ulcers. There are also risks of port breakage or leakage (Kassir et al., 2016).

1.4.3.2 Orlistat

Orlistat, a pancreatic lipase inhibitor, can stop the enzymes from digesting triglycerides. This help reduce the amount of dietary fat being absorbed by the body in order to lower calorie intake, promote weight loss and improve NAFLD (Harrison et al., 2009). Besides, a randomized controlled study found that orlistat treatment decreased alanine transaminase (ALT) level in NAFLD patients (Zelber–Sagi et al., 2006). However, orlistat causes various side effects, including abdominal cramps, oily evacuation, oily rectal leakage and steatorrhea. Also, long-term orlistat treatment may cause fat-soluble vitamin deficiency (Huang et al., 2013; Sjöström et al., 2007).

1.5 Dietary phytochemicals

Given that the current pharmacologic therapeutic agents used for NAFLD treatment possess different degrees of adverse side effects, there is a need to identify novel treatment candidates with high efficacy and safety profiles for NAFLD management. Therefore, increasing numbers of studies have focused on natural compounds for preventing and treating NAFLD. Dietary phytochemicals, such as carotenoids, phenolic compounds, alkaloids, glycosides, saponins, and terpenes,

naturally exist in plants and consumption of dietary phytochemicals has been associated with reducing the risk of various chronic metabolic diseases, including obesity, cardiovascular disease, type II diabetes mellitus, and NAFLD (Craig, 1997; Dillard and German, 2000; Y.-J. Zhang et al., 2015). In this section, recent studies on dietary phytochemicals for NAFLD management and their potential mechanisms will be summarized and discussed.

1.5.1 Carotenoids

Carotenoids are a class of lipid soluble phytochemicals. They are organic pigments that give the yellow, orange and red colour to fruits and vegetables. Since carotenoids can only be synthesized by plants, fungi, algae and bacteria, they must be consumed through the diet. Nowadays, more than 700 naturally occurring carotenoids have been identified and among them, α-carotene, β-carotene, lutein, lycopene, zeaxanthin and cryptoxanthin are the most common carotenoids in the diet. Due to their lipophilic properties, carotenoids accumulate mainly in hepatic tissue and may serve as free radicals scavengers to protect the liver against oxidative damage (Murillo et al.,

2016; Ni et al., 2016). Carotenoids are also considered as anti-inflammatory micronutrients, which may help inhibit the progression of NAFLD (Yilmaz et al., 2015). A previous human study observed that plasma levels of carotenoids, including α-carotene, β-carotene, lutein, and lycopene, were significantly decreased in NASH patients compared to healthy controls, suggesting that the depletion of carotenoids in NASH patients may be due to counterbalance of oxidative stress, and carotenoids may be a rational treatment option for NAFLD (Erhardt et al., 2011). Another study about investigating the relationship between serum carotenoid levels and the prevalence of NAFLD in Chinese adults also found that increased serum levels of α-carotene, β-carotene, lutein, zeaxanthin and total carotenoids were significantly associated with a decrease in the prevalence as well as the degree of NAFLD, suggesting that carotenoids may help reduce the risk of having NAFLD (Cao et al., 2015).

1.5.1.1 β-carotene

β-carotene, a provitamin A carotenoid, presents in red orange fruits and vegetables, especially carrots. It is a precursor for vitamin A and is the major dietary source of vitamin A. β-carotene is also considered as an antioxidant which protects body cells

against oxidative damage. Several studies have reported that β-carotene may help alleviate NAFLD. An *in vivo* study using high-fat diet-induced Sprague-Dawley rat (SD rat) model of NAFLD demonstrated that administration of β-carotene improved NAFLD via antioxidative, anti-inflammatory and lipid lowering effects. Also, it was shown to significantly reduce serum levels of ALT and aspartate transaminase (AST), and increase superoxide dismutase activity in the liver (Seif El-Din et al., 2015). Another *in vivo* study found that administration of 9-*cis* β-carotene inhibited fat accumulation and inflammation in the livers of high-fat diet-fed LDLR knockout mice. Apart from that, administration of 9-*cis* β-carotene significantly reduced high-fat diet-induced hypercholesterolemia. Cholesterol and TG levels in the liver were also decreased. The anti-inflammatory effect of 9-*cis* β-carotene treatment could be due to the reduction in the hepatic expression levels of inflammatory genes, such as toll-like receptor 2, IL-1α and vascular cell adhesion molecule-1 (Harari et al., 2008).

1.5.1.2 Lycopene

Lycopene is a non-provitamin A carotenoid and can be found in red fruits and vegetables. Tomatoes are the major dietary sources of lycopene. Epidemiologic studies

have shown an inverse association between the consumptions of tomatoes / tomato-based products and the risks of various metabolic disorders (Agarwal and Rao, 2000; Heber and Lu, 2002; Murillo et al., 2016; Rao and Agarwal, 2000). Lycopene has been suggested to play an important role in the beneficial effects brought by tomato consumption. An *in vivo* study found that treatment of lycopene improved hepatic steatosis in high-fat diet-fed C57BL/6J mice. Weight gain as well as hepatic lipid accumulation caused by high-fat diet were significantly reduced by lycopene treatment. It was believed that lycopene improved NAFLD by regulating lipid metabolism in the liver and inhibiting intracellular lipid accumulation through upregulation of miR-21 and inhibition of fatty acid binding protein 7 (Ahn et al., 2012). Another *in vivo* study using high-fat diet-induced Wistar rat model of NAFLD evaluated the hepatoprotective effect of lycopene. The results showed that lycopene treatment significantly decreased the serum levels of ALT, AST, TG and total cholesterol (TC), increased the levels of superoxide dismutase and glutathione in the liver. The study suggested that lycopene exerts a protective effect on NAFLD through downregulation of TNF-α and CYP2E1 expressions (Jiang et al., 2016).

1.5.2 Phenolic compounds

Phenolic compounds are a group of secondary metabolites of plants and are the biggest group of dietary phytochemicals. They have a common structure consisting of a hydroxyl group bonded directly on a benzene ring. Phenolic compounds comprise a wide range of molecules from simple monophenolic structures, such as phenols and phenolic acids, to polyphenolic structures, such as flavonoids, stilbenes and tannins, and also highly polymerised compounds derived from these various groups. Phenolic compounds exhibit a wide range of beneficial health effects and have been reported as potential agents for preventing and treating various metabolic diseases, such as cardiovascular diseases, type II diabetes mellitus, NAFLD, neurodegenerative diseases, and cancer (S. Li et al., 2018; D. Lin et al., 2016; Ozcan et al., 2014; Rio et al., 2013). It is believed that antioxidant properties as well as the ability to interact with proteins are the key factors that make phenolic compounds possessing different bioactivities (Ozcan et al., 2014).

1.5.2.1 Phenolic acids

Phenolic acids are a class of phenolic compounds containing at least one phenol

ring and a carboxyl group. They can be divided into two major groups, hydroxybenzoic acids and hydroxycinnamic acids. Caffeic, ferulic and *p*-coumaric acids are the most common representatives of phenolic acids and have been frequently encountered and studied for their bioactivities (Heleno et al., 2015). Several studies have reported that these phenolic acids may help ameliorate NAFLD. Caffeic acid has been shown to possess lipid lowering effect on steatotic HepG2 cells by inhibition of lipogenesis and promotion of lipolyisis via upregulation of AMPK (Liao et al., 2014). Caffeic acid has also been reported to reduce lipid accumulation and endoplasmic reticulum stress in the liver of high-fat diet-induced obese C57BL/6 mice by increasing autophagy and improving insulin sensitivity (H. M. Kim et al., 2018). Besides, a study found that combined treatment with caffeic and ferulic acids prevented body weight gain, improved hyperglycemia, hypercholesterolemia and hypertriglyceridemia in high-fat diet-fed C57BL/6 mice by improving glucose sensitivity and lipid metabolism (Bocco et al., 2016). Apart from that, in an *in vivo* study investigating the effects of ferulic acid on alleviating high-fat and high-fructose diet-induced metabolic syndrome parameters, ferulic acid treatment was shown to alleviate obesity, hyperlipidemia, hyperglycemia,

hepatic injury, and insulin resistance in high-fat and high-fructose diet-fed SD rats, suggesting that ferulic acid could be used as dietary supplements for high-fat and high-fructose diet-induced metabolic disorders (Wang et al., 2015).

1.5.2.2 Flavonoids

Flavonoids are known as one of the largest classes of phytochemicals. They are benzo-γ-pyrone derivatives consisting of two phenolic rings and one pyrane ring. Flavonoids can be further classified into different subclasses, including flavones, flavonols, flavanones, flavanonols, isoflavones, flavan-3-ols, anthocyanidins and neoflavonoids.

Quercetin, a flavonol, has been reported to ameliorate NAFLD *in vitro* and *in vivo*. In a study using FFA- and insulin-induced HepG2 cell model of NAFLD, quercetin was found to improve insulin resistance by enhancing insulin signal transduction and reduce hepatic lipid accumulation by downregulation of SREBP-1c and FAS (X. Li et al., 2013). Quercetin was also shown to significantly reduce various hepatic biomarkers and biochemical parameters, including serum albumin, total bilirubin, creatinine, urea,

uric acid and glucose levels, in rats with NASH (Surapaneni and Jainu, 2014). A study demonstrated that quercetin alleviated insulin resistance, improved serum lipid profiles, ameliorated hepatic steatosis and reduced serum ALT activities in *ob/ob* mice, and suggested that quercetin prevented NAFLD partly by overexpression of adiponectin and reduction of pro-inflammatory cytokines (H.-N. Choi et al., 2015).

Kaempferol, another flavonol, was found to reduce the hepatic accumulation of TGs and cholesterol and improved hyperlipidemia in high-fat diet-induced obese rats by increasing lipid metabolism through the downregulation of SREBPs and promoting the hepatic expressions of acyl-CoA oxidase and CYP4A1 as well as upregulation the hepatic expression of PPAR-α (C. J. Chang et al., 2011). Besides, kaempferol was shown to alleviate insulin resistance via inhibition of hepatic IKK/NF-κB pathway in diabetic SD rats induced by high-fat diet plus streptozotocin. Serum biochemical parameters, including ALT, AST, TGs, TC, and LDL-C, were also significantly reduced by kaempferol treatment (Luo et al., 2015).

Epigallocatechin gallate is a flavan-3-ol that is commonly found in tea. Increasing

evidence shows that epigallocatechin gallate is associated with benefits in treating NAFLD and these benefits are suggested to be related to the antioxidative, anti-inflammatory and anti-fibrosis effects of epigallocatechin gallate and its ability in regulating glucose and lipid metabolisms (Chen et al., 2018). An *in vivo* study investigating the protective mechanisms of epigallocatechin gallate in high-fat diet-induced SD rat model of NAFLD demonstrated that epigallocatechin gallate treatment reduced hepatic accumulation of lipid, and attenuated fibrosis, oxidative stress, and inflammation in the liver via modulation of TGF/SMAD, PI3K/Akt/FoxO1, and NF-κB pathways (J. Xiao et al., 2014). Besides, in another *in vivo* study, epigallocatechin gallate was found to improve NAFLD in high-fat diet-induced C57BL/6 mouse model of NAFLD and ameliorate insulin resistance through enhancing the insulin clearance by upregulation of hepatic insulin-degrading enzyme expression (Gan et al., 2015).

1.6 Pterostilbene

Pterostilbene (trans-3,5-dimethoxy-4′-hydroxystilbene; Figure 1.6.1) is a

naturally derived polyphenolic compound that is primarily present in blueberries, grapes and *Pterocarpus marsupium* heartwood (H. S. Lin et al., 2009; Roupe et al., 2006). Its content varies among different types of berries, ranged from 99 ng/g dry weight in *Vaccinium ashei* to 520 ng/g dry weight in *Vaccinium stamineum* (A. M. Rimando et al., 2004; Rodriguez-Bonilla et al., 2011). Pterostilbene is a stilbenoid phytochemical and is a natural dimethylated analogue of resveratrol. Resveratrol, a natural polyphenol that is found largely in grapes and barriers, has been reported to have a wide range of beneficial health effects. With a chemical structure similar to resveratrol, pterostilbene was thought to be potential to have comparable biological activities as resveratrol. Accumulating studies demonstrate that pterostilbene possesses diverse biological activities, including antioxidative, anti-diabetic, anti-inflammatory, anti-aging, anti-cancer, cardiovascular protective and neuroprotective effects. Besides, pterostilbene has no known toxicity level. Previous study demonstrated that dietary administration of 3000 mg/kg bw/day pterostilbene for 28 days did not cause any toxic effect on mice (Ruiz et al., 2009). Such properties make pterostilbene a potential preventive and therapeutic agent in variety of human diseases including metabolic

disorders, cardiovascular and neurological diseases, as well as cancer (McCormack and McFadden, 2012). Due to the presence of two methoxy groups, pterostilbene exhibits increased lipophilic and oral absorption as well as bioavailability (Athar et al., 2007; A. Bishayee, 2009; Perecko et al., 2010; Stivala et al., 2001). Pharmacokinetic studies demonstrated that the bioavailability of pterostilbene is 80% which is much higher than that of resveratrol (20%) (Kapetanovic et al., 2011). The substitution of two hydroxyl groups with two methoxy groups in pterostilbene helps increase its transportation into cells. Moreover, the methoxy groups prevent pterostilbene from being quickly glucuronidated and sulphated for excretion, and thus increase the metabolic stability of pterostilbene. This provides pterostilbene a biological advantage over resveratrol as a potential therapeutic agent.

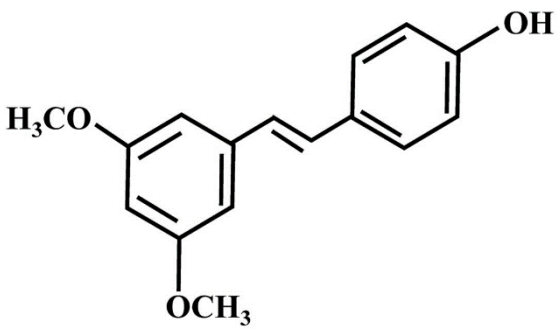

Figure 1.6.1 Chemical structure of pterostilbene.

1.6.1 Antioxidative effect

Pterostilbene has been demonstrated to exert dose-dependent antioxidant effect and its antioxidant activity is comparable to resveratrol (Amorati et al., 2004; Agnes M. Rimando et al., 2002; Stivala et al., 2001). The compound itself is an antioxidant and can serve as a direct scavenger of oxidants. Multiple studies showed that pterostilbene exhibits free radical scavenging effect in cell free system and the results are comparable to those of resveratrol. These studies demonstrated pterostilbene to be a free radical scavenger against various free radicals, including 2,2-diphenyl-1-picryl-hydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and hydrogen peroxide in a concentration-dependent manner. Pterostilbene has also been shown to inhibit lipid peroxidation *in vitro*.

Apart from that, pterostilbene has been found to be able to increase the activities of superoxide dismutase and catalase *in vivo* (Satheesh and Pari, 2006). Superoxide dismutase and catalase are the major antioxidant enzymes that are responsible for the elimination of free radicals in the body. The increase in the activities of these

antioxidant enzymes helps attenuate oxidative stress and protect cells against oxidative damage.

1.6.2 Anti-diabetic effect

Pterostilbene has been reported to have antiglycemic effect. A study found that treatment with pterostilbene significantly reversed the decreased activities of antioxidant enzymes in liver and kidney of streptozotocin (STZ)-nicotinamide-induced diabetic rats as well as normalized the increased levels of lipid peroxidation in liver and kidney of the diabetic rats. Treatment with pterostilbene also significantly reduced the pathological changes observed in liver and kidney of the diabetic rats, suggesting that pterostilbene attenuates type II diabetes mellitus by exhibiting its antioxidant property (Satheesh and Pari, 2006). Another study demonstrated that pterostilbene exhibits anti-diabetic effect and protects pancreatic β-cell against oxidative stress by activating nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated antioxidant signaling pathways (Elango et al., 2016). The activation of Nrf2 promotes the production of antioxidant enzymes (superoxide dismutase and catalase), phase 2 detoxification

enzymes (NAD(P)H quinone oxyreductase and HO-1) and glutathione biosynthesis enzymes (glutathione and glutathione S transferase). Also, pterostilbene was demonstrated to improve serum insulin levels and intraperitoneal glucose tolerance of the STZ-induced diabetic mice, suggesting that pterostilbene ameliorates type II diabetes mellitus by exhibiting antioxidant and hypoglycemic effects (Elango et al., 2016). Pterostilbene has also been shown to regulate glucose homeostasis. Treatment with pterostilbene significantly increased the activities of glycolytic enzyme, hexokinase, and significantly decreased the activities of gluconeogenic enzymes, glucose-6-phosphatase and fructose-1,6-bispgosphatase, in diabetic rats. Regulation of these hepatic enzymes promotes glycolysis and inhibits gluconeogenesis resulting in improvement of glycemic control (Pari and Satheesh, 2006). Furthermore, pterostilbene has been demonstrated to ameliorate insulin resistance in obesogenic-diet-fed rats. The expression of GLUT4 in skeletal muscle was increased in pterostilbene-treated rats, indicating that promotion of glucose uptake in skeletal muscle to facilitate glucose utilization may be involved in the anti-diabetic effect of pterostilbene (Gomez-Zorita et al., 2015).

1.6.3 Anti-inflammatory effect

Several studies have demonstrated that pterostilbene exhibits anti-inflammatory effect. As discussed before, pterostilbene was shown to promote activation of Nrf2 to protect pancreatic β-cell against oxidative stress. Another study using IL-1β-treated chondrocytes demonstrated that pterostilbene activates Nrf2 to attenuate inflammation in chondrocytes. Treatment with pterostilbene reversed IL-1β-induced increased levels of pro-inflammatory enzymes, cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS), and inflammatory mediators, nitric oxide (NO) and prostaglandin E2 (PGE2), and inhibit IL-1β-induced inflammation via stimulating the nuclear translocation of Nrf2 (Xue et al., 2017). In addition to activation of Nrf2, a study demonstrated that p38 MAPK pathway may also be involved in the anti-inflammatory action of pterostilbene. Pterostilbene exhibited anti-inflammatory effect in human colon carcinoma cell lines (HT-29) by inhibiting the cytokine-induced activation of p38 resulting in downregulation of inflammation-related genes including COX-2 and iNOS (Paul et al., 2009). A study also showed treatment with pterostilbene decreased the levels of pro-inflammatory mediators, including TNF-α, IL-1β, IL-6, matrix

metallopeptidase (MMP)-2 and MMP-9, in human corneal epithelial cells (HCECs) exposed to hyperosmotic medium (J. Li et al., 2016). Pterostilbene was found to inhibit LPS-induced pro-inflammatory mediators such as TNF-α, IL-1β, IL-6, IL-18 and NO in human rheumatoid arthritic synovial fibroblasts (E11) and human monocytic cell line (THP-1), and the *in vitro* anti-inflammatory potency of pterostilbene was shown to be stronger than that of resveratrol (Choo et al., 2014). Furthermore, pterostilbene was found to suppress phosphorylation of p-IκBα and p-p65 in TNF-α-treated murine preadipocyte cell line (3T3-L1) causing decrease in the mRNA expression of COX-2, iNOS, IL-6, and IL-1β. In the same study, treatment with pterostilbene reduced the migration of macrophages toward adipocytes in a coculture model of 3T3-L1 adipocytes and RAW 264.7 macrophages, indicating that pterostilbene inhibits pro-inflammatory responses during the interaction between 3T3-L1 adipocytes and RAW 264.7 macrophages (Hsu et al., 2013).

1.6.4 Anti-aging effect

Limited studies have been carried out to investigate the anti-aging effect of

pterostilbene. A study found that treatment with pterostilbene helped to reverse cognitive behavioral deficits in aged animals. In the experiment examining cognitive performance of the aged animals, results showed that there was a significant negative correlation between hippocampal pterostilbene levels in the aged animals and the latency and distance to find the platform, suggesting that pterostilbene improved the deleterious effects of aging on cognitive performance, particularly working memory, in a dose-dependent manner. Treatment with pterostilbene was also shown to protect against the decrease in dopamine release following an oxidative stressor (Joseph et al., 2008). Moreover, a study using senescence accelerated mouse (SAMP8) as an accelerated aging model found that treatment with pterostilbene, but not resveratrol, significantly improved radial arm water maze function in the animals, suggesting that pterostilbene may protect against age-related diseases such as Alzheimer's disease. Their findings showed that pterostilbene serves a potent neuromodulator and its effect is likely to be associated with the increase of PPAR-α expression (J. Chang et al., 2012).

1.6.5 Anti-cancer effect

A considerable number of studies have been conducted to investigate the potential anti-cancer effect of pterostilbene. It has been reported that pterostilbene showed pharmacological benefits in treating various types of cancer, including lung cancer, colon cancer, prostate cancer, and melanoma, etc.

A study using human non-small-cell lung cancer cell lines, PC9 and A549, to investigate the anticarcinogenic effect of pterostilbene on non-small-cell lung cancer demonstrated that treatment with pterostilbene could enhance endoplasmic reticulum stress in a time- and dose-dependent manner. Treatment with pterostilbene decreased the intracellular glutathione level of cancer cells as well as increased caspase-3 activity and ROS level which promotes apoptosis in cancer cells (Z. Ma et al., 2017).

Similar results were found in another study treating human oesophageal cancer. Treatment with pterostilbene reduced the viability of human oesophageal cancer cells, EC109, in a time- and dose-dependent manner. The intracellular glutathione level was decreased, and the caspase-3 activity and ROS levels were increased in

pterostilbene-treated EC109. Pterostilbene was also shown to increase endoplasmic reticulum stress in EC109, and the employments of CHOP (an endoplasmic reticulum stress-related molecule) SiRNA and N-acetylcysteine (a ROS scavenger) further confirmed that the activation of endoplasmic reticulum stress signaling pathways were involved in the mechanisms of the anti-cancer effect of pterostilbene (Feng et al., 2016).

A study compared the inhibitory effects of pterostilbene and resveratrol on three human colon cancer cells, HCT116, HT29 and Caco-2. Results showed that pterostilbene exhibited more potent inhibitory effect on colony formation as well as stronger apoptosis-inducing effect on the three cancer cells when compared with resveratrol (Nutakul et al., 2011).

Another study conducted on metastatic prostate cancer also compared the chemopreventive effects of pterostilbene and resveratrol. Results found that both pterostilbene and resveratrol inhibited the viabilities of androgen-dependent LNCaP cells and androgen-independent PC-3 cells in a time- and dose-dependent manner.

Pterostilbene was also found to possess more potent effects than resveratrol (Zook, 2014).

Treatment with pterostilbene was shown to inhibit melanoma growth *in vivo*. It downregulated pituitary production of the adrenocorticotropin hormone as well as plasma levels of corticosterone causing decrease in the glucocorticoid receptor- and Nrf2-dependent signaling or transcription, and thus reduce antioxidant defenses and inhibit growth of melanoma cells (Benlloch et al., 2016).

In a study investigating the anti-cancer effect of pterostilbene on human oral cancer, pterostilbene was demonstrated to induce cell cycle arrest and apoptosis in SAS and OECM-1 cell lines. In pterostilbene treated cells, increased formation of acidic vesicular organelles and light chain 3 (LC3)-II expression were observed, suggesting that pterostilbene induced autophagy in these human oral cancer cell lines and the autophagy-inducing effect of pterostilbene was found to be associated with activation of JNK and inhibition of Akt, ERK, and p38 MAPK (Ko et al., 2015).

1.7 The aims of the study

Pterostilbene has been demonstrated to exhibit multiple biological activities, including antioxdative, anti-inflammatory and anti-diabetic effects. Such properties of pterostilbene may be able to target the pathogenic processes in the progression of NAFLD, such as oxidative stress, inflammation and insulin resistance. To validate the hypothesis, *in vitro* and *in vivo* hepatic steatotic experimental models were established and used in this study. The objectives of the present study are,

1. To investigate the protection effect of pterostilbene against oxidative stress as well as its lipid reduction effect and the possible molecular mechanisms using steatotic hepatic cell model *in vitro*.

2. To evaluate the potential therapeutic effects of pterostilbene against NAFLD using high-fat diet-induced hepatic steatosis animal model *in vivo*.

3. To investigate the possible mechanistic pathways of pterostilbene in treating NAFLD using molecular, lipidomic and genomic approaches.

Chapter 2 Materials and Methods

2.1 Determination of free radical scavenging activity

DPPH radical scavenging assay was used to determine the free radical scavenging activity of pterostilbene. DPPH free radical solution was prepared with methanol in a concentration of 24 mg/L. 50 μL of test solutions of different concentrations (0.1 – 3,000 μM) of pterostilbene were added to 1.95 mL of DPPH free radical solution and mixed well. Then, the sample solution mixtures were incubated in the dark for an hour. After incubation, the absorbance value of the sample (A_{sample}) measured at 515 nm against a blank by UV-visible spectrometer (Perkin Elemer Lambda 35). The absorbance value of 50 μL water in 1.95 mL DPPH free radical solution ($A_{control}$) was measured as control. Ascorbic acid (Vitamin C) was used as a reference antioxidant. All tests were performed in triplicate. The percentage free radical scavenging capacity (SR%) was calculated as follows:

$$SR\% = (1 - A_{sample}/A_{control}) \times 100\%$$

2.2 *In vitro* study

Normal human hepatic cell line (L02) was used as the cell model for the *in vitro*

study of pterostilbene in treating NAFLD.

2.2.1 Cell culture

L02 cells purchased from Shanghai Institute of Biological Sciences were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) (*v/v*) and 1% Penicillin/Streptomycin (*v/v*) in a humidified incubator maintained at 37°C and 5% CO_2. Subculture of cells was carried out for every three to four days with 0.25% trypsin in a subcultivation ratio of 1:3.

2.2.2 Evaluation of the toxicity of pterostilbene in L02 cells

To determine the toxicity of pterostilbene in L02 cells, MTT cell viability assay was used. After trypsinization and collection of L02 cells, they were seeded in a 96-well microplate with 1×10^4 cells in 100 µL complete medium per well and incubated at 37°C with 5% CO_2 overnight. Then, the culture medium was replaced with complete medium containing different concentrations of pterostilbene (0.3, 1, 3, 10, 30, 100, 300, or 500 µM) and dimethyl sulfoxide (DMSO; final concentration of 0.1%). The cells were incubated at 37°C with 5% CO_2 for 24 h. For the control, cells were

treated with 0.1% DMSO only for 24 h. After 24 h incubation, the medium was removed and 100 µL MTT solution (0.5 mg/mL in serum-free high glucose DMEM) was added into each well. The cells were incubated for 2 h at 37°C and 5% CO_2. Finally, the MTT solution was removed and 100 µL DMSO was added for complete dissolution of formazan in cells. The optical density of each well was measured be reading the absorbance at 570 nm with 655 nm as reference using a microplate reader (CLARIOstar®, BMG Labtech). The results were expressed as a percentage of MTT reduction, with the absorbance exhibited by the control cells arbitrarily set as 100%. The percentage cell viability was calculated as follows:

Percentage cell viability = (Absorbance of treatment group / Absorbance of control group) × 100%.

2.2.3 Oxidative stress

2.2.3.1 Evaluation of the toxicity of H_2O_2 in L02 cells

To determine the toxicity of H_2O_2 in L02 cells, MTT cell viability assay was used. After trypsinization and collection of L02 cells, they were seeded in a 96-well microplate with 1×10^4 cells in 100 µL complete medium per well and incubated at

37°C with 5% CO_2 overnight. Then, the culture medium was replaced with complete medium containing different concentrations of H_2O_2 (0.1, 0.3, 0.5, or 1 mM). The cells were incubated at 37°C with 5% CO_2 for 24 h. The cells without H_2O_2 exposure were served as the control. After 24 h incubation, the medium was removed and 100 µL MTT solution (0.5 mg/mL in serum-free high glucose DMEM) was added into each well. The cells were incubated for 2 h at 37°C and 5% CO_2. Finally, the MTT solution was removed and 100 µL DMSO was added for complete dissolution of formazan in cells. The optical density of each well was measured be reading the absorbance at 570 nm with 655 nm as reference using a microplate reader (CLARIOstar®, BMG Labtech). The results were expressed as a percentage of MTT reduction, with the absorbance exhibited by the control cells arbitrarily set as 100%. The percentage cell viability was calculated as follows:

Percentage cell viability = (Absorbance of treatment group / Absorbance of control group) × 100%.

The concentration of H_2O_2 that caused 50% cell death was chosen as the model for evaluating the protective effect of pterostilbene in L02 cells against H_2O_2-induced

oxidative damage.

2.2.3.2 Evaluation of the protective effect of pterostilbene in L02 cells against H_2O_2-induced oxidative damage

The MTT cell viability assay was used to access the protective effect of pterostilbene in L02 cells against H_2O_2-induced oxidative damage. After trypsinization and collection of L02 cells, they were seeded in a 96-well microplate with 1×10^4 cells in 100 µL complete medium per well and incubated at 37°C with 5% CO_2 overnight. Then, L02 cells were treated with various concentrations of pterostilbene (10, 30, or 50 µM) for 2 h prior to exposure to 0.5 mM H_2O_2 for 24 h. The cells without drug treatment and H_2O_2 exposure were served as the control. The liquid was removed and replaced with 100 µL MTT solution (0.5 mg/mL in serum-free high glucose DMEM) for 2 h at 37°C and 5% CO_2. The medium was then removed and 100 µL DMSO was added to dissolve the MTT formazan. The optical density of each well was measured be reading the absorbance at 570 nm using a microplate reader (CLARIOstar®, BMG Labtech). The results were expressed as a percentage of MTT reduction, with the absorbance exhibited by the control cells arbitrarily set as 100%. The percentage cell

viability was calculated as follows:

Percentage cell viability = (Absorbance of treatment group / Absorbance of control group) × 100%.

2.2.3.3 Detection of intracellular ROS levels

Intracellular ROS levels were measured using dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay. After trypsinization and collection of L02 cells, they were seeded in 96-well black microplates at 1×10^4 cells per well and incubated overnight at 37°C and 5% CO_2. L02 cells were treated with various concentrations of pterostilbene (10, 30, or 50 μM) for 24 h. After treatment, the cells were washed with PBS twice and exposed to 20 μM DCFH-DA in phenol red-free DMEM and incubated in the dark for 30 min. The DCFH-DA was removed and the cells were washed with PBS twice. Then, 500 μM H_2O_2 in phenol red-free DMEM was added. The cells without drug treatment and H_2O_2 exposure were served as the control. The intracellular ROS levels were determined by measuring the fluorescence intensity with an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a microplate reader (CLARIOstar®, BMG Labtech).

2.2.3.4 Western blot analysis

Western blot analyses of ERK, p-ERK, JNK, p-JNK, p38, p-p38 and HO-1 protein levels were performed. L02 cells were seeded at a density of 4×10^6 cells/well in 6-well microplates and incubated overnight at 37°C and 5% CO_2. The cells were treated with various concentrations (30 or 50 μM) of pterostilbene for 2 h prior to 0.5 mM H_2O_2 exposure. After treatment, the cells were washed with PBS and cell lysis was performed in ice-cold lysis buffer consisting of 1 mL radioimmunoprecipitation assay (RIPA) buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF). The cell lysates were centrifuged at 13,000 rpm under 4°C for 10 min and the supernatants were collected. The concentration of the proteins in the cell lysates were quantified with Bradford protein assay (Bio-Rad, USA). Equal amounts of protein were subjected to 10% SDS-PAGE and the proteins were then transferred to polyvinylidene difluoride (PVDF) membranes at 100 V for 2 h. The membranes were incubated with blocking solution consisting of 5% w/v bovine serum albumin (BSA) in tris-buffered saline with Tween (TBST) for 1 h at room temperature. After 1 h blocking, the membranes were rinsed with TBST. The blots were then incubated with primary antibodies (Abcam Inc.,

USA) specific to the protein of interest overnight at 4°C. The membranes were subsequently probed with secondary goat anti-rabbit or goat anti-mouse IgG horseradish peroxidase conjugated antibodies (Kangchen Bio-tech, Shanghai, China) at a dilution of 1:5,000 and 1:10,000 respectively for 1 h at room temperature. The immunoreactive protein bands were visualized using enhanced chemiluminescence (ECL) in a ChemiDoc XRS machine and analyzed using Quantity One 4.6.7 for Windows (Bio-Rad, USA) software. Housekeeping protein, β-actin (42 kDa) or GAPDH (36 kDa), was chosen as loading control. Each reported value was derived from the ratio between arbitrary units obtained by protein band of interest and the respective β-actin or GAPDH band.

2.2.4 Lipid accumulation

2.2.4.1 Preparation of FFA

Stock solutions of oleic acid (OA; 18:1 cis-9), an unsaturated fatty acid and palmitic acid (PA; 16:0), a saturated fatty acid were prepared by dissolving 10 mM OA or PA in 10% fatty acid-free BSA and homogenized at 45 – 50°C. The homogenized fatty acid-BSA solutions were then filtered with 0.22 μm filter.

The FFA mixture used for establishment of steatotic hepatocyte model was composed with OA and PA in an OA/PA molar ratio of 2:1.

2.2.4.2 Nile Red staining and intracellular lipid quantification

To observe and quantify the intracellular lipid droplets in the L02 cells with different treatments, Nile Red was used for staining. After different treatments, cells were washed with phosphate-buffered saline (PBS) twice, fixed in 4% paraformaldehyde for 20 min, washed with PBS twice again and stained with 1 μM Nile Red (Sigma-Aldrich) in PBS for 15 min at room temperature. Finally, stained cells were rinsed with PBS twice to remove excess dye.

After staining with Nile Red, the intracellular lipid levels in L02 cells with different treatments were quantified with a microplate reader (CLARIOstar®, BMG Labtech) by measuring fluorescence at excitation wavelength of 488 nm and emission wavelength of 520 nm.

2.2.4.3 Determination of the concentration of FFA used as steatotic hepatocyte model

L02 cells were trypsinized and collected. The collected cells were then seeded in a

96-well black microplate as well as a 96-well microplate with 1×10^4 cells in 100 μL complete medium per well and incubated at 37°C with 5% CO_2 overnight. After that, the culture medium was replaced with complete medium containing different concentrations of fatty acids (0.001, 0.003, 0.01, 0.03, 0.1, or 0.3 mM). The cells were further incubated at 37°C with 5% CO_2 for 24 h. The cells without FFA treatment were served as the control. Same treatments for both the 96-well black microplate and the 96-well microplate. After 24 h incubation, the medium was removed. For the 96-well black microplate, the cells were stained with Nile Red for quantification of the intracellular lipid levels. For the 96-well microplate, MTT assay was applied to determine the cell viability after different FFA treatments.

The concentration of FFA that led to significant increase in the intracellular lipid levels of L02 cells compared with the control without affecting the cell viability was chosen as the model for evaluating the intracellular lipid lowering effect of pterostilbene in steatotic L02 cells.

2.2.4.4 Evaluation of the toxicity of pterostilbene in L02 cells in the present of FFA

To determine the toxicity of pterostilbene in L02 cells in the present of 0.3 mM FFA, MTT cell viability assay was used. After trypsinization and collection of L02 cells, they were seeded in a 96-well microplate with 1×10^4 cells in 100 µL complete medium per well and incubated at 37°C with 5% CO_2 overnight. Then, the culture medium was replaced with complete medium containing 0.3 mM FFA and different concentrations of pterostilbene (0.3, 1, 3, 10, 30, or 100 µM) and DMSO (final concentration of 0.1%). For the control, the cells were treated with 0.1% DMSO only; for the model, the cells were treated with 0.3 mM FFA and 0.1% DMSO. The cells were incubated at 37°C with 5% CO_2 for 24 h. After 24 h incubation, the medium was removed and 100 µL MTT solution (0.5 mg/mL in serum-free high glucose DMEM) was added into each well. The cells were incubated for 2 h at 37°C and 5% CO_2. Finally, the MTT solution was removed and 100 µL DMSO was added for complete dissolution of formazan in cells. The optical density of each well was measured be reading the absorbance at 570 nm with 655 nm as reference using a microplate reader (CLARIOstar®, BMG Labtech). The results were expressed as a percentage of MTT reduction, with the absorbance

exhibited by the control cells arbitrarily set as 100%. The percentage cell viability was calculated as follows:

Percentage cell viability = (Absorbance of treatment group / Absorbance of control group) × 100%.

The concentrations of pterostilbene that did not caused significant decrease in the cell viability of L02 cells in the present of 0.3 mM FFA were chosen for evaluating the intracellular lipid lowering effect of pterostilbene in steatotic L02 cells.

2.2.4.5 Evaluation of the intracellular lipid lowering effect of pterostilbene in steatotic L02 cells

L02 cells were trypsinized and collected. The collected cells were then seeded in a 96-well black microplate as well as a 96-well microplate with 1×10^4 cells in 100 μL complete medium per well and incubated at 37°C with 5% CO_2 overnight. After that, the culture medium was replaced with complete medium containing 0.3 mM FFA and different concentrations of pterostilbene (0.3, 1, 3, 10, or 30 μM) and DMSO (final concentration of 0.1%). The cells were further incubated at 37°C with 5% CO_2 for 24 h. The cells without FFA treatment were served as the control and the cells treated with

0.3 mM FFA only were served as the model. Same treatments for both the 96-well black microplate and the 96-well microplate. After 24 h incubation, the medium was removed. For the 96-well black microplate, the cells were stained with Nile Red for quantification of the intracellular lipid levels. For the 96-well microplate, MTT assay was applied to determine the cell viability after different FFA treatments.

2.2.4.6 Confocal microscopy

L02 cells were trypsinized and collected. The collected cells were then seeded in glass bottom dishes with complete medium and incubated at 37°C with 5% CO_2 overnight. After that, the culture medium was replaced with complete medium containing 0.3 mM FFA and different concentrations of pterostilbene (10 or 30 μM) and DMSO (final concentration of 0.1%). The cells were further incubated at 37°C with 5% CO_2 for 24 h. The cells without FFA treatment were served as the control and the cells treated with 0.3 mM FFA only were served as the model. After 24 h incubation, the medium was removed and the cells were stained with Nile Red.

The Nile Red stained L02 cells with different treatments were observed directly

under a confocal microscope (Leica SPE Confocal Microscope) to evaluate intracellular lipid droplets qualitatively.

2.2.4.7 Western blot analysis

Western blot analyses of p-ACC, FAS, HMGCR, PPAR-α, LC3-II, p62, AMPK and p-AMPK protein levels were performed. L02 cells were seeded at a density of 4×10^6 cells/well in 6-well microplates and incubated overnight at 37°C and 5% CO_2. The cells were treated with 0.3 mM FFA and various concentrations (10 or 30 μM) of pterostilbene for 24 h. After treatment, the cells were washed with PBS and cell lysis was performed in ice-cold lysis buffer (1 mL RIPA buffer supplemented with 1 mM PMSF). The cell lysates were centrifuged at 13,000 rpm under 4°C for 10 min and the supernatants were collected. The concentration of the proteins in the cell lysates were quantified with Bradford protein assay (Bio-Rad, USA). Equal amounts of protein were subjected to 7.5% and 15% SDS-PAGE and the proteins were then transferred to PVDF membranes at 100 V for 2 h. The membranes were incubated with blocking solution (5% w/v BSA in TBST) for 1 h at room temperature. After 1 h blocking, the membranes were rinsed with TBST. The blots were then incubated with primary antibodies (Abcam

Inc., USA) specific to the protein of interest overnight at 4°C. The membranes were subsequently probed with secondary goat anti-rabbit or goat anti-mouse IgG horseradish peroxidase conjugated antibodies (Kangchen Bio-tech, Shanghai, China) at a dilution of 1:5,000 and 1:10,000 respectively for 1 h at room temperature. The immunoreactive protein bands were visualized using ECL in a ChemiDoc XRS machine and analyzed using Quantity One 4.6.7 for Windows (Bio-Rad, USA) software. Housekeeping protein, β-actin (42 kDa), was chosen as loading control. Each reported value was derived from the ratio between arbitrary units obtained by protein band of interest and the respective β-actin band.

2.3 *In vivo* study

2.3.1 Animals and experimental treatment

Male SD rats (150 ± 10 g) were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). Normal rat chow composed of 14% protein, 10% fat and 76% carbohydrate, and high-fat rat chow which is a standard rat chow supplemented with 1% cholic acid, 2% pure cholesterol and 5.5% oil (Kwok et al.,

2010; Q. Li et al., 2009) were purchased from Guangdong Provincial Medical Laboratory Animal Center (Guangzhou, China). Pioglitazone was applied as positive control for the animal experiment. All rats were housed under standard conditions (temperature 25 ± 2°C, humidity 60 ± 10%, light from 6 am to 6 pm) with free access to water and rat chow. After acclimation for a week in the laboratory environment, the rats were randomly assigned to one of the eight different experimental groups (each with 8). These groups were (1) Control: a control group that fed with normal rat chow for 8 weeks; (2) Model: a model group that fed with high-fat rat chow for 4 weeks followed by normal rat chow for 4 weeks; (3) PiL: a low-dose pioglitazone treatment group that fed with high-fat rat chow for 4 weeks followed by normal rat chow as well as pioglitazone (2 mg/kg bw per day, *p.o.*) for 4 weeks; (4) PiM: a middle-dose pioglitazone treatment group that fed with high-fat rat chow for 4 weeks followed by normal rat chow as well as pioglitazone (4 mg/kg bw per day, *p.o.*) for 4 weeks; (5) PiH: a high-dose pioglitazone treatment group that fed with high-fat rat chow for 4 weeks followed by normal rat chow as well as pioglitazone (8 mg/kg bw per day, *p.o.*) for 4 weeks; (6) PtL: a low-dose pterostilbene treatment group that fed with high-fat rat

chow for 4 weeks followed by normal rat chow as well as pterostilbene (30 mg/kg bw per day, *p.o.*) for 4 weeks; (4) PtM: a middle-dose pterostilbene treatment group that fed with high-fat rat chow for 4 weeks followed by normal rat chow as well as pterostilbene (100 mg/kg bw per day, *p.o.*) for 4 weeks; (5) PtH: a high-dose pterostilbene treatment group that fed with high-fat rat chow for 4 weeks followed by normal rat chow as well as pterostilbene (300 mg/kg bw per day, *p.o.*) for 4 weeks. After 4 weeks of steatotic model development, the rats were undergone diet modification along with administration of distilled water (vehicle) or their corresponding treatments by oral gavage once every morning for 4 weeks. At the end of the experimental period, the rats were fasted overnight and killed by carbon dioxide euthanasia. Blood, livers, and guts were then collected for further analysis. The experimental protocol was conducted under the animal license issued by the Health Department of the Hong Kong SAR Government and the Animal Subjects Ethics Sub-committee (ASESC no. 14-15/45-ABCT-R-GRF) of The Hong Kong Polytechnic University. All procedures were consistent with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and the

principles outlined in the Declaration of Helsinki. Every effort was made to limit animal suffering and the number of animals used in this study.

2.3.2 Oral glucose tolerance test

An oral glucose tolerance test (OGTT) was performed on all rats on the 4th week (after feeding with 4 weeks of high-fat diet) and the 8th week (after 4 weeks of treatments). The rats were food deprived overnight before the OGTT in the morning. All the rats were given glucose (0.6 g/kg body weight) orally using gavage tube. Blood samples were obtained at 0, 30, 60, 90, and 120 minutes of time intervals from rats' tail vein by tail snipping. The blood glucose levels of the samples were assessed with a glucometer (ACCU-CHEK® Performa test strips in ACCU-CHEK® Performa blood glucose meter, Roche Diagnostics, Germany).

2.3.3 Analysis of serum biochemical changes

Blood was collected in chilled centrifuge tubes by cardiac puncture immediately after carbon dioxide euthanasia. The collected blood samples were allowed to clot for 2 h at 4°C. The clotted blood samples were then centrifuged at 1,500 g under 4°C for 15

min. After that, serum was collected and was stored at -80°C until further analysis. The ALT, AST, TG, TC, LDL-C, and high-density lipoprotein cholesterol (HDL-C) levels in serum were measured by the ALYCON systems using Roche Reagents. The serum insulin levels were measured with Rat/Mouse Insulin ELISA Kit (EMD Millipore, USA) according to the manufacturer's instructions. HOMA-IR index was calculated as follows:

HOMA-IR index = fasting insulin (μU/mL) × fasting glucose (mmol/L) / 22.5

2.3.4 Liver lipid content evaluation

Liver samples (around 1 g) were dissected, accurately weighed and homogenized with chloroform-methanol (2:1, *v/v*) mixture to a final dilution of 1:20 w/v using Ultra-turrax T-10 homogenizer. After filtration, 10 mL of individual filtrate was added to 2 mL water and the mixture was centrifuged at 900 g for 20 min. The lower phase was dried and its weight was measured. Liver lipid content was expressed as weight of lipid per g of liver.

2.3.5 Liver histopathological examination

Hepatic tissues from all animals were fixed in 4% paraformaldehyde and embedded in paraffin wax. Paraffin sections (5 μm) were stained with haematoxylin and eosin (H&E) and observed with a light microscope (Olympus BX43, Olympus, Tokyo, Japan) and photomicrographs (400×) were taken. The degree of NAFLD was graded according to NAFLD Activity Score (ranges from 0 to 8) which is based on the sum of three components: steatosis (% of liver cells containing fat; 0: < 5%, 1: 5 – 33%, 2: > 33 – 66%, 3: > 66%), lobular inflammation (no. of foci / 200×; 0: no foci, 1: < 2 foci, 2: 2 – 4 foci, 3: > 4 foci), and hepatocyte ballooning (amount of ballooned cells; 0: none, 1: few, 2: many) (Kleiner et al., 2005). The areas of lipid vacuoles were measured by ImageJ (National Institutes of Health, Bethesda, Maryland, USA) at 100× magnification and were expressed as the percentage area of lipid vacuoles (Massart et al., 2012).

2.3.6 Western blot analysis

Western blot analyses of p-ACC, FAS, SREBP-2, HMGCR, ERK, p-ERK, JNK,

p-JNK, p38, p-p38, HO-1, AMPK, p-AMPK, p-mTOR, LC3-II and PPAR-α protein levels in liver were performed. Liver samples were homogenized and lysed in ice-cold lysis buffer (1 mL RIPA buffer supplemented with 1 mM PMSF). The lysates were centrifuged at 13,000 rpm under 4°C for 10 min and the supernatants were collected. The concentration of the proteins in the lysates were quantified with Bradford protein assay (Bio-Rad, USA). Equal amounts of protein were subjected to 7.5%, 10% and 15% SDS-PAGE and the proteins were then transferred to PVDF membranes at 100 V for 2 h. The membranes were incubated with blocking solution (5% w/v BSA in TBST) for 1 h at room temperature. After 1 h blocking, the membranes were rinsed with TBST. The blots were then incubated with primary antibodies (Abcam Inc., USA) specific to the protein of interest overnight at 4°C. The membranes were subsequently probed with secondary goat anti-rabbit or goat anti-mouse IgG horseradish peroxidase conjugated antibodies (Kangchen Bio-tech, Shanghai, China) at a dilution of 1:5,000 and 1:10,000 respectively for 1 h at room temperature. The immunoreactive protein bands were visualized using ECL in a ChemiDoc XRS machine and analyzed using Quantity One 4.6.7 for Windows (Bio-Rad, USA) software. Housekeeping protein, β-actin (42 kDa),

was chosen as loading control. Each reported value was derived from the ratio between arbitrary units obtained by protein band of interest and the respective β-actin band.

2.3.7 Lipidomics study

2.3.7.1 Chemical and Reagent

Ammonium acetate (≥ 98%), ammonium formate (≥ 99.0%) were purchased form Sigma-Aldrich (St. Louis, MO, USA). Cholic acid-D_4 was purchased from Cambridge Isotope Laboratory Inc. (Cambridge, UK). HPLC-graded 2-propanol, methyl tert-butyl ether (MTBE), chloroform, acetonitrile and methanol were obtained from Fisher Scientific (Hampton, NH, USA). Formic acid was purchased from VWR (Radnor, Pennsylvania, USA). Water was purified in-house using a Milli-Q Advantage A10 water purification system (Millipore, Bedford, MA, USA).

2.3.7.2 Quality control sample preparation method

An aliquot of 20 μL of each serum sample was pooled, vortexed and aliquoted to provide quality control (QC) samples, and kept at -80°C until use. For each analytical batch, QC samples went through extraction protocols as described below similar to all

other samples. Before the start of the chemical analysis, five repeated injections of the same QC sample were used to verify the working condition of the instruments. Afterwards, a QC sample was injected to monitor the stability of the instruments after every five sample runs.

2.3.7.3 Serum sample extraction

Serum samples of the Control, the Model, the pioglitazone and the pterostilbene treatment groups were thawed at 4°C before preparation. Each 50 μL serum sample was mixed with 225 μL chilled methanol and vortexed for 30 s, and the mixture was stood at -20°C overnight. Then, 750 μL chilled MTBE were added, and the mixture was stood at -20°C for another 2 h. After that, 188 μL of water contains 0.1% ammonium acetate were added, followed by centrifugation at 14,000 rpm under 4°C for 5 min. 360 μL upper phase of each serum sample was transferred into a new microcentrifuge tube and evaporated to dryness under a gentle stream of nitrogen gas. The dried residue was finally reconstituted in 100 μL of 2-propanol/methanol/chloroform (4:2:1, *v/v/v*) with 7.5 mM ammonium acetate and 1 ppm cholic acid-D_4 as internal standard, and vortexed for further 30 s. After centrifugation at 14,000 rpm under 4°C for 15 min, 90

μL of supernatant was collected for ultra-performance liquid chromatography (UPLC)-Orbitrap mass spectrometry (MS) analysis.

2.3.7.4 UPLC-Orbitrap MS condition

A 2 μL aliquot was injected into a Waters ACQUITY UPLC system. The separation was performed on a Waters ACQUITY UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm) with HSS T3 pre-column (2.1 mm × 5 mm, 1.8 μm, Waters Corporation, Milford, USA). The mobile phase for positive mode consisted of combinations of A (60% acetonitrile in water, *v/v*, with 0.1% formic acid) and B (9:1 2-propanol/acetonitrile, *v/v*, with 10 mM ammonium formate). The mobile phase for negative mode consisted of combinations of A (60% acetonitrile in water, *v/v*, with 10 mM ammonium acetate) and B (9:1 2-propanol/acetonitrile, *v/v*, with 10 mM ammonium acetate). The other conditions are the same for both modes, flow rate at 0.3 mL/min with elution gradient as follows: 0 min, 15% B; 2 min, 30% B; 3 min, 48% B; 13 min, 82% B; 14 – 17 min, 99% B. A 3-min post-run time was set to fully equilibrate the column between injections. Column and sample chamber temperature were set at 40°C and 6°C, respectively.

MS was performed on a Thermo Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) operating in a heated electrospray ion (H-ESI) source in both positive and negative modes. Nitrogen gas (≥ 99.999%) were used as sheath and Aux with flow rate of 40 and 3 arbitrary unit, respectively, The spray voltages in positive and negative modes were 3.2 kV and 2.8 kV, respectively. The instrument was operated in data-dependent acquisition mode, with full MS scans over a mass range of m/z 100 to 1,200 acquired in the Orbitrap at 120,000 resolution using an automatic gain control (AGC) target value of 200,000 and a maximum injection time of 100 ms. In each cycle (3 s) of data-dependent acquisition analysis, the most intense ions with intensity threshold above 50,000 were selected for fragmentation at normalized collision energy of 27 ± 3% higher energy collisional. The number of selected precursor ions for fragmentation was determined by the "Top Speed" acquisition algorithm. Fragment ion spectra were acquired in the Orbitrap (30,000 resolution) with an AGC target at 50,000 and a maximum injection time at 100 ms for Orbitrap MS^2 detection.

2.3.7.5 Data processing and analysis

Peak picking and alignment of all raw data were conducted by Progenesis QI software (Nonlinear Dynamics, United Kingdom) in both ionization modes in two separate analyses. Data were imported in the following setting prior to peak picking: resolution (full width at half maximum), 16,000; adduct, $[M+H]^+$ for positive mode, $[M-H]^-$ for negative mode; retention time limit, 0 to 20 min; data type, centroided while other settings were set default. All ion abundance was normalized by internal standards of its own sample to generate a data matrix that consisted of the retention time, m/z value, and the normalized ion abundance. Quality screening was done by filtering out those metabolites which coefficient of variations (CV) was greater than 30% in quality control samples to reduce the contribution of unstable peaks and eliminate noise from the dataset (Dunn et al., 2011).

The resultant data matrices were introduced to Extended Statistical tool EZinfo v2.0 software (Umetrics AB, Sweden) for multivariate statistics. The data were scaled to unit variance for principal component analysis (PCA), to give an overview of the repeatability of QC samples. The QC samples with high repeatability should cluster

together in the score plot of PCA. The samples excluding QC samples were pareto-scaled for partial least squares-discriminant analysis (PLS-DA) and orthogonal partial least squares-discriminant analysis (OPLS-DA). Potential markers of interest were extracted from the variable importance in the projection (VIP) values (threshold of VIP $\geq$ 1) and the location in S-loading plot of OPLS-DA based on their contribution to the variation and correlation in the data set. The markers were identified by MS^2 and matched with the METLIN (http://metlin.scripps.edu), the MassBank (www.massbank.jp) and the Human Metabolome Databases (www.hmdb.ca) and/or confirmed by literatures and authentic standards based on retention times, mass fragmentation pattern and accurate masses (mass error $\leq$ 5 ppm).

Statistical analyses were performed using SPSS PASW Statistics 18 (Chicago, IL, USA). After logarithmic transformation [log2 (normalized ion abundance)] followed by filtering out outliers (1.5 times of the interquartile range) and, statistical differences were analyzed at a univariate level by one-way analysis of variance (ANOVA); Tukey's honestly significant difference (HSD) or Tamhane's T2 *post-hoc* test was used based on homogeneity of variances with a $p < 0.05$ considered as statistically significant.

2.3.8 Gut microbiota analysis

2.3.8.1 Fecal samples collection

Fresh fecal samples were collected from all animals on the 8th week (after feeding with 4 weeks of high-fat diet followed by 4 weeks of treatments). The collected fecal samples were stored at -80°C until further analysis.

2.3.8.2 Fecal microbiota analysis

DNA extraction was performed using fecal DNA extraction kit (Solarbio, Beijing, China) according to the manufacturer's instructions. The final concentration and the purity of DNA were determined by NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA), and the DNA quality was checked by 1% agarose gel electrophoresis. Polymerase chain reaction (PCR) amplification of the V3-V4 region of the gut bacterial 16S rRNA gene was performed by thermocycler PCR system (GeneAmp 9700, ABI, USA) using the universal primers of the forward 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and the reverse 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The conditions of the PCR reactions included 3 min of denaturation at 95°C, followed by 27 cycles at 95°C for 30 s,

annealing at 55°C for 30 s, elongation at 72°C for 45 s, and a final extension at 72°C for 10 min. PCR reactions were performed in triplicate. A 20 μL reaction mixture was prepared with 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase and 10 ng of template DNA. The resulted PCR products were extracted using 2% agarose gel, further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, USA) according to the manufacturer's protocol. The purified amplicons were then pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). After sequencing, taxonomical classification and microbial diversity detection as well as statistical analyses were performed in the cloud platform of majorbio (http://www.i-sanger.com) for the determination and identification of the community structure of the gut microbiota. The gut microbiota changes between groups were compared to explore the associations between the community structure of the gut microbiota and the development of NAFLD as well as the effects of high-fat diet and

treatments on the community structure of the gut microbiota.

2.3.8.3 Gut histopathological examination

Gut tissue samples from all animals were fixed in 4% paraformaldehyde and embedded in paraffin wax. Paraffin sections (5 μm) were stained with H&E and observed with a light microscope (Olympus BX43, Olympus, Tokyo, Japan) and photomicrographs (100×) were taken.

2.4 Statistical analysis

All data were reported as the mean ± standard deviation and were analyzed using one-way ANOVA and Bonferroni's *post-hoc* test for multiple comparisons between groups. Analysis was performed by the statistical software GraphPad Prism 5.02 (San Diego, CA, USA) for Windows. A value of probability (p) < 0.05 was considered statistically significant.

Chapter 3 Results

3.1 Determination of free radical scavenging activity

To determine the free radical scavenging activity of pterostilbene, DPPH radical scavenging assay was conducted. Ascorbic acid was used as a reference antioxidant for comparison. The results were shown in Figure 3.1.1. Pterostilbene exhibited a scavenging activity against DPPH free radicals with EC_{50} of 45.76 ± 1.025 μM in a concentration-dependent manner. The EC_{50} of ascorbic acid was determined to be 16.09 ± 1.025 μM.

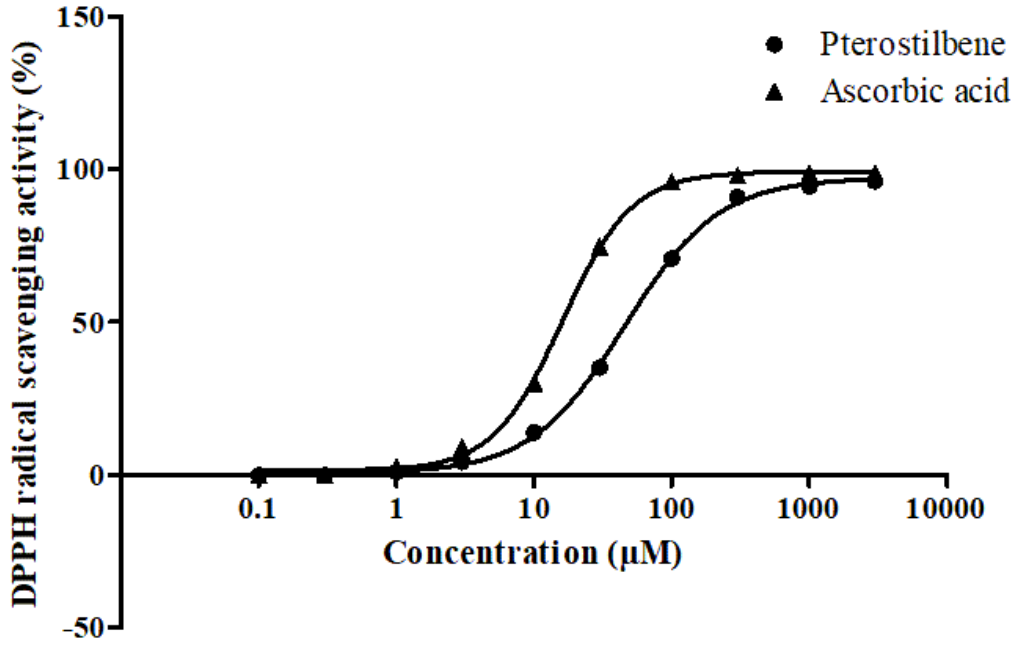

Figure 3.1.1 DPPH radical scavenging activity of pterostilbene.
Ascorbic acid was used as a reference antioxidant. The data are expressed as the mean ± standard deviation, n = 3.

3.2 *In vitro* study

3.2.1 Evaluation of the toxicity of pterostilbene in L02 cells

MTT cell viability assay was conducted to evaluate the toxicity of pterostilbene in L02 cells. Results (Figure 3.2.1) showed that 0.3 μM – 100 μM pterostilbene 24-h treatments gave no significant toxic effect to L02 cells. 300 μM pterostilbene 24-h treatment significantly decreased the percentage cell viability when compared with the Control ($p < 0.001$). When the concentration of pterostilbene increased to 500 μM, almost all the cells died. Therefore, concentrations of pterostilbene higher than 300 μM were regarded as unsuitable for 24-h drug treatment in the L02 cells.

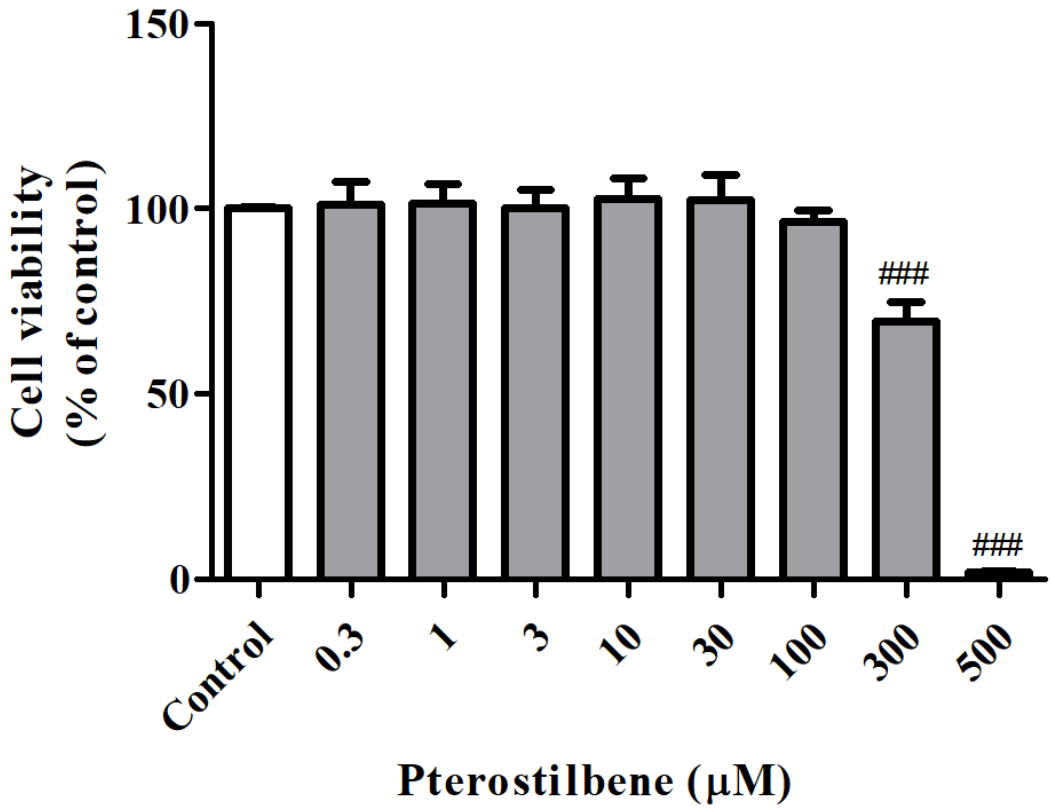

Figure 3.2.1 Results of MTT cell viability assay showing the toxicity of different concentrations of pterostilbene on L02 cells.

The data are expressed as the mean ± standard deviation, n = 3. ### $p < 0.001$ versus Control.

3.2.2 Oxidative stress

3.2.2.1 Evaluation of the toxicity of H_2O_2 in L02 cells

MTT cell viability assay was conducted to evaluate the toxicity of H_2O_2 in L02 cells. Results (Figure 3.2.2) showed that H_2O_2 24-h treatment resulted in a concentration-dependent loss of cell viability in L02 cells and 0.5 mM H_2O_2 24-h

treatment caused 50% cell death in L02 cells. Therefore, 0.5 mM H_2O_2 24-h treatment was chosen as the model for evaluating the protective effect of pterostilbene in L02 cells against H_2O_2-induced oxidative damage.

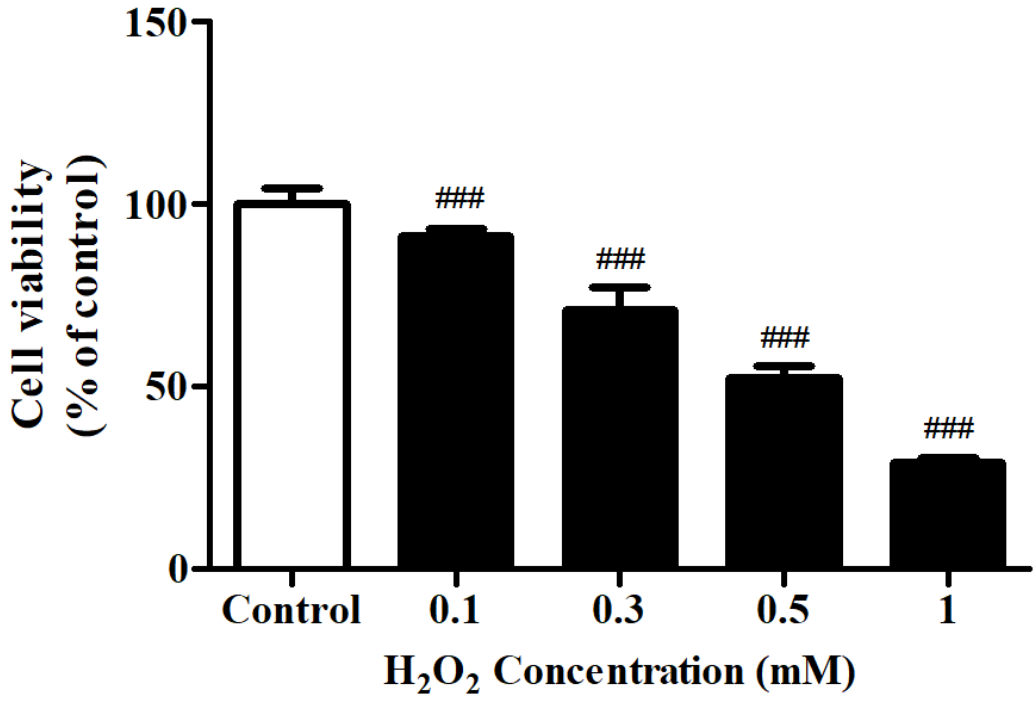

Figure 3.2.2 Results of MTT cell viability assay showing the toxicity of different concentrations of H_2O_2 on L02 cells.

The data are expressed as the mean ± standard deviation, n = 6. ### $p < 0.001$ versus Control.

3.2.2.2 Evaluation of the protective effect of pterostilbene in L02 cells against H_2O_2-induced oxidative damage

MTT cell viability assay was conducted to evaluate the protective effect of

pterostilbene in L02 cells against H_2O_2-induced oxidative damage. Results (Figure 3.2.3) showed that 2-h pretreatment with 30 μM and 50 μM pterostilbene significantly reduced H_2O_2-induced cytotoxicity L02 cells ($p < 0.05$ and $p < 0.01$ respectively), suggesting that pterostilbene may help protect L02 cells against H_2O_2-induced oxidative damage.

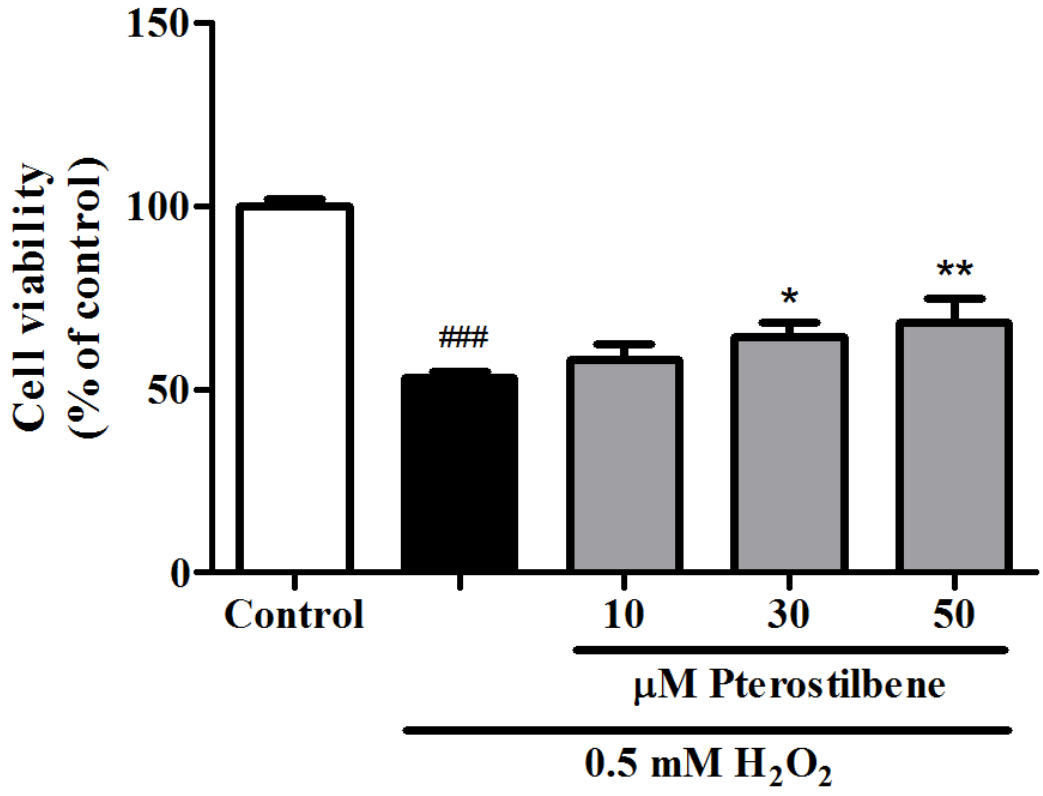

Figure 3.2.3 Pterostilbene attenuates H_2O_2-induced cytotoxicity in L02 cells. Data are expressed as the mean ± standard deviation, n = 4. ### $p < 0.001$ versus Control; * $p < 0.05$ and ** $p < 0.01$ versus 0.5 mM H_2O_2 group.

3.2.2.3 Detection of intracellular ROS levels

To measure the intracellular ROS levels, DCFH-DA assay was conducted. Results (Figure 3.2.4) showed that 0.5 mM H_2O_2 treatment significantly increased the increased intracellular ROS levels ($p < 0.001$). 30 μM and 50 μM pterostilbene 24-h treatment significantly reduced the increased intracellular ROS levels induced by 0.5 mM H_2O_2 treatment ($p < 0.001$).

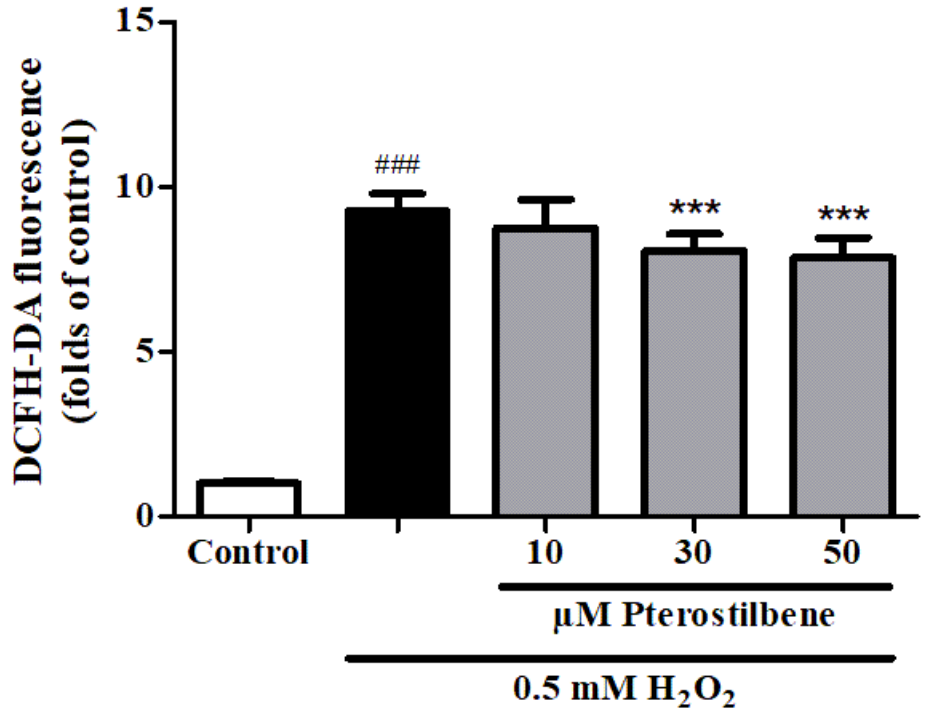

Figure 3.2.4 Effects of pterostilbene on intracellular ROS production in L02 cells.

The data are expressed as the mean ± standard deviation, n = 12. ### $p < 0.001$ versus Control; *** $p < 0.001$ versus 0.5 mM H_2O_2 group.

3.2.2.4 Western blot analysis

The effects of pterostilbene on H_2O_2-induced phosphorylation of ERK, JNK and p38 MAPK were shown in Figure 3.2.5. Exposure to 0.5 mM H_2O_2 for 24 h significantly increased the phosphorylated levels of ERK, JNK, and p38 MAPK in L02 cells ($p < 0.001$). Pretreatment with 30 μM pterostilbene significantly reduced H_2O_2-induced phosphorylation of ERK and JNK in L02 cells ($p < 0.01$) while pretreatment with 50 μM pterostilbene significantly reduced H_2O_2-induced phosphorylation of ERK, JNK and p38 MAPK in L02 cells ($p < 0.001$, $p < 0.001$ and $p < 0.01$ respectively). Pretreatment with 30 μM and 50 μM pterostilbene alone for 2 h did not give significant changes on the phosphorylated levels of ERK, JNK and p38 MAPK.

In addition, L02 cells were treated with different concentrations of pterostilbene for 24 h for the determination of HO-1 expression level. Results (Figure 3.2.6) showed that 10 μM – 50 μM pterostilbene 24-h treatments significantly increased the expression level of HO-1 in L02 cells.

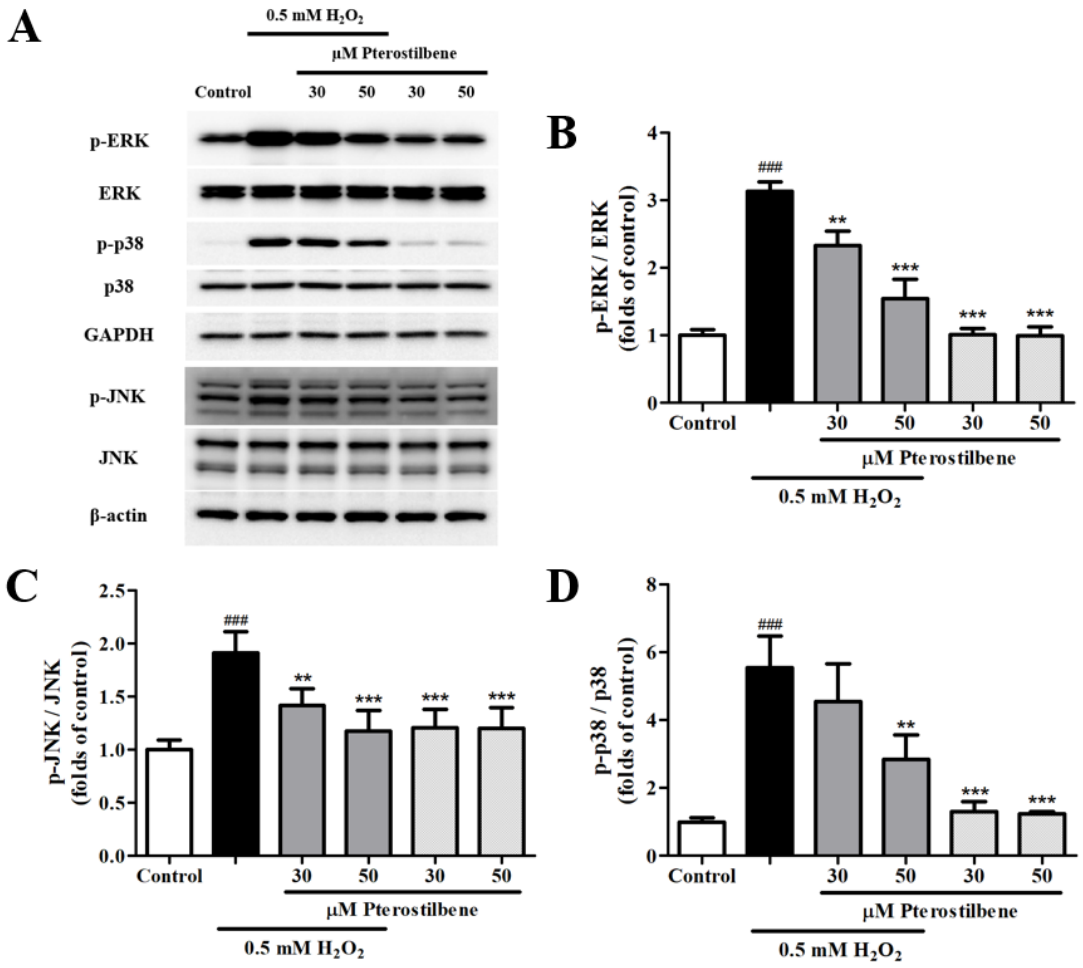

Figure 3.2.5 Pterostilbene attenuates H_2O_2-induced phosphorylation of ERK, JNK and p38 MAPK in a dose-dependent manner.

L02 cells were pretreated with pterostilbene for 2 h followed by 0.5 mM H_2O_2 exposure. The expressions of ERK, p-ERK, JNK, p-JNK, p38 and p-p38 were determined by western blot. **(A)** The representative data and **(B – D)** densitometric analysis of the blots are shown. The data are expressed as the mean ± standard deviation, n = 3. $^{###}$ $p < 0.001$ versus Control; ** $p < 0.01$ and *** $p < 0.001$ versus 0.5 mM H_2O_2 group.

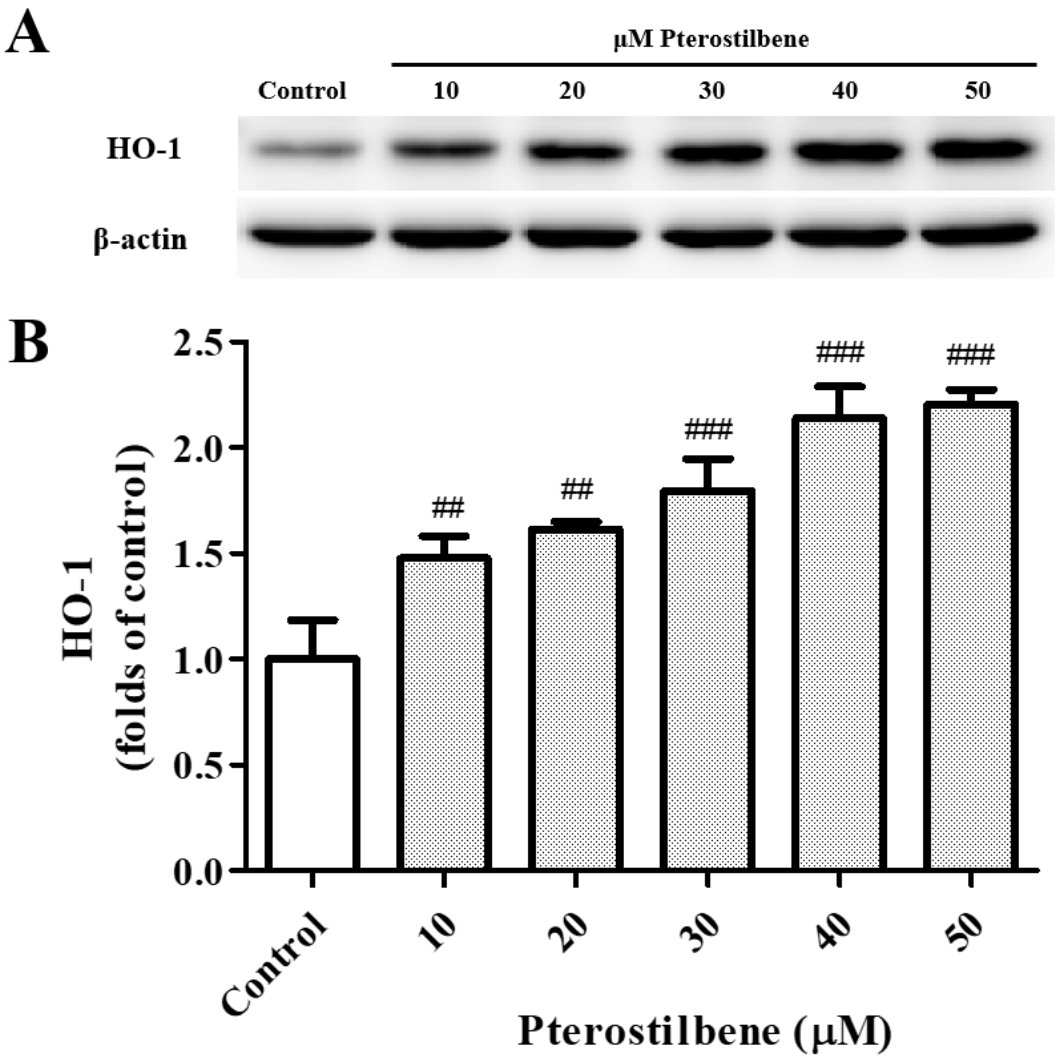

Figure 3.2.6 Pterostilbene upregulates HO-1 protein expression in L02 cells in a dose-dependent manner.

L02 cells were treated with pterostilbene for 24 h. The expression of HO-1 was determined by western blot. **(A)** The representative data and **(B)** densitometric analysis of the blots are shown. The data are expressed as the mean ± standard deviation, n = 3. $^{\#\#}$ $p < 0.01$ and $^{\#\#\#}$ $p < 0.001$ versus Control.

3.2.3 Lipid accumulation

3.2.3.1 Determination of the concentration of FFA used as steatotic hepatocyte model

MTT cell viability assay was conducted to evaluate the toxicity of FFA in L02 cells. Results (Figure 3.2.7A) showed that 24-h treatments with FFA concentrations ranged from 0.001 mM – 0.3 mM showed no significant toxic effect to L02 cells. On the other hand, results of Nile Red staining of intracellular lipid (Figure 3.2.7B) showed that 24-h treatments with 0.1 mM and 0.3 mM FFA significantly increased the intracellular lipid content of L02 cells ($p < 0.001$) when compared with the Control. In order to provide better distinguishment to show the intracellular lipid lowering ability of a drug, a higher FFA concentration with no significant toxic effect, 0.3 mM, was chosen for the development of steatotic hepatocyte model.

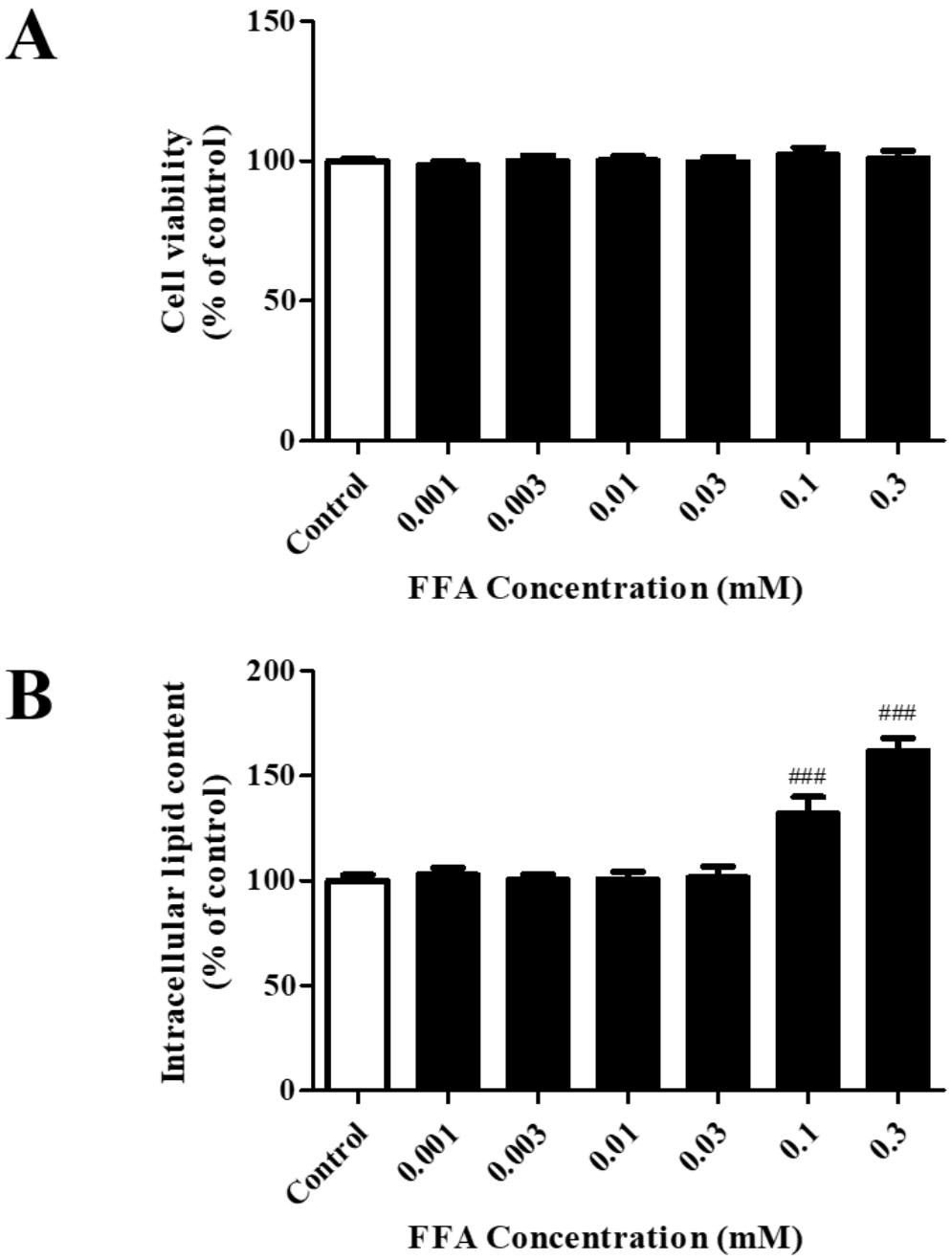

Figure 3.2.7 Determination of the concentration of FFA used for the development of steatotic hepatocyte model.

(A) Results of MTT cell viability assay showing the toxicity of different concentrations of FFA 24-h treatments to L02 cells. **(B)** Lipid contents of L02 cells after different concentrations of FFA 24-h treatments. The data are expressed as the mean ± standard deviation, n = 3. $^{###}$ $p < 0.001$ versus Control.

3.2.3.2 Evaluation of the toxicity of pterostilbene in L02 cells in the present of FFA

Results of MTT cell viability assay (Figure 3.2.8) showed that 0.3 μM – 30 μM pterostilbene 24-h treatments gave no significant toxic effect to the steatotic L02 hepatocyte model (developed with 0.3 mM FFA). 100 μM pterostilbene 24-h treatment significantly decreased the percentage cell viability of the steatotic L02 hepatocyte model when compared with the Control ($p < 0.05$) and the Model ($p < 0.05$). Therefore, 100 μM pterostilbene 24-h treatment was regarded as unsuitable for 24-h drug treatment in the steatotic L02 hepatocyte model.

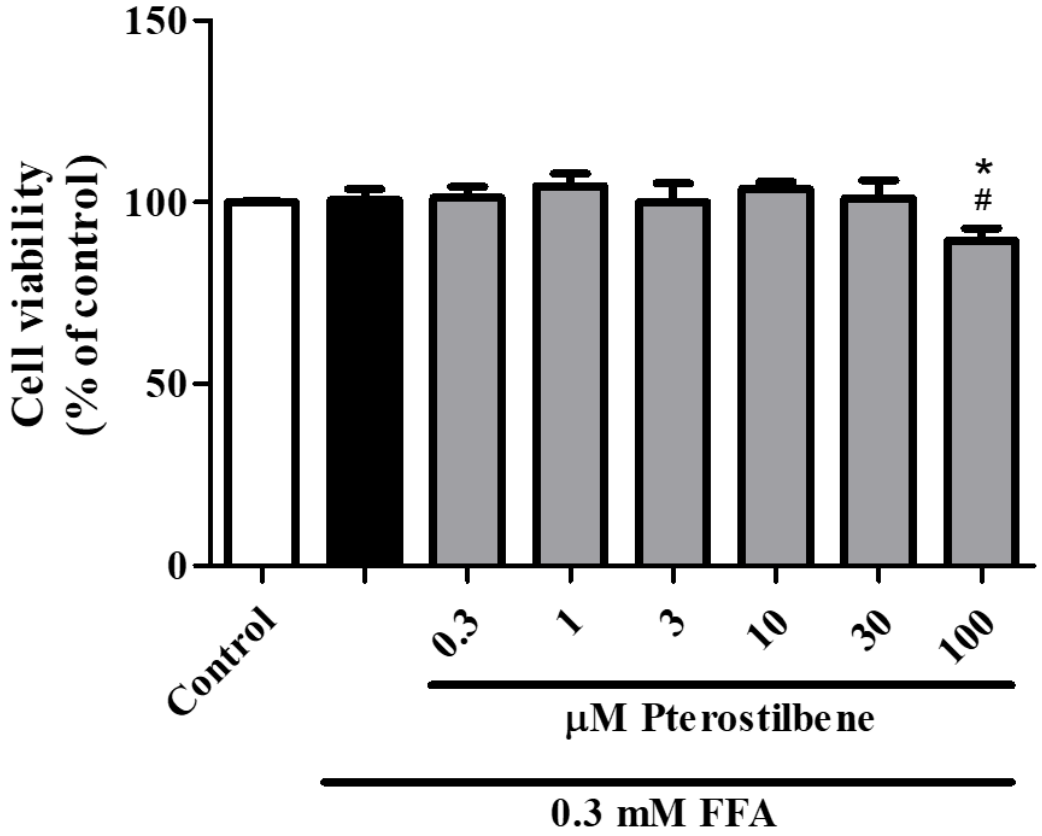

Figure 3.2.8 Results of MTT cell viability assay showing the toxicity of different concentrations of pterostilbene plus 0.3 mM FFA 24-h treatments to L02 cells.

The data are expressed as the mean ± standard deviation, n = 3. $^{\#}$ $p < 0.05$ versus Control; * $p < 0.05$ versus 0.3 mM FFA group.

3.2.3.3 Evaluation of the intracellular lipid lowering effect of pterostilbene in steatotic L02 cells

According to the results of Nile Red staining of intracellular lipid (Figure 3.2.9), concentrations of pterostilbene ranged from 0.3 μM – 3 μM showed no significant effect on the intracellular lipid content of the steatotic L02 hepatocyte model. 10 μM

pterostilbene 24-h treatment showed significant decrease in the intracellular lipid content of the steatotic L02 hepatocyte model ($p < 0.05$) while 30 μM pterostilbene 24-h treatments showed significant decrease in the intracellular lipid content of the steatotic L02 hepatocyte model ($p < 0.01$).

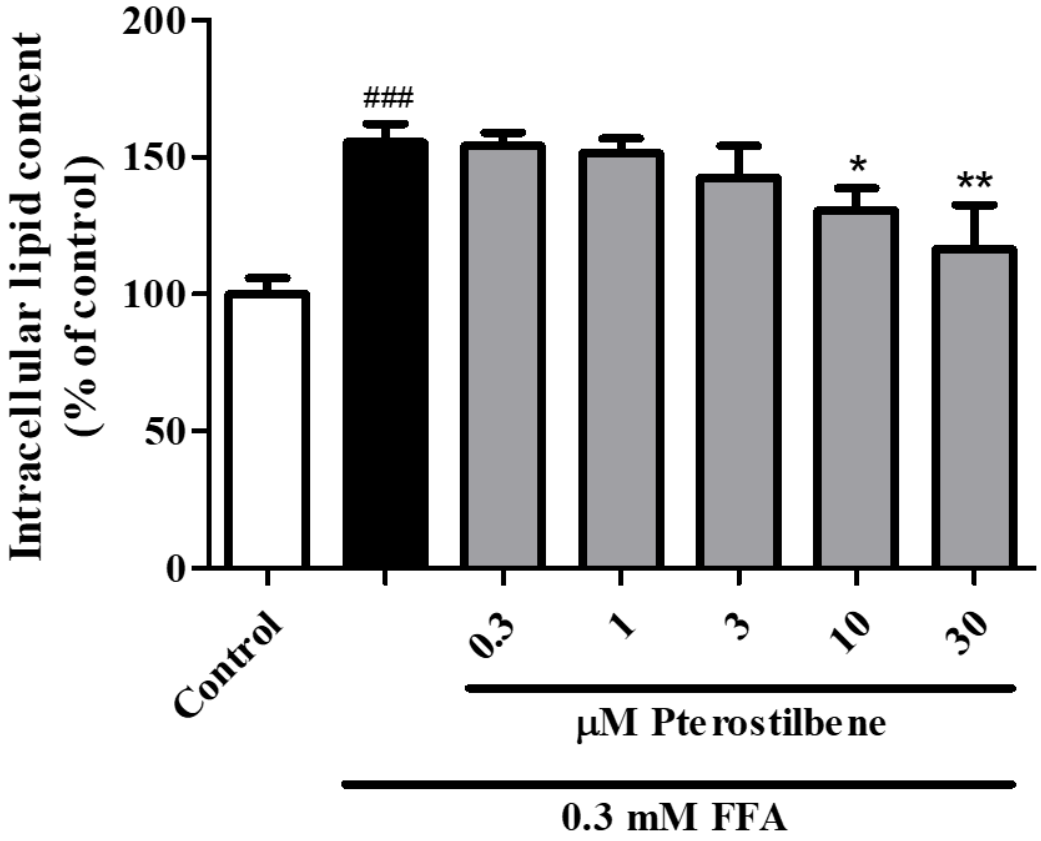

Figure 3.2.9 Intracellular lipid contents of L02 cells after different concentrations of pterostilbene plus 0.3 mM FFA 24-h treatments.

The data are expressed as the mean ± standard deviation, n = 3. ### $p < 0.001$ versus Control; * $p < 0.05$ and ** $p < 0.01$ versus 0.3 mM FFA group.

Confocal microscopy was applied to observe the intracellular lipid droplets in L02 cells (Figure 3.2.10). When compared the Control and the Model, the number of lipid droplets increased after 24-h treatment of 0.3 mM FFA. After treating with 10 μM and 30 μM pterostilbene, the number of lipid droplets decreased.

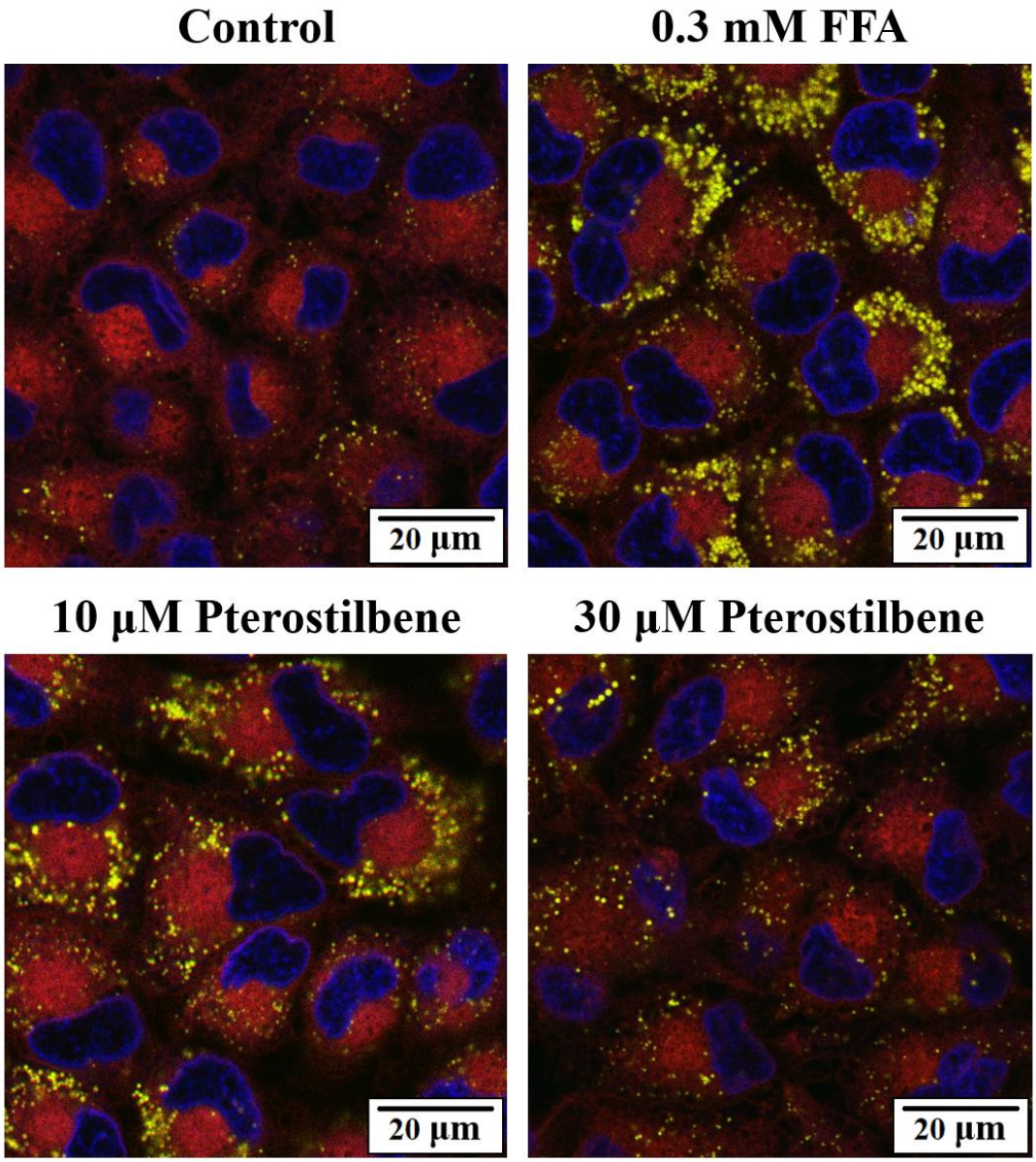

Figure 3.2.10 The distribution of intracellular lipid droplets in L02 cells with different treatments observed by laser scanning confocal microscopy.

L02 cells were stained with Nile Red. The polar membrane lipids are shown in red and the cell nuclei are shown in blue. Yellow spots indicate the neutral intracellular lipid droplets.

3.2.3.4 Western blot analysis

To evaluate the effects of pterostilbene on lipid metabolism, proteins related to fatty acid synthesis, p-ACC and FAS; cholesterol synthesis, HMGCR; and fatty acid β-oxidation, PPAR-α, were detected with western blot. Results were shown in Figure 3.2.11. Treatment with 0.3 mM FFA for 24 h significantly reduced the protein expression level of p-ACC ($p < 0.001$) and increased the protein expression level of FAS ($p < 0.01$) as well as that of HMGCR ($p < 0.001$) in L02 cells. The expression level of PPAR-α in L02 cells after 0.3 mM FFA 24-h treatment was also decreased, but there was no significant difference when compared with the Control. Treatment with 30 μM pterostilbene significantly increased the protein expression level of p-ACC ($p < 0.01$) and reduced that of FAS ($p < 0.001$) in steatotic L02 cells. Both 10 μM and 30 μM pterostilbene treatments decreased of the protein expression level of HMGCR ($p < 0.01$ and $p < 0.001$ respectively) in steatotic L02 cells. The expression level of PPAR-α in steatotic L02 cells was significantly upregulated by 30 μM pterostilbene treatment ($p < 0.01$).

Figure 3.2.12 showed the results of western blot of autophagy related proteins.

The protein expression level of LC3-II was significantly decreased in L02 cells after 0.3 mM FFA 24-h treatment ($p < 0.01$). 30 μM pterostilbene 24-h treatment significantly reversed the reduced LC3-II protein expression level in steatotic L02 cells ($p < 0.01$). In addition, the protein expression level of p62 was significantly increased in L02 cells after 0.3 mM FFA 24-h treatment ($p < 0.05$). Both 10 μM and 30 μM pterostilbene 24-h treatment significantly decreased the p62 protein expression level in steatotic L02 cells ($p < 0.001$).

Previous studies suggested that activation of AMPK inhibits hepatic lipogenesis and gluconeogenesis which helps improve lipid accumulation and insulin resistance. The results showed that treatment with pterostilbene resulted in a dose-dependent phosphorylation of AMPK in L02 cells (Figure 3.2.13). The phosphorylated level of AMPK was significantly elevated by 40 μM and 50 μM pterostilbene treatments ($p < 0.01$ and $p < 0.001$ respectively).

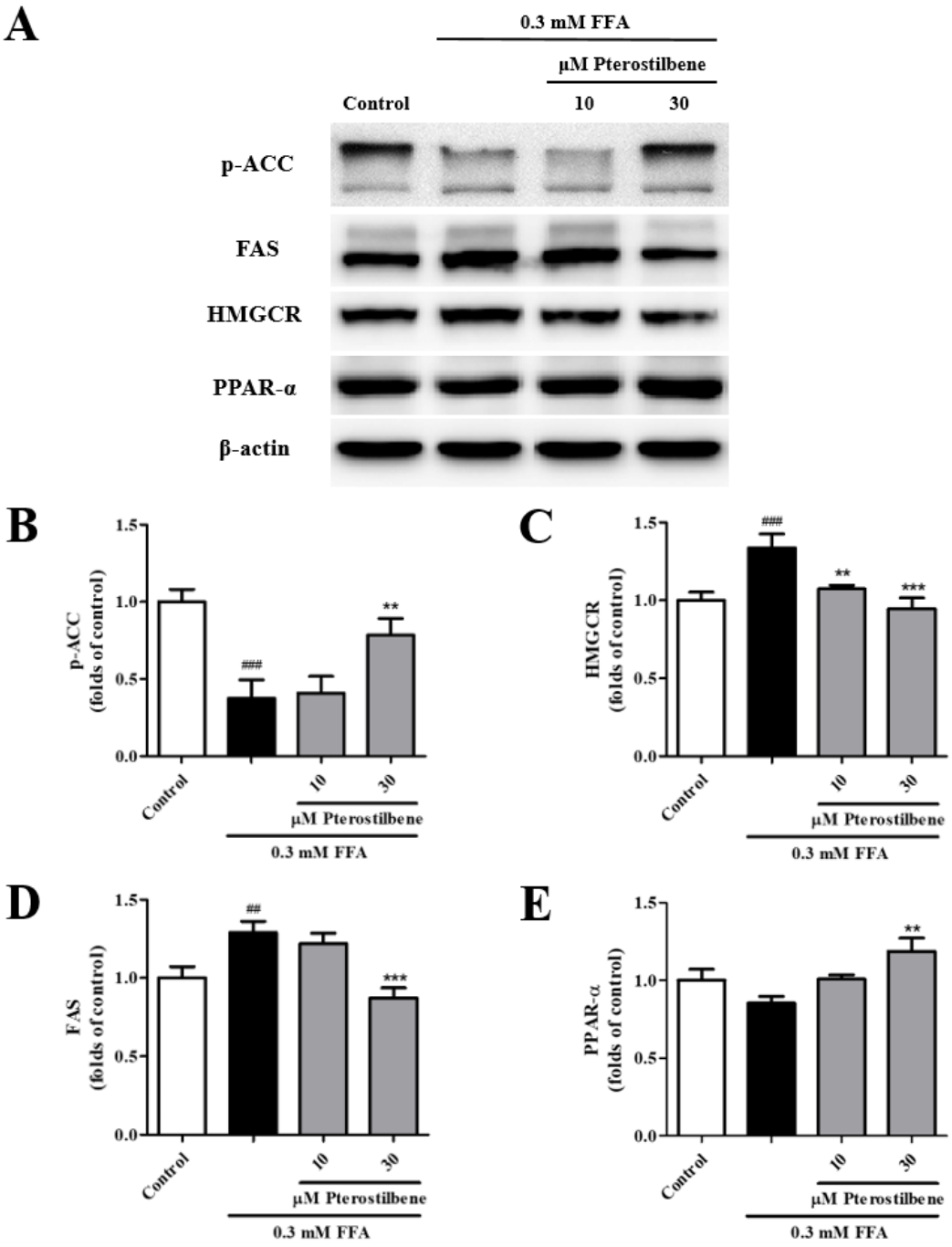

Figure 3.2.11 Effects of pterostilbene on the expression levels of p-ACC, FAS, HMGCR and PPAR-α in steatotic L02 cells.

L02 cells were treated with pterostilbene and 0.3 mM FFA for 24 h. The expressions of p-ACC, FAS, HMGCR and PPAR-α were determined by western blot. **(A)** The representative data and **(B – E)** densitometric analysis of the blots are shown. The data are expressed as the mean ± standard deviation, n = 3. $^{\#\#}$ $p < 0.01$ and $^{\#\#\#}$ $p < 0.001$ versus Control; ** $p < 0.01$ and *** $p < 0.001$ versus 0.3 mM FFA group.

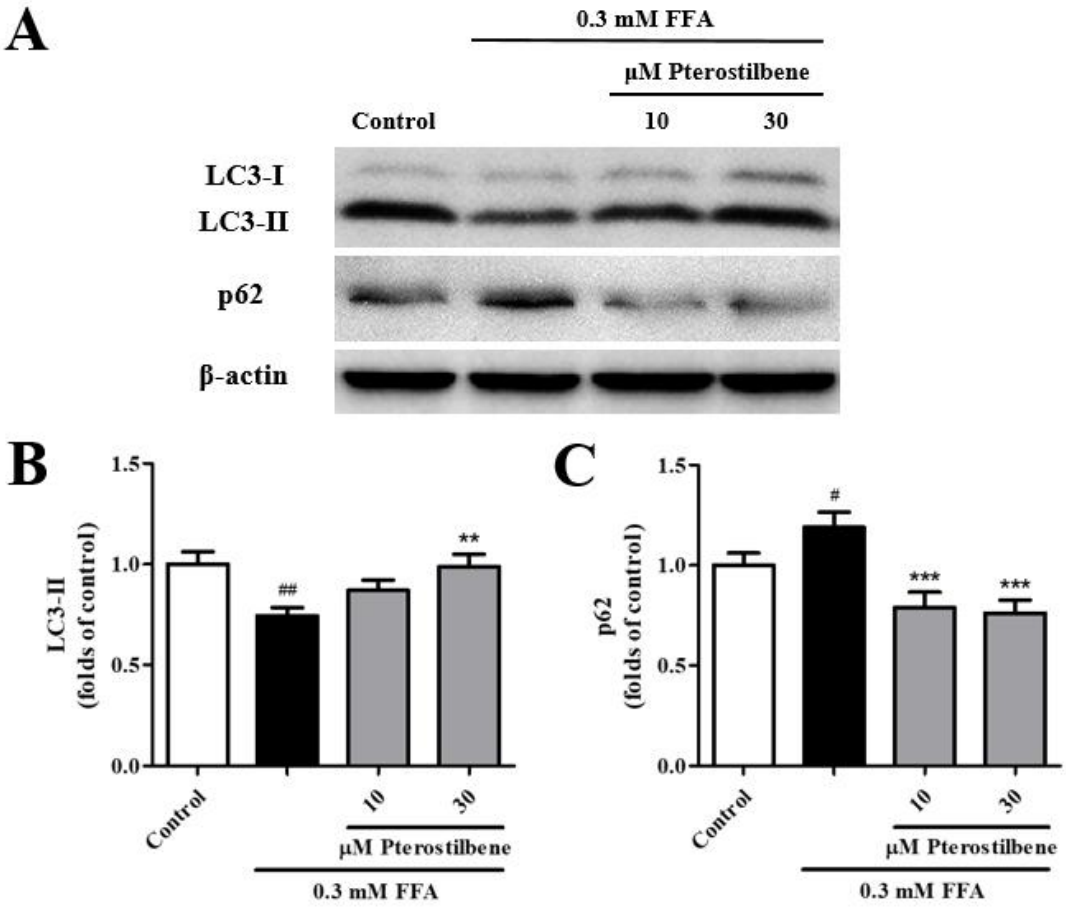

Figure 3.2.12 Effects of pterostilbene on the expression levels of LC3-II and p62 in steatotic L02 cells.

L02 cells were treated with pterostilbene and 0.3 mM FFA for 24 h. The expressions of LC3-II and p62 were determined by western blot. **(A)** The representative data and **(B – C)** densitometric analysis of the blots are shown. The data are expressed as the mean ± standard deviation, n = 3. $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ versus Control; ** $p < 0.01$ and *** $p < 0.001$ versus 0.3 mM FFA group.

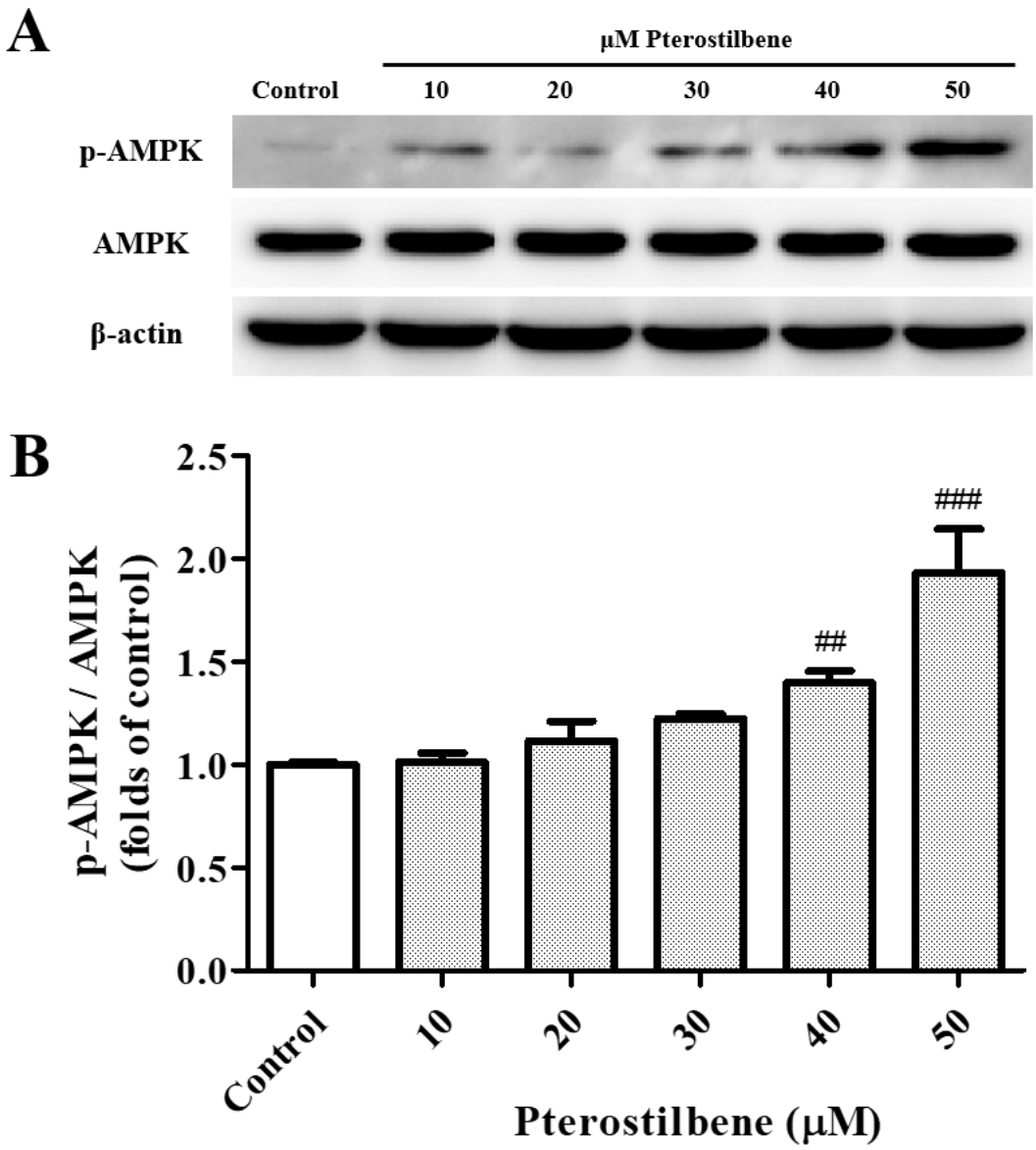

Figure 3.2.13 Pterostilbene upregulates the phosphorylated level of AMPK in L02 cells in a dose-dependent manner.

L02 cells were treated with pterostilbene for 24 h. The expressions of AMPK and p-AMPK were determined by western blot. **(A)** The representative data and **(B)** densitometric analysis of the blots are shown. The data are expressed as the mean ± standard deviation, n = 3. $^{\#\#}$ $p < 0.01$ and $^{\#\#\#}$ $p < 0.001$ versus Control.

3.3 *In vivo* study

3.3.1 Body weight record

As shown in Figure 3.3.1, the fasting body weights of the SD rats in various groups were not significantly different at the beginning of the experiment. At the end of the experiment, the fasting body weights of the SD rats in the PiH group was higher than those of the SD rats in other groups, and the fasting body weights of the SD rats in the Model group were slightly higher than than those of the SD rats in the Control group and the pterostilbene treatment groups, suggesting that administration of pterostilbene did not influence the growth of rats.

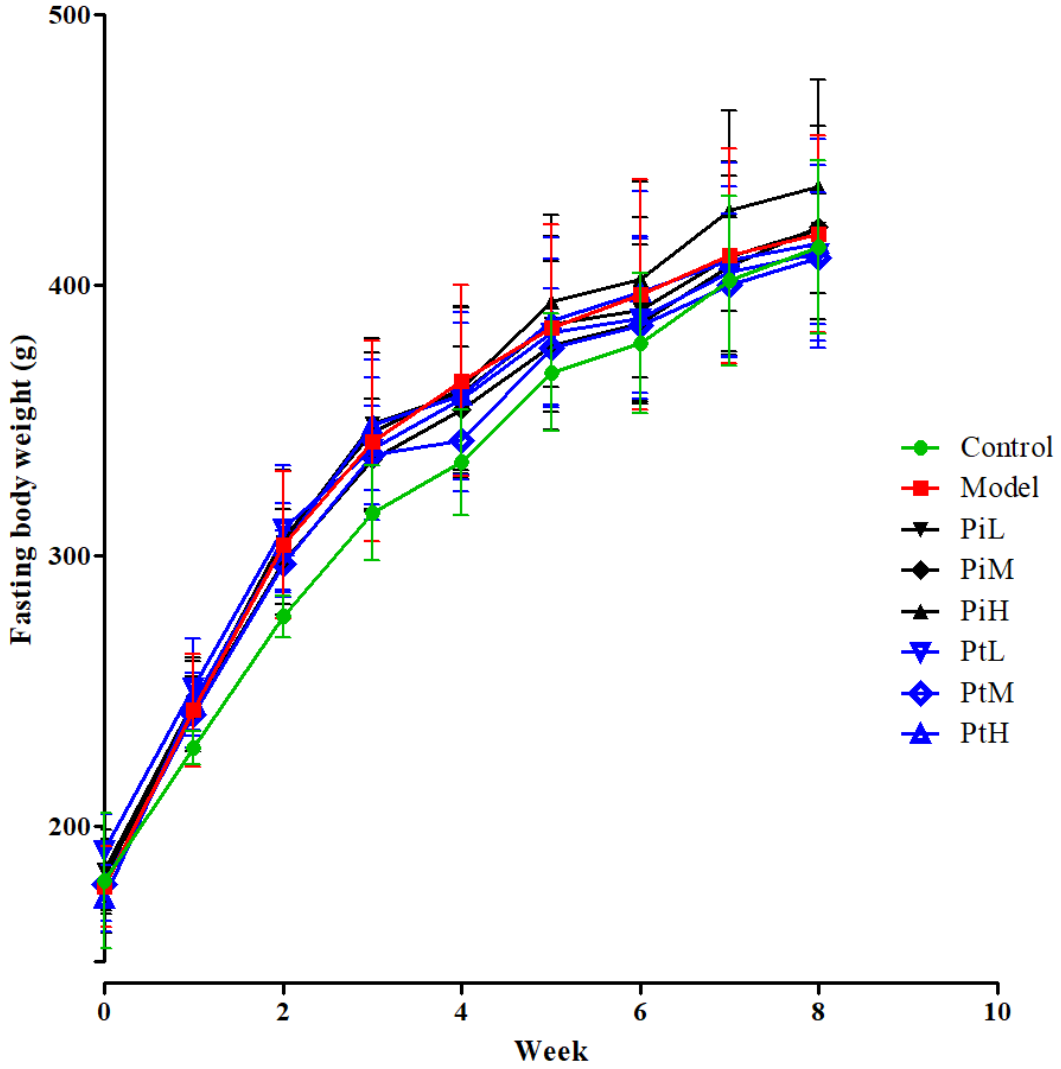

Figure 3.3.1 Fasting body weights of the SD rats in various groups.

The data are expressed as the mean ± standard deviation, n = 8.

3.3.2 Oral glucose tolerance test

The results of OGTT were shown in Figure 3.3.2. At week 4, blood glucose levels at 120 min of high-fat diet-fed rats (the Model group, the pioglitazone treatment groups and the pterostilbene treatment groups) were significantly higher than those of the rats in the Control group. At week 8, blood glucose levels at 120 min of the rats in the pioglitazone treatment groups and the pterostilbene treatment groups were siginificantly decreased when compared with those of the rats in the Model group, suggesting that treatments with pioglitazone and pterostilbene help improve glucose metabolism in high-fat diet-induced steatotic rats.

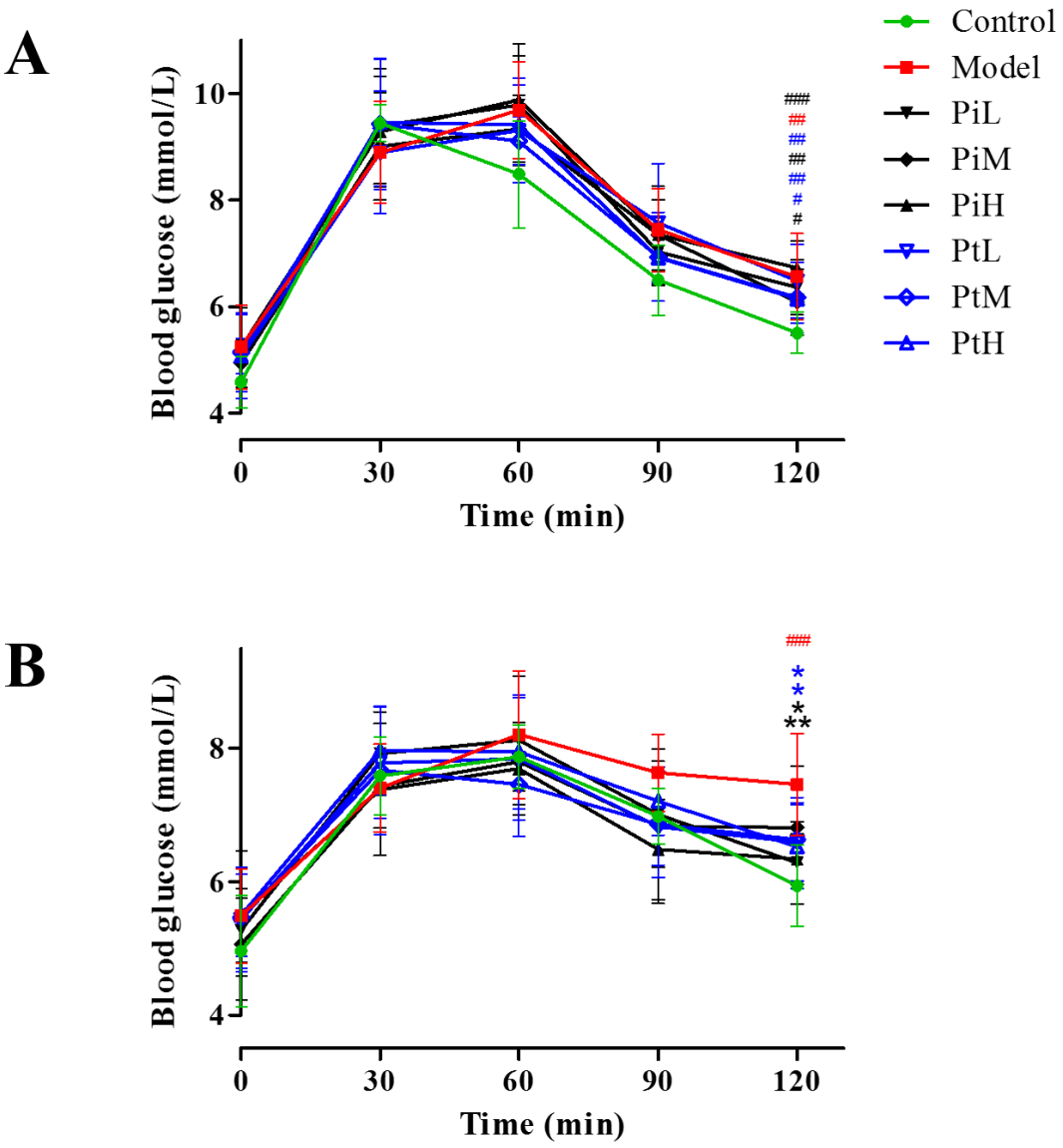

Figure 3.3.2 Results of OGTT showing the blood glucose levels of the SD rats in various groups.

Results of OGTT at **(A)** week 4 and **(B)** week 8 are shown. The data are expressed as the mean ± standard deviation, n = 8. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ and $^{\#\#\#}$ $p < 0.001$ versus Control; * $p < 0.05$ and ** $p < 0.01$ versus Model.

3.3.3 Analysis of serum biochemical changes

Figure 3.3.3 showed the changes of the serum ALT, AST, TG, TC, LDL-C and HDL-C levels of the rats in various group at the end of the *in vivo* experiment. There were significant increases in the serum ALT and TC levels of the rats in the Model group when compared with the Control group ($p < 0.001$). The serum ALT levels of the rats were significanlt decreased in PiL, PiM, PtM and PtH groups compared with the Model group ($p < 0.01$, $p < 0.001$, $p < 0.001$ and $p < 0.001$ respectively). The serum TG levels of the rats in PtH group were significantly lower than those in the Model group ($p < 0.05$). Moreover, the serum TC levels of the rats were significanlt decreased in all of the pioglitazone ($p < 0.01$) and the pterostilbene ($p < 0.05$) treatment groups. The serum LDL-C levels of the rats were significanlt decreased in PiH and PtM groups compared with the Model group ($p < 0.05$).

Figure 3.3.4 showed the fasting serum glucose and insulin levels as well as the homeostasis model assessment of insulin resistance (HOMA-IR) indices of the rats in various group at the end of the *in vivo* experiment. For the fasting serum glucose levels,

no statistically significant difference was found between groups. However, there were significant increases in the serum insulin levels of the rats in the Model group when compared with the Control group ($p < 0.001$). The serum insulin levels in all of the pioglitazone and the pterostilbene treatment groups were significantly reduced when compared with the Model group ($p < 0.001$). Similarly, there were significant increases in the HOMA-IR indices of the rats in the Model group when compared with the Control group ($p < 0.001$). The HOMA-IR indices in all of the pioglitazone and the pterostilbene treatment groups were significantly reduced when compared with the Model group ($p < 0.001$), suggesting that treatments with pioglitazone and pterostilbene help improve insulin sensitivity in high-fat diet-induced steatotic rats.

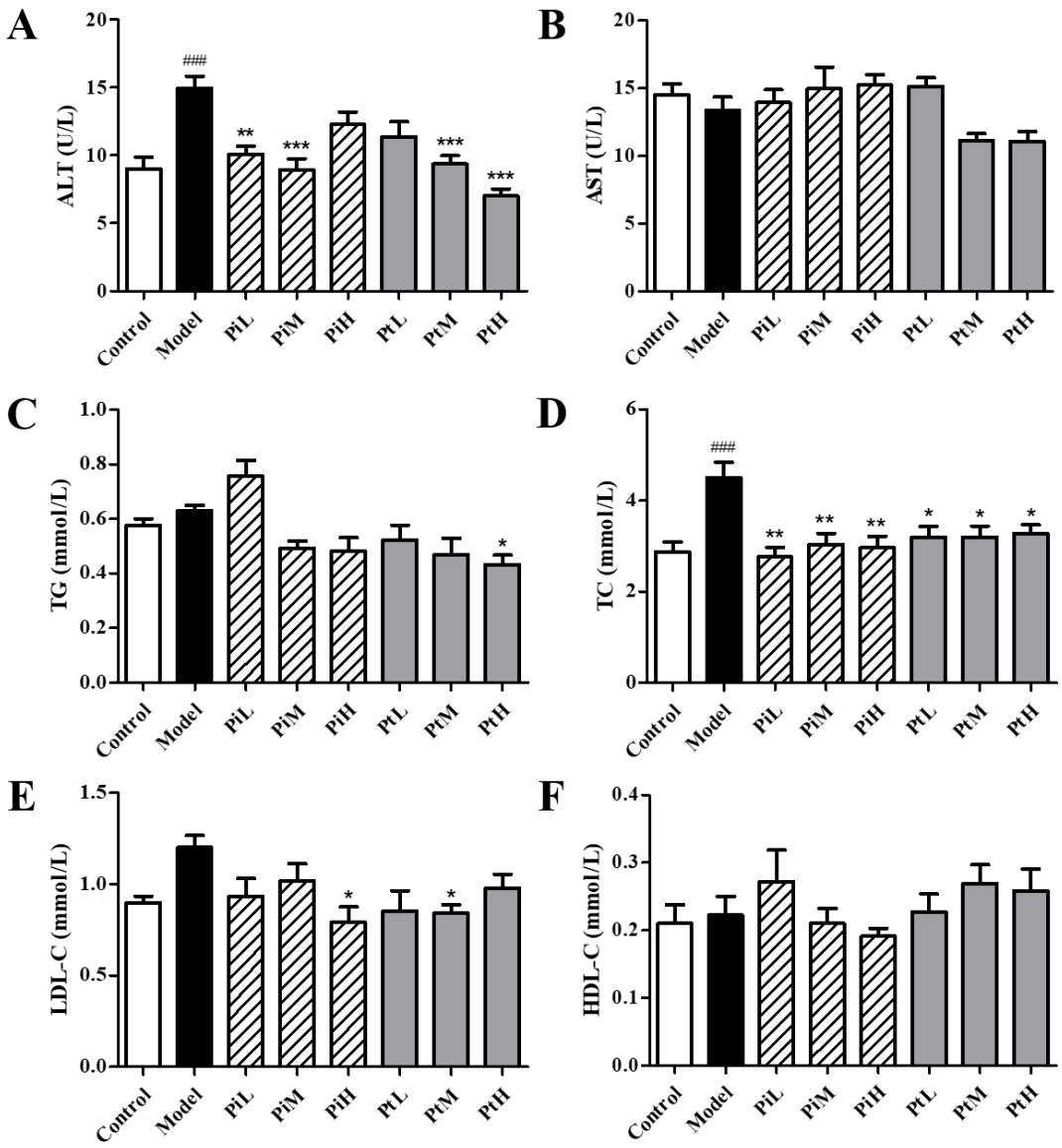

Figure 3.3.3 Effects of pterostilbene treatments on serum biochemical parameters.

The serum **(A)** ALT, **(B)** AST, **(C)** TG, **(D)** TC, **(E)** LDL-C and **(F)** HDL-C levels of the SD rats in various groups are shown. The data are expressed as the mean ± standard deviation, n = 8. ### $p < 0.001$ versus Control; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus Model.

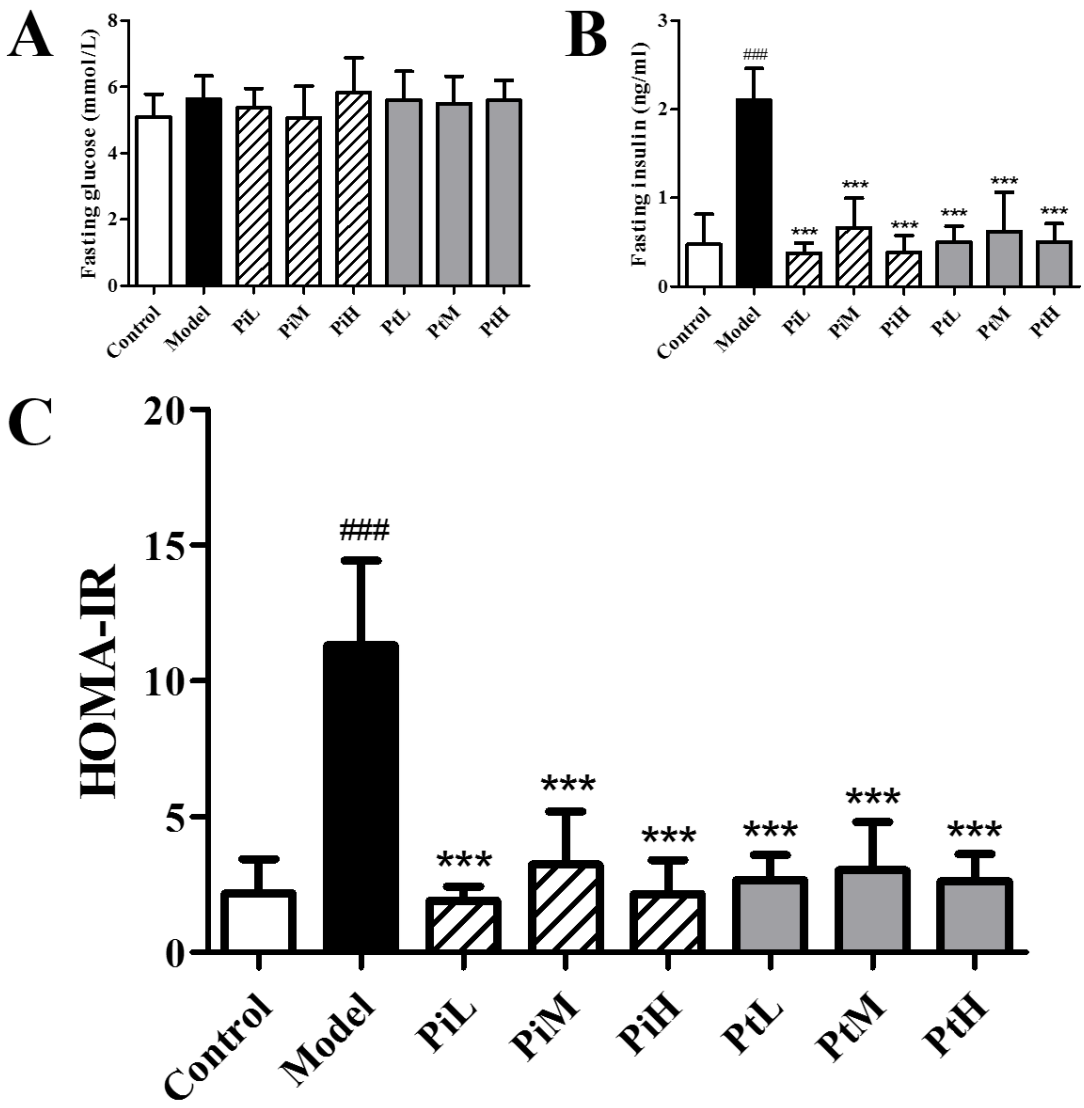

Figure 3.3.4 Effects of pterostilbene treatments on the fasting serum glucose and insulin levels and HOMA-IR index.

The **(A)** fasting serum glucose levels, **(B)** insulin levels, and **(C)** HOMA-IR indices of the SD rats in various groups are shown. The data are expressed as the mean ± standard deviation, n = 8. $^{###}$ $p < 0.001$ versus Control; *** $p < 0.001$ versus Model.

3.3.4 Liver lipid content evaluation

As shown in Figure 3.3.5A, no obvious pathological changes could be observed from the gross appearance of the liver from the Control group; the liver gave a reddish-brown colour with a normal surface morphology. For the gross appearance of the liver from the Model group, pathological abnormalities including a yellowish-brown liver colour and lipid accumulation were observed. Treatments with pioglitazone as well as pterostilbene improved the colour and surface morphology of the livers when compared with the Model group.

Determination of the liver lipid content was performed to assess the effects of pterostilbene treatments on hepatic lipid accumulation. Results (Figure 3.3.5B) showed that at the end of the experiment, the liver lipid content was significantly higher in the Model group than in the Control group ($p < 0.001$). Treatments with pioglitazone as well as pterostilbene significantly reduced lipid accumulation in the liver when compared with the Model group.

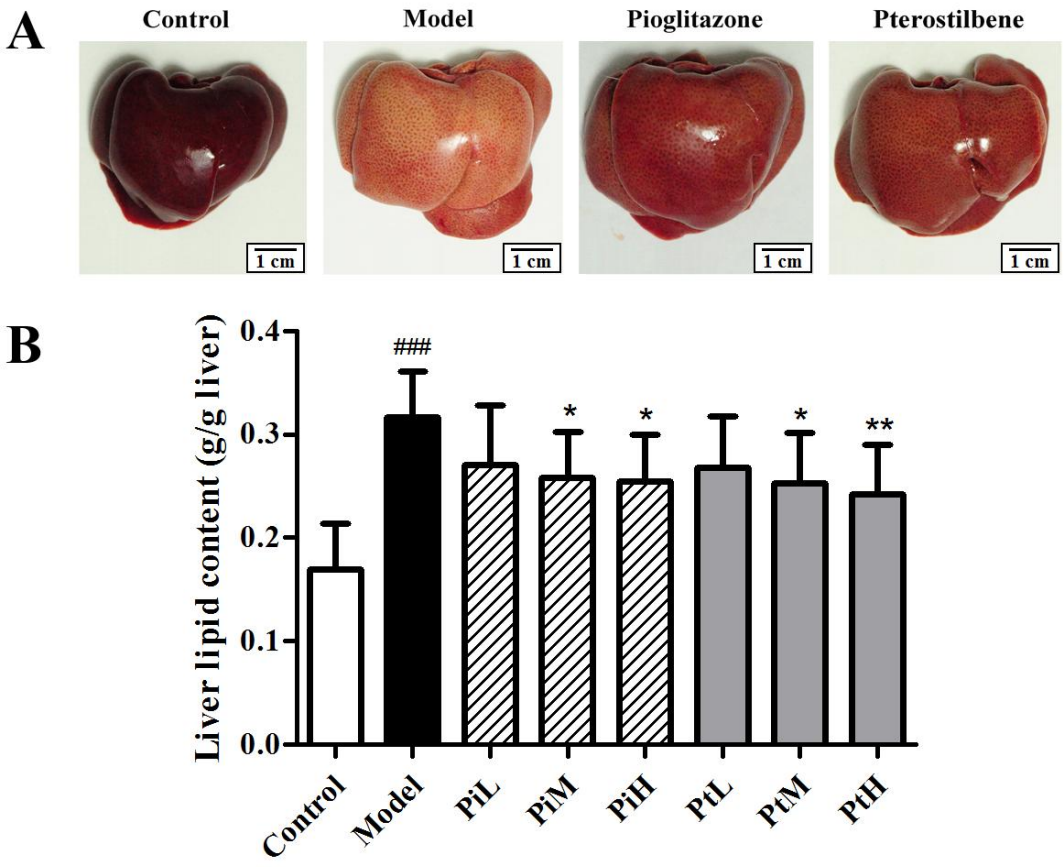

Figure 3.3.5 Pterostilbene treatment ameliorates hepatic lipid accumulation. **(A)** The gross appearance and **(B)** the lipid content of the liver from the rats in various groups. The data are expressed as the mean ± standard deviation, n = 8. $^{###}$ $p < 0.001$ versus Control; * $p < 0.05$ and ** $p < 0.01$ versus Model.

3.3.5 Liver histopathological examination

The histopathological results of the liver tissues from the rats in various groups following H&E staining were shown in Figure 3.3.6. The livers of the rats in the Model group showed significant steatosis. The hepatocytes were enlarged with severe hepatic microvesicular and macrovesicular lipid. It could be observed that the formation of macrovesicular lipid in the cytoplasm of hepatocytes resulting in pushing of the nucleus to the side. Compared with the Model group, treatments with pioglitazone as well as pterostilbene significantly reduced the number of lipid droplets in hepatocytes. The sizes of cells as well as lipid droplets were also reduced.

Figure 3.3.7 showed the NAFLD Activity Score and the percentage area of lipid vacuoles in various groups. The NAFLD Activity Score and the percentage area of lipid vacuoles in the Model group were significantly increased ($p < 0.001$). Compared with the Model group, treatments with pioglitazone as well as pterostilbene significantly reduced the NAFLD Activity Score and the percentage area of lipid vacuoles in a dose-dependent manner.

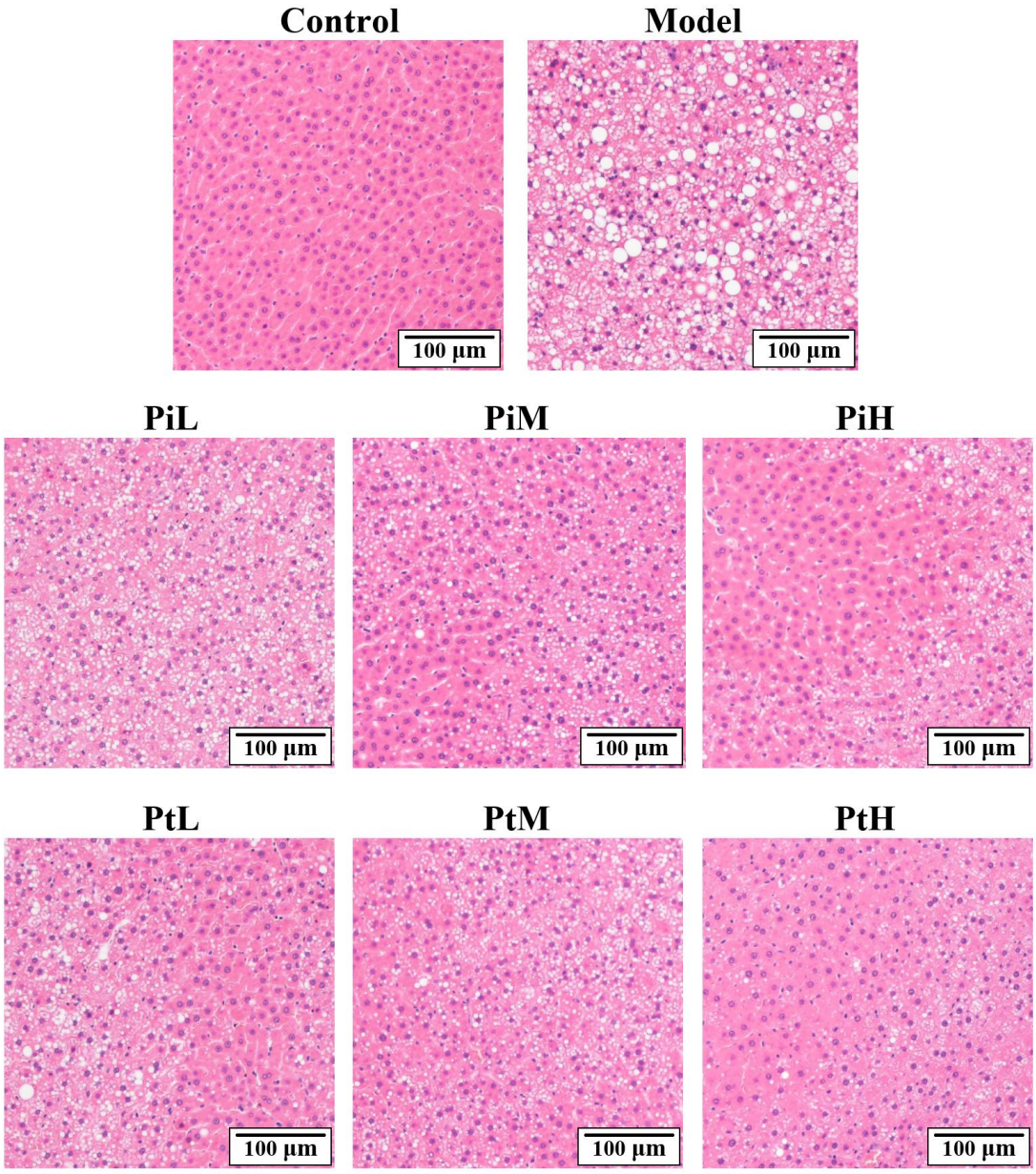

Figure 3.3.6 Comparison of histomorphology of the liver tissues among each group detected by H&E staining.

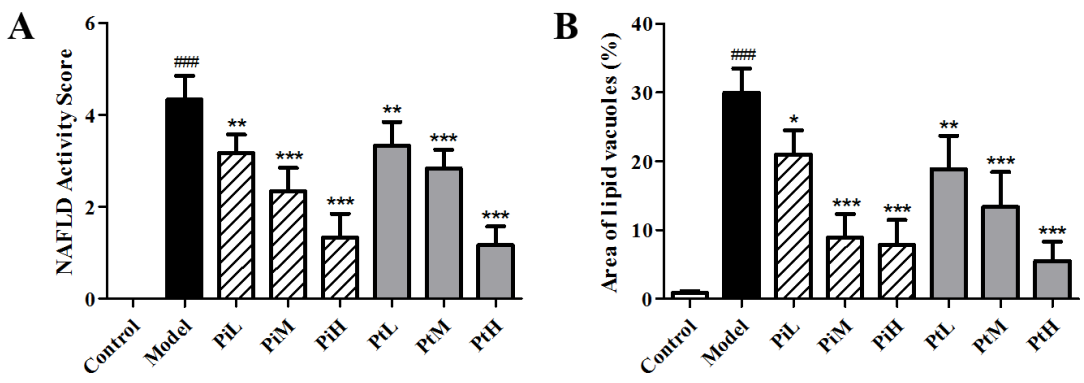

Figure 3.3.7 Semi-quantitative analyses of the liver histopathological results. **(A)** The NAFLD Activity Score and **(B)** the percentage area of lipid vacuoles in various groups. The data are expressed as the mean ± standard deviation, n = 6. ### $p < 0.001$ versus Control; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus Model.

3.3.6 Western blot analysis

To verify and investigate the molecular mechanisms of pterostilbene in ameliorating NAFLD, western blot analysis of fatty acid synthesis-, cholesterol synthesis-, oxidative stress-, autophagy-, and fatty acid β-oxidation-related proteins was conducted

Figure 3.3.8 showed the results of fatty acid synthesis-related proteins. The hepatic expression levels of FAS and p-ACC were significantly elevated in the Model group ($p < 0.001$). Compared with the Model group, the hepatic expression levels of FAS were significantly reduced in PtM and PtH groups ($p < 0.05$ and $p < 0.001$

respectively) while those of p-ACC were significantly reduced in PtH group ($p < 0.01$).

Figure 3.3.9 showed the results of cholesterol synthesis-related proteins. The hepatic expression levels of SREBP-2 and HMGCR were significantly elevated in the Model group ($p < 0.001$). Compared with the Model group, the hepatic expression levels of SREBP-2 were significantly reduced in PtM and PtH groups ($p < 0.05$ and $p < 0.001$ respectively). Those of HMGCR were also significantly reduced in PtH group ($p < 0.05$).

Figure 3.3.10 showed the results of oxidative stress-related proteins. The hepatic expression levels of p-ERK, p-JNK and p38 were significantly elevated in the Model group ($p < 0.001$). Compared with the Model group, the phosphylated levels of MAPKs were significantly reduced in the pterostilbene treatment groups ($p < 0.01$). In the Model group, the hepatic expression levels of HO-1 were significantly reduced ($p < 0.01$). The reduced hepatic expression levels of HO-1 were significantly elevated in the pterostilbene treatment groups ($p < 0.05$).

As shown in Figure 3.3.11, the hepatic phosphorylated levels of AMPK were

decreased in the Model group ($p < 0.001$). Treatment with pterostilbene significantly increased the hepatic phosphorylated levels of AMPK in a dose-dependent manner. The hepatic expression levels of p-mTOR in the Model group were significantly increased ($p < 0.05$). Compared with the Model group, the hepatic expression levels of p-mTOR were significantly decreased in PtM and PtH groups ($p < 0.05$ and $p < 0.01$ respectively). In addition, the hepatic expression levels of LC3-II, which were significantly decreased in the Model group ($p < 0.05$), were significantly elevated in the pterostilbene treatment groups. Moreover, the hepatic expression levels of PPAR-α were significantly increased in the Model group ($p < 0.05$) and were further elevated in the pterostilbene treatment groups.

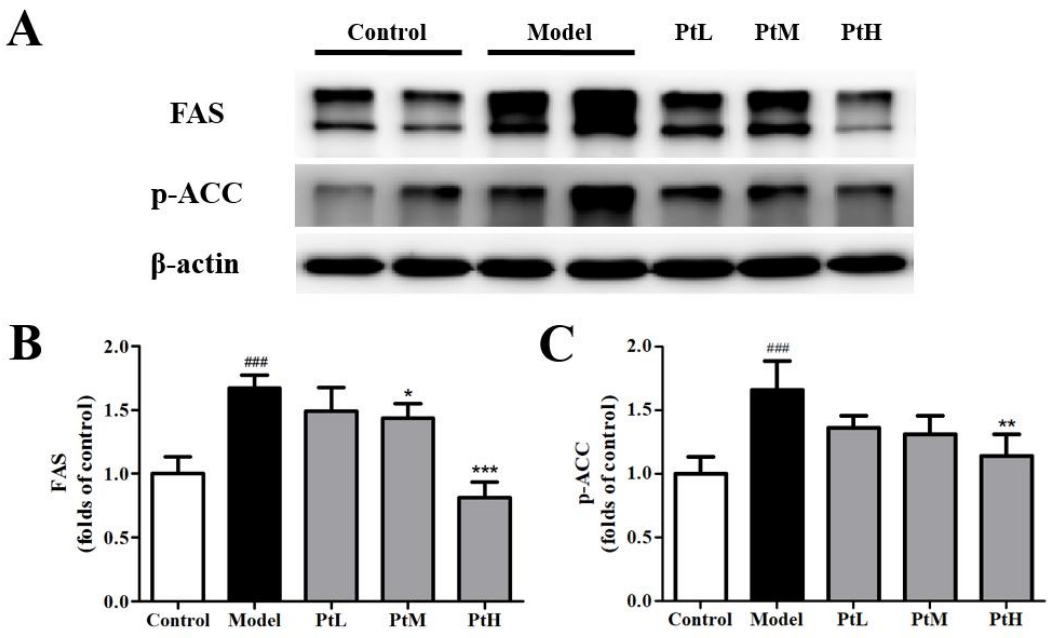

Figure 3.3.8 Effects of pterostilbene on the hepatic expression levels of p-ACC and FAS in steatotic SD rats.

The expressions of p-ACC and FAS in the liver of the rats in various groups were determined by western blot. **(A)** The representative data and **(B – C)** densitometric analysis of the blots are shown. The data are expressed as the mean ± standard deviation, n = 6 for Control and Model, n = 3 for PtL, PtM, and PtH. $^{###}$ $p < 0.001$ versus Control; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus Model.

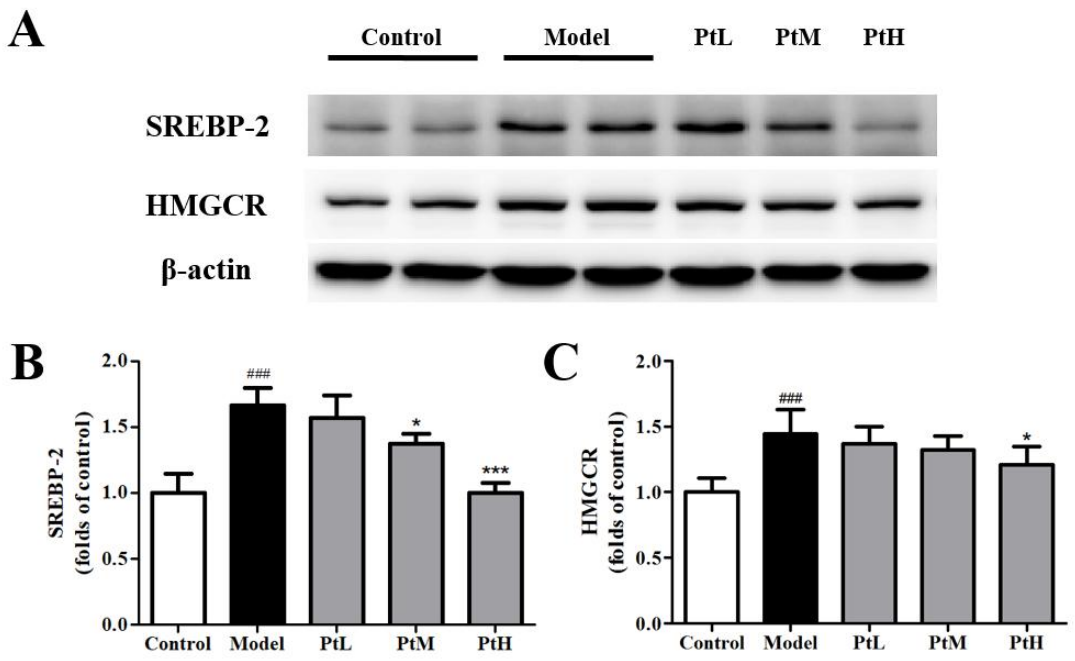

Figure 3.3.9 Effects of pterostilbene on the hepatic expression levels of SREBP-2 and HMGCR in steatotic SD rats.

The expressions of SREBP-2 and HMGCR in the liver of the rats in various groups were determined by western blot. **(A)** The representative data and **(B – C)** densitometric analysis of the blots are shown. The data are expressed as the mean ± standard deviation, n = 6 for Control and Model, n = 3 for PtL, PtM, and PtH. $^{###}$ $p < 0.001$ versus Control; * $p < 0.05$ and *** $p < 0.001$ versus Model.

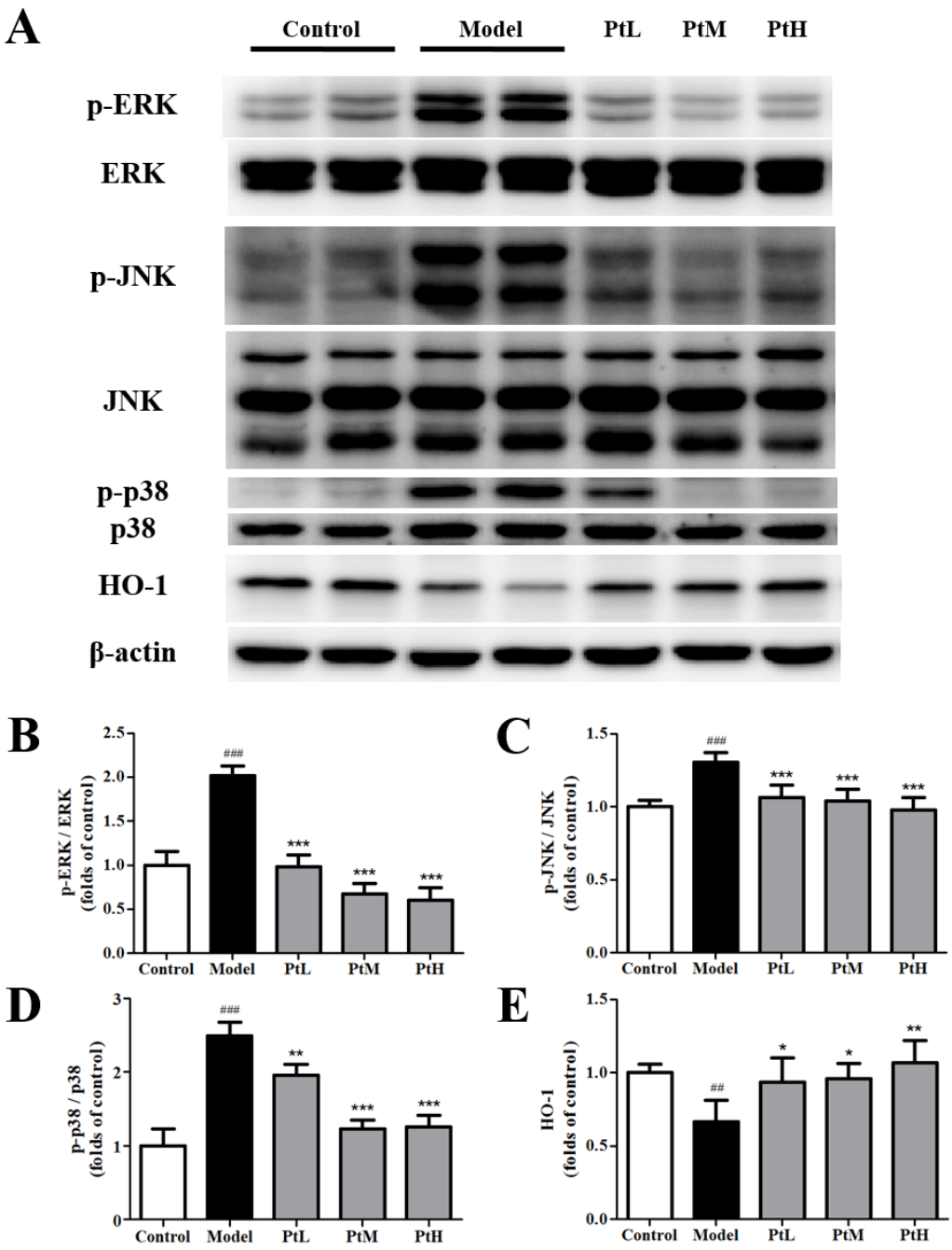

Figure 3.3.10 Effects of pterostilbene on the hepatic expression levels of MAPKs and HO-1 in steatotic SD rats.

The expressions of ERK, p-ERK, JNK, p-JNK, p38, p-p38 and HO-1 in the liver of the rats in various groups were determined by western blot. **(A)** The representative data and **(B – E)** densitometric analysis of the blots are shown. The data are expressed as the mean ± standard deviation, n = 6 for Control and Model, n = 3 for PtL, PtM, and PtH. $^{\#\#}$ $p < 0.01$ and $^{\#\#\#}$ $p < 0.001$ versus Control; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus Model.

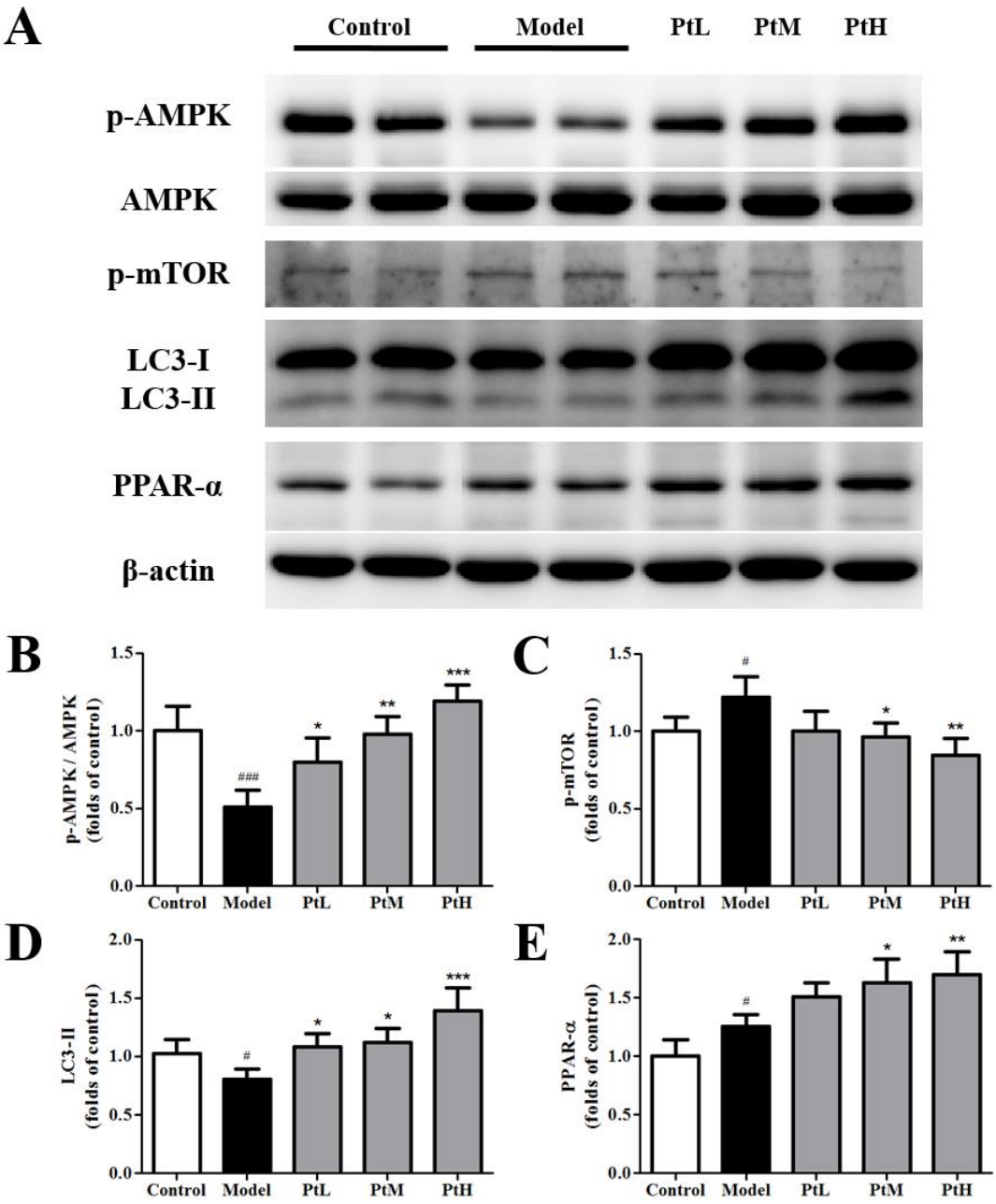

Figure 3.3.11 Effects of pterostilbene on the hepatic expression levels of AMPK, p-mTOR, LC3-II and PPAR-α in steatotic SD rats.

The expressions of AMPK, p-AMPK, p-mTOR, LC3-II and PPAR-α 1 in the liver of the rats in various groups were determined by western blot. **(A)** The representative data and **(B – E)** densitometric analysis of the blots are shown. The data are expressed as the mean ± standard deviation, n = 6 for Control and Model, n = 3 for PtL, PtM, and PtH. $^{\#}$ $p < 0.05$ and $^{\#\#\#}$ $p < 0.001$ versus Control; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus Model.

3.3.7 Lipidomics study

The stability of UPLC-Orbitrap MS detection were assessed by inter-day measurement of the pooled QC sample injections and 2 internal standards. The results showed that the CV of the analytes was less than 15% (Table 3.3.1). Figure 3.3.12A showed the PCA score plot evaluating the stability of the analytical instrument. The QC sample injections of inter-day experiments were clustered together in the PCA score plot, indicating that high reproducibility was achieved across the runs.

The changes in the serum metabolites of lysophospholipids in the Control and the Model groups were investigated using OPLS-DA. Referring to the OPLS-DA score plot (Figure 3.3.12B), a clear separation was shown between the Control group and the Model group. This model was described by high values of the R^2Y and Q^2, confirming its strength. PLS-DA was further carried out to investigate the differences of metabolite profiles in various groups and study the effect of pterostilbene on high-fat diet-induced alterations of serum metabolites. The PLS-DA score plot (Figure 3.3.12C) showed a clear distinction between groups. The Control group and the Model group were

separated along the first principal component. The pterostilbene treatment (PtH) group was deviated from the Model group to the Control group along the first principal component and was separated from the Control and the Model groups along the second principal component.

The changes in the serum metabolites of lysophospholipids in various groups were summarized in Table 3.3.1 and Figure 3.3.13. The results revealed that eight lysophosphatidylcholines (lysoPCs; C15:1, C16:1, C17:1, C18:1, C18:2, C20:2, C20:3 and C20:5) were increased in the Model group when compared with the Control group. Treatments with pioglitazone as well as pterostilbene reduced the levels of these lysoPCs.

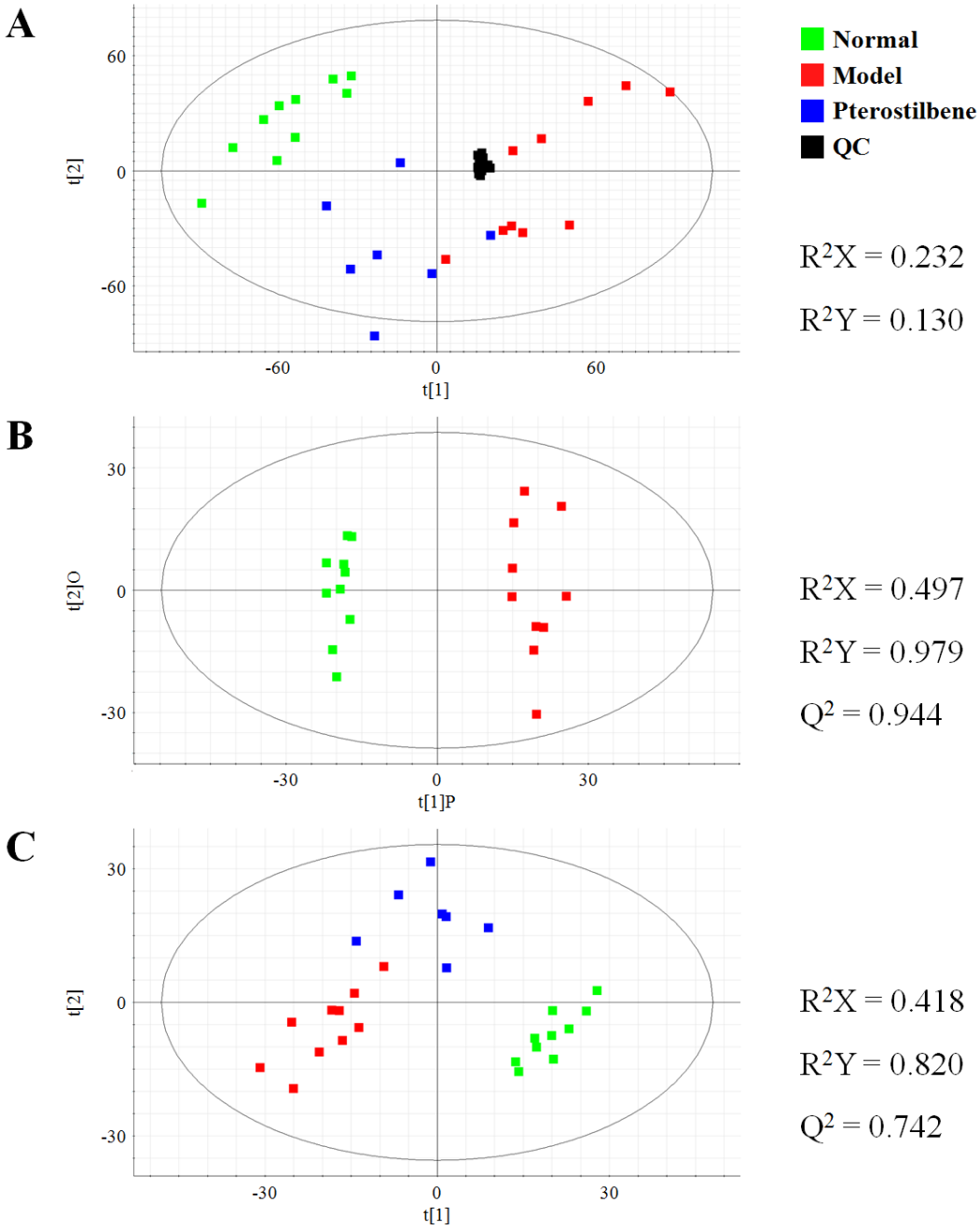

Figure 3.3.12 PCA, OPLS-DA and PLS-DA of serum metabolites in the Control, the Model and the pterostilbene treatment groups.

(A) PCA, **(B)** OPLS-DA and **(C)** PLS-DA score plots of serum metabolites acquired in positive ionization mode for UPLC-Orbitrap MS. t[1] and t[2] represents the first and the second principal components respectively, t[1]P represents principal component of OPLS-DA, and t[2]O represents orthogonal component of OPLS-DA. R^2X and R^2Y represent the fraction of the variance of the X and Y variables respectively. Q^2 represents the predictive capability of the model.

Table 3.3.1 Fold changes of metabolites identified by UPLC-Orbitrap MS

Metabolites	Adducts	Retention time (min)	Theoretical m/z	Measured m/z	Mass error / ppm	Fold change		QC CV (%)
						Control / Model	Pterostilbene / Model	
LysoPC15:1	$[M+H]^+$	3.90	480.3085	480.3091	1.25	$0.57^{\#\#}$	$0.60^{\#\#}$	5.43
LysoPC16:1	$[M+H]^+$	2.62	494.3241	494.3248	1.43	0.58*	0.49**	7.08
LysoPC17:1	$[M+H]^+$	3.24	508.3398	508.3403	0.95	0.67*	0.57**	7.31
LysoPC18:0	$[M-H_2O]^+$	4.56	506.3610	506.3611	0.20	$1.56^{\#\#\#}$	$1.42^{\#\#}$	9.08
LysoPC18:1	$[M+H]^+$	3.79	522.3554	522.3562	1.55	$0.70^{\#\#}$	$0.63^{\#\#}$	10.24
LysoPC18:2	$[M+H]^+$	2.99	520.3403	520.3406	0.62	$0.70^{\#\#}$	0.79	8.94
LysoPC20:0	$[M+H]^+$	4.82	552.4024	552.4042	3.23	$1.58^{\#}$	0.98	9.93
LysoPC20:2	$[M+H]^+$	4.00	548.3716	548.3718	0.34	$0.58^{\#\#}$	$0.69^{\#}$	7.97
LysoPC20:3	$[M+H]^+$	3.41	546.3560	546.3562	0.35	0.38***	0.79	6.20
LysoPC20:5	$[M+H]^+$	2.27	542.3241	542.3251	1.80	$0.54^{\#\#\#}$	$0.61^{\#\#}$	6.76
LysoPC22:5	$[M+H]^+$	3.48	570.3554	570.3562	1.34	1.87**	0.77	5.92
LysoPE18:2	$[M+Na]^+$	3.09	500.2748	500.2755	1.34	$0.76^{\#}$	0.88	11.43
Stearoylcarnitine	$[M+H]^+$	4.40	428.3734	428.3738	1.00	$1.46^{\#\#\#}$	1.02	9.36

One-way ANOVA, Tukey's HSD for equal variances: $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ and $^{\#\#\#}$ $p < 0.001$; Tamhane's T2 for unequal variances: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. n = 10 for Control and Model, n = 7 for pterostilbene treatment group.

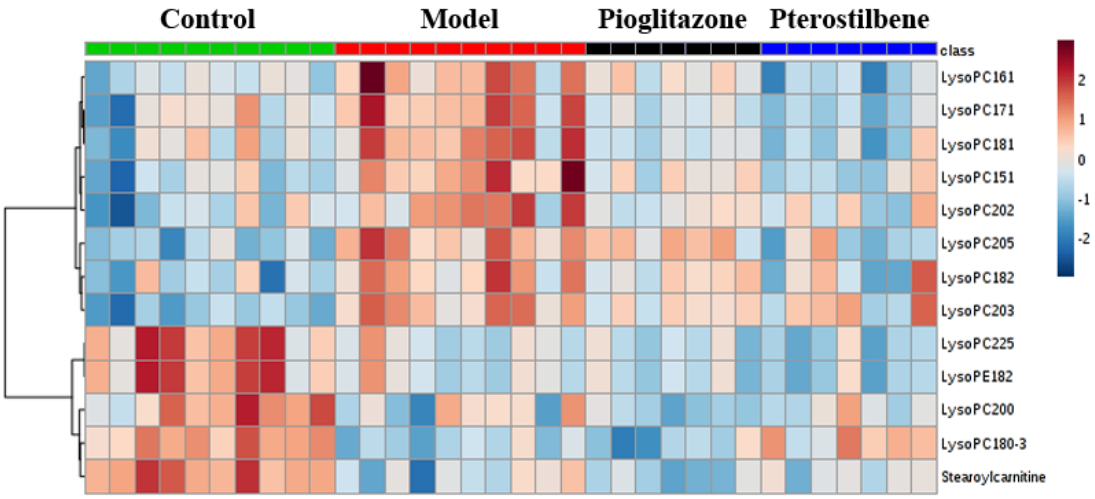

Figure 3.3.13 Serum metabolite profiles in the Control, the Model, the pioglitazone and the pterostilbene treatment groups.

Heatmap showing the top 13 identified metabolites acquired in positive ionization mode for UPLC-Orbitrap MS contributing to the classification of PLS-DA. The colour code represents the row z-score.

3.3.8 Gut microbiota analysis

The changes in the community structure of gut microbiota at the phylum level and the family level in various groups were shown in Figure 3.3.14. PLS-DA was applied to investigate the the differences of community structure of gut microbiota. The PLS-DA score plots (Figure 3.3.15) showed clear separation among the Control, the Model and the pterostilbene treatment (PtH) groups. The results (Figure 3.3.16) revealed that the proportions of *Lactobacillaceae*, *Peptostreptococcaceae*, *Coriobacteriaceae*, *Desulfovibrionaceae*, and *Streptococcaceae* were increased and those of *Ruminococcaceae* and *Lachnospiraceae* were decreased in the Model group when compared with the Control group. Treatments with pterostilbene restored the proportions of *Lactobacillaceae*, *Ruminococcaceae*, *Peptostreptococcaceae*, *Coriobacteriaceae*, *Desulfovibrionaceae*, and *Streptococcaceae*.

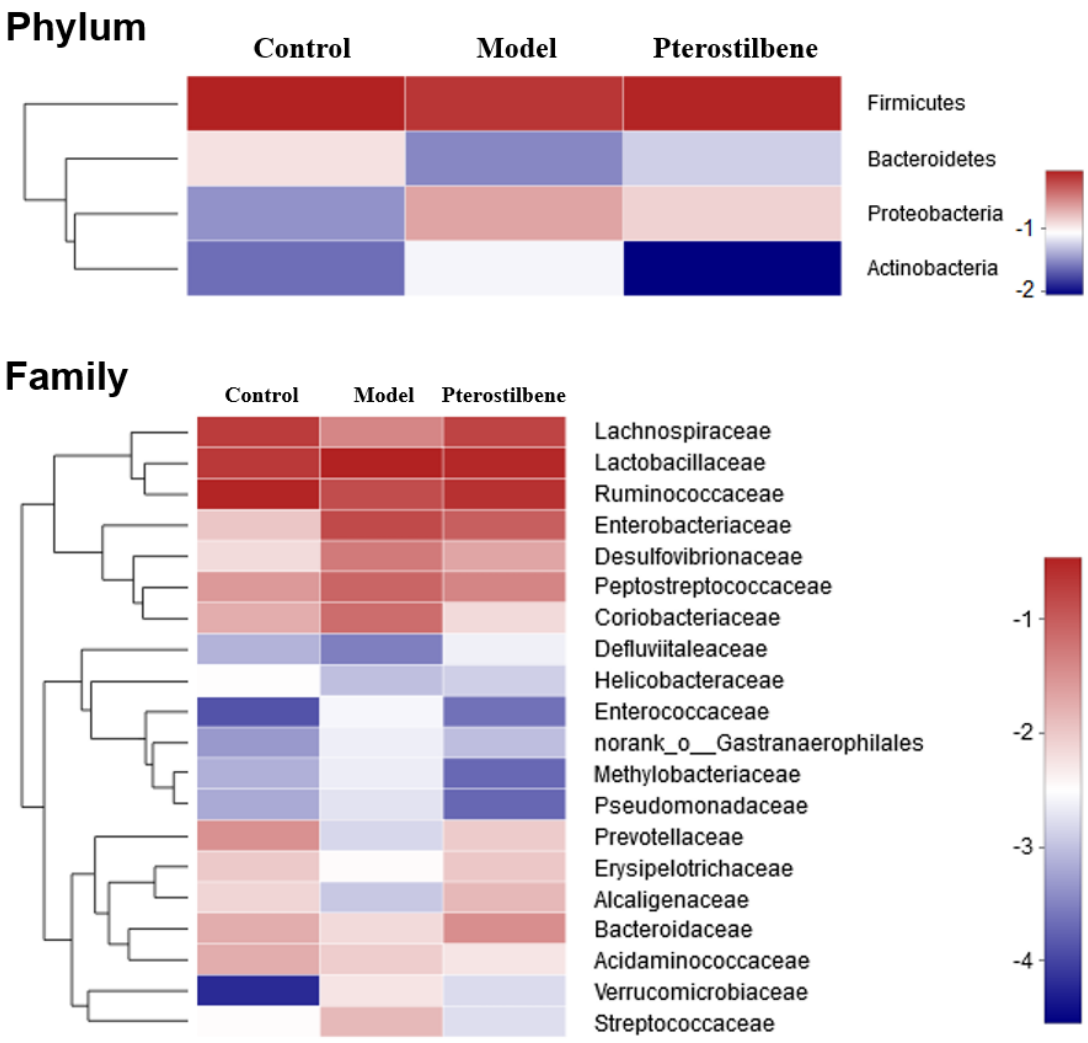

Figure 3.3.14 The community structure of gut microbiota in the Control, the Model and the pterostilbene treatment groups.

Heatmaps showing the relative abundance of gut microbial communities at the phylum level and the family level in each group. The colour code represents the log10-transformed relative abundance.

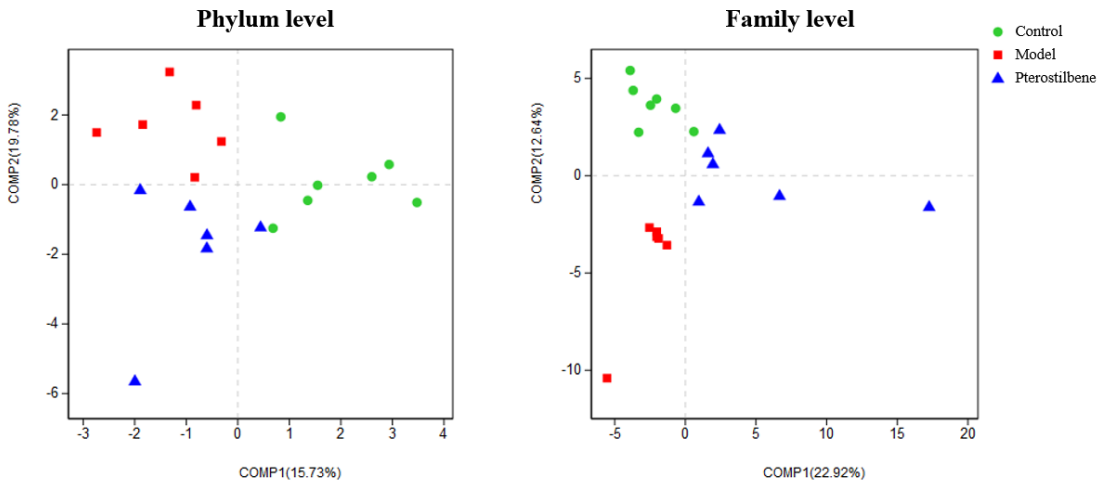

Figure 3.3.15 PLS-DA of the community structure of gut microbiota in the Control, the Model and the pterostilbene treatment groups.

PLS-DA score plots of the community structure of gut microbiota at the phylum level and the family level in each group.

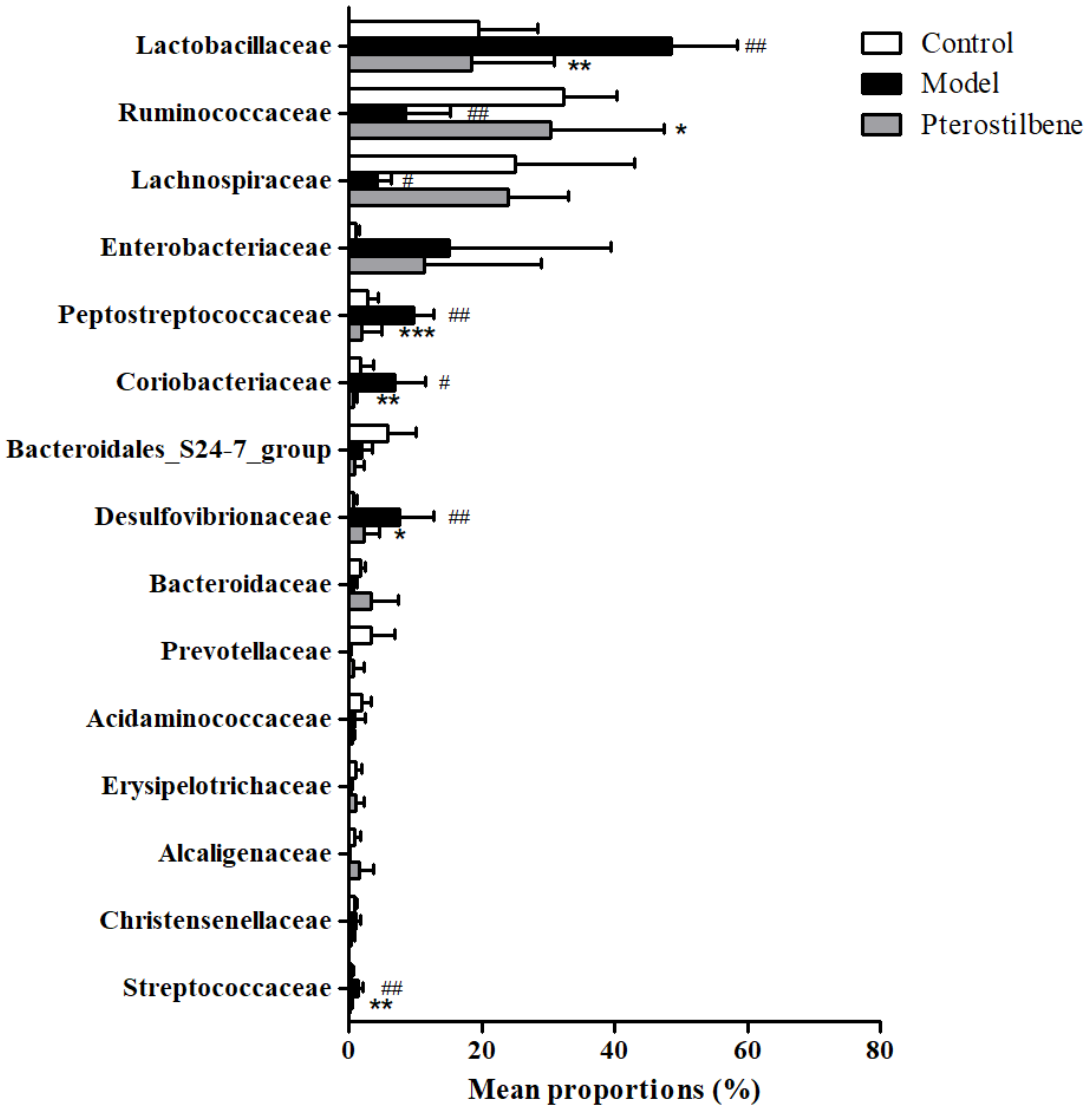

Figure 3.3.16 Effects of pterostilbene on the community structure of gut microbiota in steatotic SD rat model.

The data are expressed as the mean ± standard deviation. n = 7 for Control, n = 6 for Model and pterostilbene treatment group. $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ versus Control; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus Model.

3.3.9 Gut histopathological examination

Figure 3.3.17 showed the histopathological results of the small intestine tissues from the rats in various groups following H&E staining. Results revealed that there was no intestinal inflammation in all the groups.

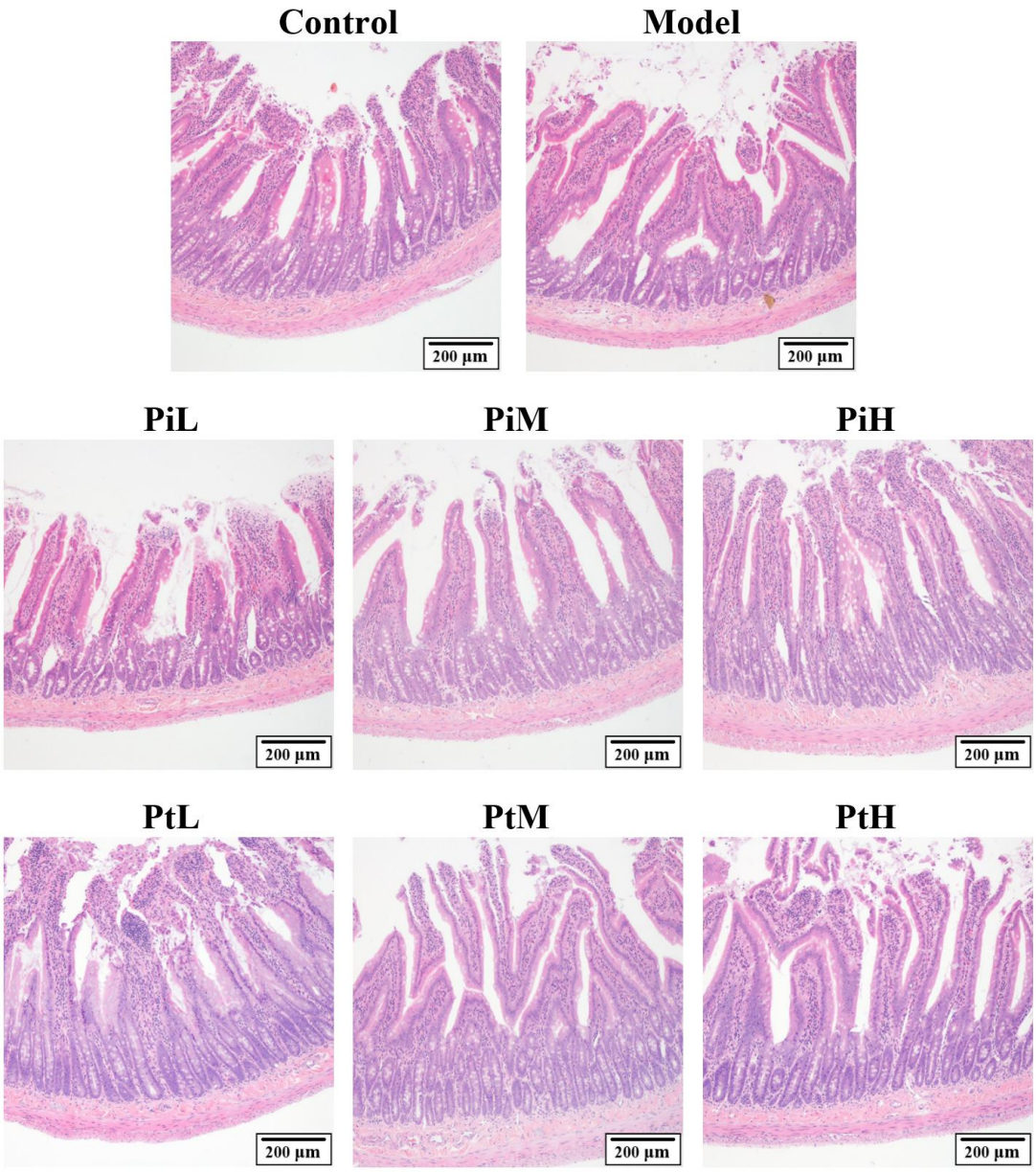

Figure 3.3.17 Comparison of histomorphology of the small intestine tissues among each group detected by H&E staining.

Chapter 4 Discussion

4.1 *In vitro* study

4.1.1 Oxidative stress

Oxidative stress refers to an imbalance between the production of free radicals and the ability of the biological system to detoxify and remove these free radicals, resulting in elevated intracellular ROS in the body which causes oxidative damage to DNA, proteins and phospholipids. Oxidative stress has been considered to play an important role in the pathogenesis of NAFLD and is associated with mitochondrial dysfunction and inflammation induction in hepatocytes. Protection of hepatocytes against oxidative damage may help alleviate the development and the progression of NAFLD.

Pterostilbene is a naturally derived polyphenolic compound and has been shown to exhibit dose-dependent antioxidant effect. In DPPH radical scavenging assay, pterostilbene possessed free radical scavenging ability in a concentration dependent manner. Moreover, pterostilbene has been demonstrated to attenuate oxidative stress and protect various types of cells against oxidative stress by increasing the activities of antioxidant enzymes, such as superoxide dismutase and catalase. Pterostilbene has

potential to protect hepatocytes against oxidative damage in order to attenuate NAFLD.

In this study, L02 cell line was used as the hepatocyte cell model and H_2O_2 was used to induce oxidative stress to study the antioxidative effect of pterostilbene in protection of hepatocytes *in vitro* and its possible underlying mechanisms.

4.1.1.1 Establishment of oxidative stress *in vitro* hepatocyte model

In the establishment of oxidative stress *in vitro* model, H_2O_2 was used. H_2O_2 is a by-product generated from superoxide produced by mitochondria during aerobic metabolism. It is recognized as a central mediator of oxidative stress. Therefore, H_2O_2 has been used to induce oxidative stress in various *in vitro* studies.

In the present study, the toxicities of treating various concentrations of H_2O_2 on L02 cells for 24 hr were evaluated based on the cell viability. In order to provide better distinguishment to show the protective effect of pterostilbene on L02 cells against oxidative stress-mediated cell death, the concentration of H_2O_2 that caused 50% cell death was chosen as the model for the corresponding experiments.

4.1.1.2 Pterostilbene protected L02 cells against H_2O_2-induced oxidative stress

In the present study, pretreatment of 30 and 50 μM pterostilbene for 2 h prior to 0.5 mM H_2O_2 24-h exposure significantly reduced H_2O_2-induced cell death in L02 cells, suggesting that pterostilbene helps protect L02 cells against H_2O_2-induced oxidative stress. Furthermore, pretreatment with pterostilbene was demonstrated to reduce intracellular ROS formation in L02 cells when challenging with H_2O_2. That means pterostilbene protect L02 cells against oxidative stress-mediated cell death by inhibition of intracellular ROS formation.

MAPKs are a family of protein kinases that convert extracellular stimuli into a wide range of intracellular responses. Three subgroups of MAPKs, including the ERKs, the JNKs, and the p38 MAPKs, have been well-defined in mammalian cells. As discussed before, these subgroups of MAPKs regulate diverse cellular functions including cell survival, proliferation, differentiation, and apoptosis (Cargnello and Roux, 2011; E. K. Kim and Choi, 2010; W. Zhang and Liu, 2002). Thus, MAPKs play an important role in various physiological processes. Various studies demonstrated that oxidative stress could lead to activation of MAPKs resulting in inflammation and

apoptosis. In the present study, pterostilbene was demonstrated to attenuate H_2O_2-induced phosphorylation of ERKs, JNKs and p38 MAPKs in L02 cells, suggesting that the mechanism of pterostilbene in protection of L02 cells against H_2O_2-induced oxidative stress involved inhibition of MAPKs activation.

HO is an enzyme that is responsible for catalyzing the degradation of heme to biliverdin, free iron and carbon monoxide. Besides, HO is highly upregulated in response to various stimili, including oxidative stress and inflammation. HO-1 is one of the two isoforms of HO that found in mammals. It has been shown that upregulation of HO-1 expression exhibit protection effect against cellular oxidative stress. Result of western blot analysis demonstrated that pterostilbene treatment upregulate HO-1 expression in L02 cells in a dose-dependent manner, suggesting that upregulation of HO-1 expression may be involved in the mechanism of pterostilbene in protection of L02 cells against oxidative stress.

4.1.2 Lipid accumulation

NAFLD is a hepatic manifestation of the metabolic syndrome including a

spectrum of conditions ranging from nonalcoholic hepatic steatosis to NASH and is characterized as the deposition of intracellular fat in hepatocytes. Excess amount of nutrient uptake or impaired lipid metabolism lead to increased FFA levels resulting in elevated hepatic lipid production and storage. Prolonged accumulation of lipid in hepatocytes can cause hepatic lipotoxicity and insulin resistance, and is considered as one the important factors in the pathogenesis of NAFLD.

Reduction of hepatic lipid accumulation is thus a basic way to attenuate NAFLD. In the present study, the effect of pterostilbene in lowering hepatic lipid accumulation as well as the possible underlying mechanisms were investigated.

4.1.2.1 Establishment of steatotic hepatocyte model

In the establishment of steatotic hepatocyte model, OA and PA were used. The reason of using OA and PA for the establishment of steatotic hepatocyte model is that OA and PA were found to be the most abundant FFAs in liver TGs in both normal subjects and patients with NAFLD. Previous studies have investigated the intracellular levels of lipid accumulation, cytotoxicity and apoptosis in hepatocytes exposed to the

FFA mixtures by incubating hepatocytes with FFA mixtures consisting of different proportions of OA and PA to induce fat-overloading and to achieve similar intracellular levels of lipid accumulation as in the human steatotic liver. Finally, it was found that the FFA mixture consisting of OA and PA in an OA/PA molar ratio of 2:1 is associated with minor toxic and apoptotic effects. Therefore, the FFA mixture consisting of OA and PA in an OA/PA molar ratio of 2:1 was used for the establishment of steatotic hepatocyte model that mimics benign chronic steatosis (Gomez-Lechon et al., 2007).

4.1.2.2 Pterostilbene reduced lipid accumulation in steatotic L02 cells

In the present study, pterostilbene 24-h treatment significantly reduces lipid accumulation in steatotic L02 cells. From the results of confocal microscopy, it can be observed that there was a reduction in the amount of neutral lipid droplets in pterstilbene treated steatotic L02 cells when compared with the model. In order to investigate the possible molecular mechansims of pterostilbene in reducing lipid accumulation in steatotic L02 cells, western blot analysis on proteins related to lipid synthesis, utilization and clearance was conducted.

ACC and FAS are the enzymes regulating the metabolism of fatty acids. ACC catalyzes the carboxylation of acetyl-CoA to produce malonyl-CoA, which is considered as the first committed step in fatty acid synthesis. Phosphorylation of ACC inactivates the enzyme. Therefore, enhancement of phosphorylation of ACC inhibits lipogenesis in the liver and helps reduce hepatic lipid accumulation. FAS catalyzes the synthesis of palmitate, which is considered as the last step in fatty acid synthesis. Downregulation of FAS expression also helps reduce hepatic lipogenesis and lipid accumulation. Results of western blot analysis showed that FFA exposure decreased the protein expression of p-ACC and increased that of FAS. Treatment with pterostilbene increased p-ACC protein expression level and downregulated the protein expression of FAS, suggesting that the lipid accumulation reduction effect of pterostilbene may involve the regulation of fatty acid synthesis.

HMGCR is enzyme that is responsible to convert HMG-CoA into mevalonate, which is the rate-limiting step in cholesterol synthesis. Downregulation of HMGCR inhibits cholesterol synthesis and helps improve hepatic steatosis. Results of western blot analysis showed that FFA exposure increased the protein expression of HMGCR.

Treatment with pterostilbene downregulated the protein expression of HMGCR, suggesting that the lipid accumulation reduction effect of pterostilbene may also involve the regulation of cholesterol synthesis.

AMPK is an enzyme that acts as a central regulator of multiple metabolic pathways. Phosphorylation at the threonine 172 of the α-subunit activates AMPK which promotes glycolysis as well as fatty acid oxidation and inhibits the synthesis of glycogen, fat and cholesterol. AMPK also plays a role in antioxidant response and anti-inflammatory response. Therefore, AMPK is considered as an attractive pharmacological target for NAFLD therapy. Previous study had demonstrated that resveratrol protected the liver from NAFLD by reducing lipid accumulation and alleviating insulin resistance via activation of AMPK (Shang et al., 2008). Therefore, the ability of pterostilbene, a natural dimethylated analogue of resveratrol, on AMPK activation was examined in this study. Results of western blot analysis showed that pterostilbene significantly increased the phosphorylation of AMPK in L02 cells in a dose-dependent manner, suggesting that AMPK activation may contribute to the mechanism by which pterostilbene can improve NAFLD.

PPAR-α is a nuclear receptor protein that belongs to PPARs family. It is mainly responsible to regulate lipid metabolism and modulate transportation and β-oxidation of fatty acids. According to the results of western blot analysis, the protein expression of PPAR-α was decreased in L02 cells after FFA exposure. Treatment with pterostilbene increased PPAR-α protein expression level in a dose-dependent manner. Upregulation of PPAR-α promotes fatty acid β-oxidation and inhibits lipogenesis. The result suggested that pterostilbene may help promote lipid utilization and inhibit lipid synthesis to reduce hepatic lipid accumulation via upregulation of PPAR-α.

Lipid droplets are regared as organelles for lipid storage. Excess FFAs were converted into neutral lipid and stored in lipid droplets in hepatocytes. The stored lipid in lipid droplets is consumed regularly. Lipid droplets undergo autophagy to achieve lipid droplet destruction and lipolysis for generation of energy. The process of lipid droplet autophagy is termed as lipophagy. Increasing evidences showed that impairment of autophagy in hepatocytes contributes to NAFLD. Therefore, the expressions of autophagy-related proteins were also evaluted in the present study. Results of western blot analysis showed that FFA exposure significantly reduced the

protein expression of LC3-II and increased that of p62 in L02 cells. LC3-II is a marker protein for autophagy. Ubiquitin-binding protein p62 is an autophagosome cargo protein that targets other proteins that bind to it for selective autophagy. Decrease in LC3-II protein expression level and increase in p62 protein expression level means that there should be lack of autophagy, indicating that autophagy may be impaired by FFA. Treatment with pterostilbene reversed the condition and upregulated LC3-II protein expression level as well as reduced p62 protein expression level in a dose-dependent manner, suggesting that the mechanism of pterostilbene in clearance of lipid droplets in hepatocytes may involve the promotion of autophagy or prevention of FFA-induced impairment of autophagy.

4.2 *In vivo* study

4.2.1 Establishment of steatotic *in vivo* model and experimental design

To verify the amelioration effect of pterosilbene on hepatic steatosis, *in vivo* study was conducted. In the present study, male SD rats were used as the experimental animal and high-fat rat chow was fed to induce hepatic steatosis.

After developing steatotic animal model by feeding high-fat rat chow for 4 weeks, all the animals were changed to be fed with normal rat chow. At the same time, drug adminstration was started and was lasted for 4 weeks. These changes mimic the therapeutic treatment of diet modification in combination with drug administration after diagnosis of NAFLD in patients.

4.2.2 Pterostilbene ameliorated high-fat diet-induced hepatic steatosis *in vivo*

In the present study, treatment with pterostilbene was found to ameliorate high-fat diet-induced hepatic steatosis *in vivo*. Results showed that pterostilbene reduced high-fat diet-induced increase in liver lipid content in a dose-dependent manner. Both the gross appearance and histopathological observation of the livers revealed that pterostilbene treatments alleviated hepatic steatosis as compared with the Model group. Pioglitazone treatments, the positive control, were also demonstrated to alleviate hepatic steatosis as compared with the Model group. However, body weight gain, one of the adverse side effects of pioglitazone, was observed in pioglitazone treatment groups according to the weight record.

The present study revealed that pterostilbene treatments reduced the serum TC and LDL-C levels. There were also reductions on the serum ALT levels in pterostilbene treatment groups, indicating that liver function was improved. These results suggested that treatment with pterostilbene may have beneficial effects on NAFLD.

Insulin resistance is considered as one of the characteristic features of NAFLD. It is developed along with NAFLD and is involved in the pathogenesis of NAFLD. Results of OGTT and HOMA-IR showed that pterostilbene treatments helped to attenuate insulin resistance in the steatotic animals. Elevated insulin sensitivity improves glucose metabolism and reduces hepatic *de novo* lipogenesis which helps ameliorate NAFLD.

To further explore and verify the underlying molecular mechanisms, the hepatic protein expressions associated with lipid metabolism, oxidative stress, and lipophagy were detected in this study.

For fatty acid synthesis related proteins, pterostilbene treatments downregulated the hepatic protein expression of FAS, which is consistent with the *in vitro* result,

indicating that treatment with pterostilbene may attenuate NAFLD by inhibiting hepatic fatty acid synthesis. Unlike the *in vitro* results, the hepatic protein expression of p-ACC was decreased in pterostilbene treatment groups, suggesting that pterostilbene may not exhibit inhibition effect on ACC *in vivo*.

For cholesterol synthesis related proteins, pterostilbene treatment downregulated the hepatic protein expression of SREBP-2 as well as HMGCR, indicating that treatment with pterostilbene may also inhibit hepatic cholesterol synthesis to attenuate NAFLD.

The hepatic protein expressions of MAPKs, including the ERKs, the JNKs, and the p38 MAPKs, were also detected. Various studies have demonstrated that the phosphorylated levels of MAPKs in liver were increased in high-fat diet-induce steatotic animals. The phosphorylation of MAPKs is associated with increased oxidative stress, inflammatory response, as well as apoptosis. In the present study, there were also elevations in the phosphorylated levels of the ERKs, the JNKs, and the p38 MAPKs in the liver of rats in the Model group. Treatment with pterostilbene

significantly reduced the phosphorylation of the ERKs, the JNKs, and the p38 MAPKs, which are consistent with the *in vitro* result of western blot analysis. These results suggested that pterostilbene may alleviate NAFLD by protecting the liver against oxidative stress and reducing the level of inflammation.

Consistent with the *in vitro* results of western blot analysis, the hepatic protein expression of HO-1 was upregulated in pterostilbene treatment group compared with the Model group. As discussed above, upregulation of HO-1 expression exhibit protection effect against cellular oxidative stress. Moreover, multiple studies recognized that HO-1 helps reduce inflammatory stress and endoplamic reticulum stress. These effects are considered beneficial to NAFLD.

AMPK is an enzyme that regulates cellular energy metabolism. Activation of AMPK modulates glucose and lipid metabolisms and is considered beneficial to NAFLD. In the present study, increase in the phosphorylation of AMPK was also found in the liver of rats in pterostilbene treatment groups. This is consistent with the *in vitro* result of western blot analysis that pterostilbene promotes activation of AMPK.

Apart from regulating cellular energy metabolism, AMPK was also found to be a positive regulator of autophagy in mammalian cells. Activation of AMPK inhibits mammalian target of rapamycin (mTOR), finally resulting in activation of autophagy (Mao et al., 2016). In the present study, phosphorylation of mTOR was found to be elevated in the liver of rats in the Model group. Treatment with pterostilbene reduced the phosphorylation of mTOR in the liver in a dose-dependent manner. Furthermore, LC3-II, a marker protein for autophagy, its hepatic protein expression was also upregulated by treatment with pterostilbene in a dose-dependent manner, which is consistent with the *in vitro* result of western blot analysis. These results suggested that pterostilbene may reduce lipid accumulation via activation of AMPK and inhibition of mTOR.

PPAR-α is a nuclear receptor protein that regulates lipid metabolism and modulate transportation as well as β-oxidation of fatty acids. Unlike the *in vitro* result of western blot analysis that FFA exposure to L02 cells decreased the protein expression of PPAR-α, high-fat diet was found to increase the hepatic protein expression of PPAR-α. Treatment with pterostilbene further enhanced the hepatic protein expression of

PPAR-α, suggesting that pterostilbene may improve lipid utilization and reduce lipid accumulation by promoting β-oxidation of fatty acids.

In the current study, the changes in the serum metabolites of lysophospholipids in various groups of animals were determined. Eight lysoPCs (C15:1, C16:1, C17:1, C18:1, C18:2, C20:2, C20:3 and C20:5) were found to be increased in the Model group compared with the Control group. LysoPCs are one of the major plasma lipid classes that are derived from hydrolysis of phosphatidylcholines by phospholipase A_1 or phospholipase A_2. They are the major phospholipid components of oxidized low-density lipoproteins. LysoPCs are recognized as cell signaling molecules involved in various cellular activities exerting pro-inflammatory effects and are believed to play an important role in atherosclerosis and inflammatory diseases (Lee et al., 2013; Matsumoto et al., 2007; Schmitz and Ruebsaamen, 2010). Recent evidence showed that elevated serum lysoPC levels were found in high-fat diet fed animal models (Hanhineva et al., 2013; Y.-T. Liu et al., 2014; Wu and Xiao, 2014). Therefore, lysoPCs may serve as a biomarker of dyslipidemia progression. A previous study investigating alterations of serum lysophospholipid levels in dyslipidemic

hamsters found that 15 day-high-fat diet feeding increased serum levels of lysoPC C18:0, lysoPC C18:2, lysoPC C18:3, lysoPC C20:1 and lysoPC C20:2, while 30-day-high-fat diet feeding increased serum levels of lysoPC C18:0, lysoPC C18:1, lysoPC C20:3 and lysoPC C20:4 (Suárez-García et al., 2017). Some of these lysoPCs were also found to be increased in the present study, indicating that high-fat diet feeding could lead to elevated serum levels of certain lysoPCs. Treatment with pterostilbene reduced the high-fat diet-induced elevated serum levels of lysoPCs, suggesting that pterostilbene may help ameliorate high-fat diet-induced alterations.

The community structure of gut microbiota is highly affected by its host environment (Burcelin, 2012; Nicholson et al., 2012). Diet is known as one of the crucial factors that influences the community structure of gut microbiota, and increasing evidence shows that the impacts of diet on the community structure of gut microbiota are associated with the pathogenesis of various metabolic diseases, such as obesity, diabetes mellitus and NAFLD (Federico et al., 2017; Power et al., 2014; Y. Sanz et al., 2010). Numerous studies have indicated that consumption of high-fat diet caused an increase in the proportion of *Firmicutes* and a decrease in the proportion of

Bacteroidetes (Bibbò et al., 2016; Ley et al., 2005; Mujico et al., 2013; Serino et al., 2012; M. Zhang and Yang, 2016). In the present study, the proportion of *Bacteroidetes* was found to be decreased in the Model group compared with the Control group, which was consistent with the results of previous studies. However, the proportion of *Firmicutes* was found to be decreased in the Model group compared with the Control group. A previous study determining the effects of piceatannol on the composition of gut microbiota in high-fat diet-fed mice revealed that the proportion of *Firmicutes* was decreased in the high-fat diet-fed group (Tung et al., 2016). Similar results were also observed in a study investigating the changes in gut microbiota in rats fed with a high-fat diet, with increased abundances of *Firmicutes* as well as *Proteobacteria* in the high-fat diet-fed rats (Lecomte et al., 2015). Another study investigating the effect of high-fat diet on the composition of gut microbiota showed an increase in the proportion of *Proteobacteria* in the high-fat diet-fed group (Hildebrandt et al., 2009). In the present study, the proportion of *Proteobacteria* was also found to be increased in the Model group compared with the Control group. Treatment with pterostilbene was demonstrated to reverse the proportions of *Bacteroidetes*, *Firmicutes* and

Proteobacteria compared with the Model group. Apart from the changes of the composition of gut microbiota at the phylum level, treatment with pterostilbene was demonstrated to restore the proportions of gut microbiota at family level compared with the Model group, suggesting that pterostilbene may help recover the alterations of gut microbiota structure induced by high-fat diet.

4.3 Conclusion

In summary (Figure 4.3.2), treatment with pterostilbene was demonstrated to improve liver gross appearance and histology, reduced liver lipid content, decreased the serum TC, LDL-C and ALT levels in high-fat diet-induced steatotic rat model. Besides, treatment with pterostilbene alleviated insulin resistance. The beneficial effects of pterostilbene on NAFLD may be associated with amelioration of oxidative stress, reduction of hepatic lipid synthesis, and promotion of lipid utilization as well as lipophagy (Figure 4.3.1). Apart from that, treatment with pterostilbene was found to restore the high-fat diet-induced changes in the serum metabolites of lysophospholipids as well as the high-fat diet-induced alterations of gut microbiota structure to a certain extent. These may also provide benefits to NAFLD. Further studies are required to determine the potential of pterostilbene as therapeutic agents for NAFLD in clinical settings. The mechanisms of pterostilbene in the treatment of NAFLD should also be further investigated to facilitate clinical application of pterostilbene.

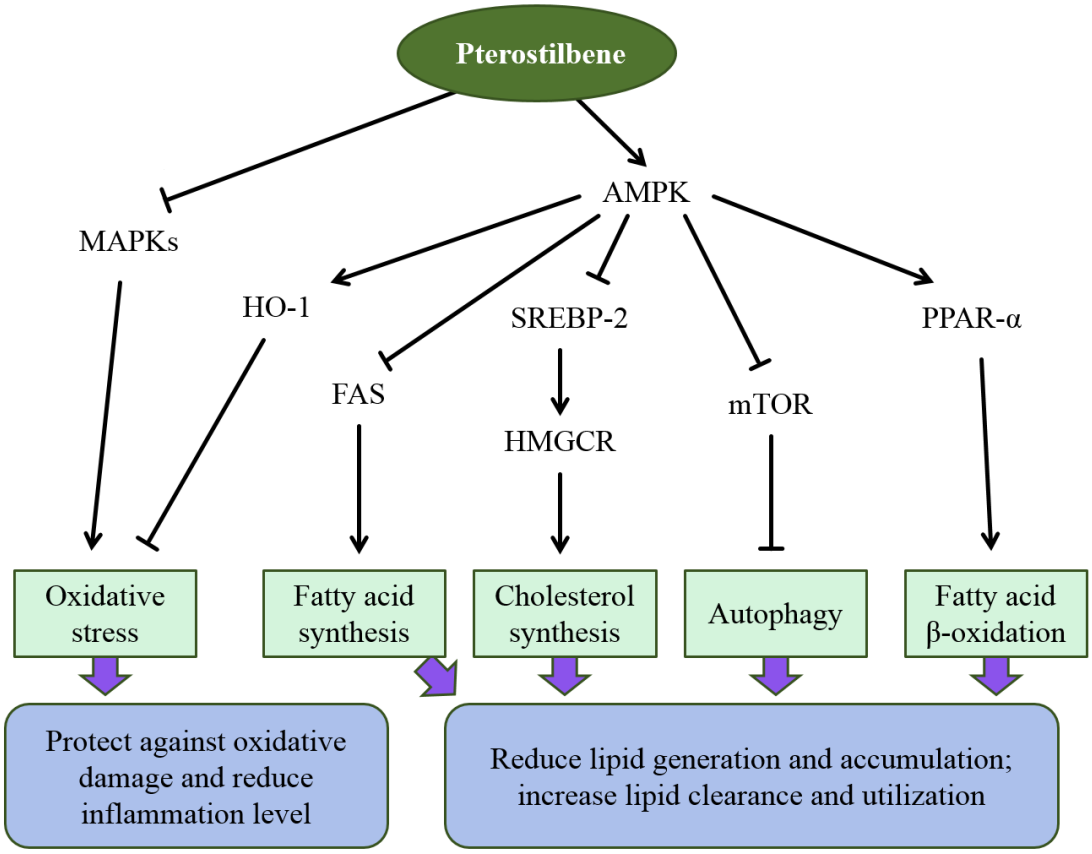

Figure 4.3.1 Schematic illustration of the proposed molecular pathways of pterostilbene involved in the protection against oxidative stress and the regulation of lipid metabolism in NAFLD.

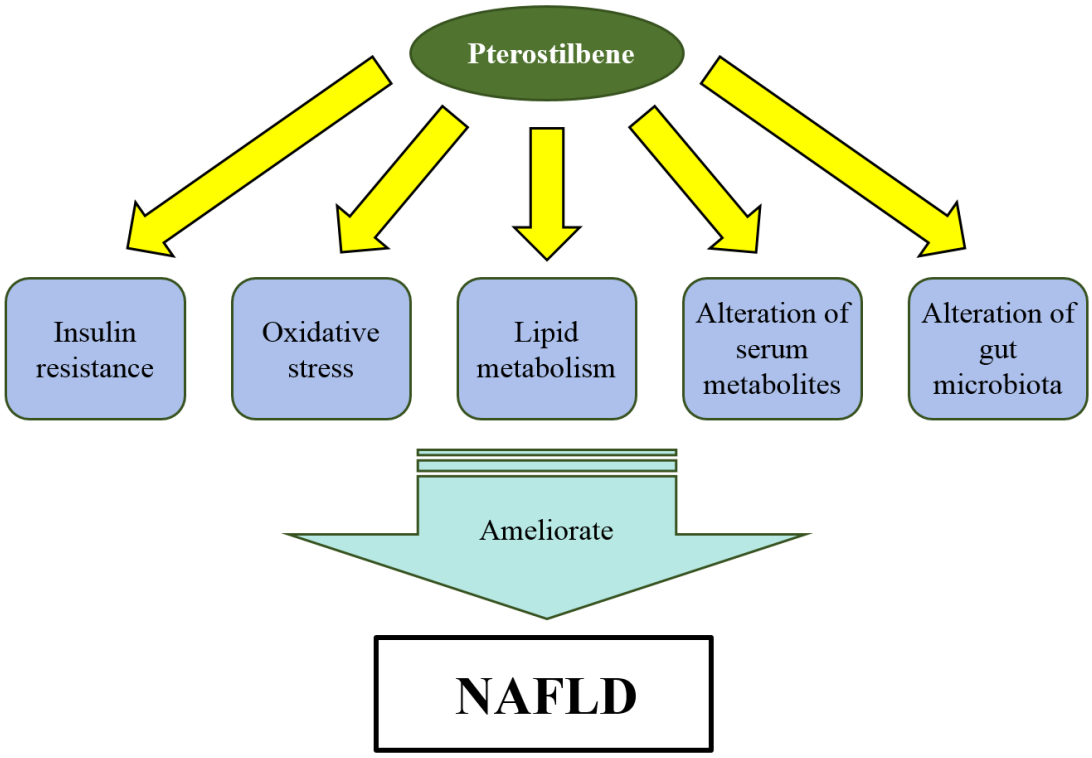

Figure 4.3.2 Summary of the potential mechanisms of pterostilbene in ameliorating NAFLD.

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