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UNTARGETED METABOLOMICS ANALYSIS OF THE INTERVENTION EFFECTS OF *PARKIA SPECIOSA* POD EXTRACT (PSPE) ON 3T3-L1 ADIPOCYTES DIFFERENTIATION

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MSc

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CONFIRMATION BY PANEL OF EXAMINERS

I certify that a Panel of Examiners has met on 23 December 2025 to conduct the final examination of Anas bin Abdullah on his Masters of Health Sciences (Medical Laboratory Technology) thesis entitled "Untargeted Metabolomics Analysis on The Intervention Effects of *Parkia speciosa* Pod Extract (PSPE) on 3T3-L1 Adipocytes Differentiation" in accordance with Universiti Teknologi MARA Act 1976 (Akta 173). The Panel of Examiners recommends that the student be awarded the relevant degree. The Panel of Examiners was as follows:

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I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Teknologi MARA. It is original and is the results of my own work, unless otherwise indicated or acknowledged as referenced work. This thesis has not been submitted to any other academic institution or non-academic institution for any degree or qualification.

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ABSTRACT

Obesity and hyperlipidemia have emerged as significant global health challenges, driving the prevalence of chronic metabolic disorders such as type 2 diabetes, cardiovascular diseases, and hypertension. These pathologies were directly contributed by the excessive accumulation of lipids in adipose tissue, primarily regulated by adipogenesis. While conventional lipid-lowering pharmacotherapies, including statins and fibrates, are widely prescribed, their long-term use is often accompanied by undesirable side effects, prompting the search for safer, plant-based alternatives. *Parkia speciosa* pods, typically discarded as waste, have emerged as a potential natural source of bioactive compounds with anti-adipogenic and lipid-lowering effects. However, the scientific understanding of the underlying mechanisms by which *P. speciosa* pod modulates adipogenesis remains limited. Therefore, this study aimed to elucidate the mechanism of anti-adipogenic activity of *Parkia speciosa* pod extract (PSPE) on 3T3-L1 adipocyte differentiation via metabolomics approach. The extract was prepared, and phytochemical analysis of PSPE including gallic acid and p-coumaric acid was analysed and quantified using HPLC. The total phenolic content (TPC) and total flavonoid content (TFC) were determined, and antioxidant activities were assessed using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. Following that, 3T3-L1 preadipocytes were induced to differentiate and treated with PSPE (62.5 µg/mL), along with simvastatin (4.19 µg/ml) as a reference drug. Lipid accumulation was evaluated through oil red O staining and triglyceride assays, while metabolomic analysis was performed via LC-QTOF-MS. HPLC analysis revealed the presence of gallic acid (53.97 ± 0.76 µg/mL) and p-coumaric acid (1.74 ± 0.11 µg/mL) in PSPE. The IC_{50} values for DPPH scavenging were 57.05 ± 0.22 µg/mL, and the highest FRAP value was determined to be 325.3 ± 4.85 µg FeSO4/mL. *In vitro* studies demonstrated that PSPE treatment significantly reduced lipid droplet formation and triglyceride levels in a dose-dependent manner ($p < 0.05$). Metabolomics analysis further revealed that PSPE intervention altered several metabolites associated with adipogenesis, including metabolites in lipid metabolism (linoleic acid, gamma-linolenic acid, and arachidonic acid), amino acid metabolism (tryptophan and phenylalanine), carbohydrate metabolism (glyoxylate and dicarboxylate pathway), nucleotide metabolism (pantothenate and CoA biosynthesis), and cofactor and vitamin metabolism (biotin and riboflavin metabolism). These metabolite alterations suggest that PSPE inhibits adipogenesis by regulating lipid signalling, enhancing fatty acid oxidation, modulating redox balance, and suppressing key adipogenic transcription factors. In conclusion, PSPE exhibits significant anti-adipogenic and lipid-lowering properties by modulating multiple metabolic pathways involved in adipocyte differentiation. These findings provide valuable mechanistic insights into the potential of *Parkia speciosa* pod extract as a natural therapeutic agent for the prevention and management of obesity and related metabolic disorders.

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Abbreviations

9,10-EpOME	9,10-Epoxyoctadecenoic Acid
AA	Arachidonic Acid
AhR	Aryl Hydrocarbon Receptor
AMPK	AMP-Activated Protein Kinase
AUC	Area Under The Curve
AUROC	Area Under Receiving Operator Characteristic
BAT	Brown Adipose Tissue
BCAA	Branched-Chain Amino Acids
C/EBPs	CCAAT/Enhancer-Binding Proteins
CE	Capillary Electrophoresis
CVD	Cardiovascular Disease
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
FAS	Fatty Acid Synthase
FFAs	Free Fatty Acids
FRAP	Ferric Reducing Antioxidant Power
GC-MS	Gas Chromatography-Mass Spectrometry
GLA	Gamma-Linolenic Acid

HMG-CoA	Hydroxymethylglutaryl-Coa
HPLC	High-Performance Liquid Chromatography
HPLC-MS/MS	High-Performance Liquid Chromatography Coupled With Tandem Mass Spectrometry
HRMS	High-Resolution Mass Spectrometry
IAA	Indoleacetic Acid
IBMX	3-Isobutyl-1-Methylxanthine
LC-MS	Liquid Chromatography-Mass Spectrometry
MCE	Mitotic Clonal Expansion
MetPA	Metabolomics Pathway Analysis
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium
NMR	Nuclear Magnetic Resonance
ORO	Oil Red O
PBS	Phosphate-Buffered Saline
PCA	Principal Component Analysis
PLS-DA	Partial Least Squares Discriminant Analysis
PPARγ	Peroxisome Proliferator-Activated Receptor Γ
PSPE	*Parkia speciosa* Pod Extract
QC	Quality Control
QTOF	Quadrupole Time-Of-Flight
ROC	Receiver Operating Characteristic
ROS	Reactive Oxygen Species

SEM	Standard Error Mean
SREBP	Sterol Regulatory Element-Binding Protein
T2DM	Type 2 Diabetes Mellitus
TCA	Tricarboxylic Acid
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
UCP1	Uncoupling Protein-1
VIP	Variable Importance In Projection
WAT	White Adipose Tissue
WHO	World Health Organization

CHAPTER 1
INTRODUCTION

1.1 Research Background

Obesity represents a multifaceted chronic medical condition that has been recognised as a serious global public health issue. Its prevalence has steadily risen, affecting individuals of all ages, including adults, children, and adolescents. This condition is strongly associated with an elevated risk of several serious chronic diseases, including type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), hypertension, and, in some circumstances, cancer (Alfaris et al., 2023). As obesity rates continue to rise, experts are attempting to understand its origins and find effective ways to manage it. Lifestyle changes, particularly increasing physical activity and reducing calorie intake, are the main strategies recommended for preventing and treating obesity. While many studies have shown that these approaches help with weight management and overall health (Jakicic et al., 2019; Kerkadi et al., 2019), obesity cases remain on the rise. Recent studies have highlighted the growing number of overweight and obese individuals in Malaysia. According to the 2019 National Health and Morbidity Survey (NHMS), 50.1% of Malaysian adults fall into these categories (Chong et al., 2023; Tat et al., 2023). By 2023, the figure had risen to 54.4%, indicating a continuous upward trend. If this pattern persists, this country may face a serious public health crisis with wide ranging effects including economic and societal consequences (Koliaki et al., 2023), as the rising number of obesity-related diseases will increase the demand for hospitalizations, medications, and long-term medical treatments, putting a heavy financial strain on the healthcare system all at once.

According to the World Health Organization (WHO), obesity is defined as an excessive accumulation of body fat that poses serious health risks and plays a key role in the development of metabolic disorders, including hyperlipidemia (Dhalla et al., 2021; Štempeľová et al., 2023). Hyperlipidemia is a well-known risk factor for cardiovascular disease. It describes increased blood lipid levels, specifically triglycerides and cholesterol, with both environmental variables and genetic predisposition affecting their development (Abbasi et al., 2024). Yanai and Yoshida (2021) claim that the distinction between primary (genetic) and secondary (acquired)

types of hyperlipidemias reflects the complexity of the condition. Primary hyperlipidemia is often marked by specific metabolite profiles associated with impaired lipoprotein processing (Alwahsh et al., 2024). In contrast, secondary hyperlipidemia, which is commonly related to obesity and diabetes, is characterised by disruptions in fatty acid metabolism, including altered polyunsaturated fatty acid metabolism. Metabolomics studies have identified significant metabolic changes associated with obesity and hyperlipidemia, particularly in lipid, amino acid, and carbohydrate metabolism (Rangel-Huerta et al., 2019; Abdullah et al., 2024; Alwahsh et al., 2024). Several metabolites have been identified as potential biomarkers for the early diagnosis and risk assessment of hyperlipidemia, such as indole-3-propionic acid, glycocholic acid, and docosahexaenoic acid (Du et al., 2020; Alwahsh et al., 2024). These insights highlight the complex relationship between obesity, hyperlipidemia, and metabolic changes, emphasising the need for early detection and targeted interventions to reduce associated health risks. Given the central role of lipid and fatty acid dysregulation in these conditions, there is a growing need for analytical approaches that can provide a detailed and integrative view of metabolic alterations at the molecular level. In this regard, metabolomics offers a particularly powerful platform for uncovering biochemical signatures, elucidating disease mechanisms, and guiding the development of precision-based therapeutic strategies (Wang et al., 2020; Rakusanova & Cajka, 2024).

Metabolomics can be defined as an analytical tool used to analyse metabolic pathways in biological systems by identifying changes in small metabolites caused by external stimuli or gene mutations. It provides a new perspective on cellular and tissue biochemical processes (Wang et al., 2019). Unlike other "omics" parameters, metabolites and their concentrations directly reflect the underlying biochemical status of cells and tissues. Despite being a relatively new omics technology, metabolomics has been widely used in various life science fields, resulting in significant advances in biomarker discovery, novel metabolite identification, and precise characterisation of metabolic processes in various organisms (Aderemi et al., 2021). Metabolomics has emerged as a powerful tool in biomedical research, particularly for studying complex diseases like diabetes, cardiovascular diseases, and cancer. This approach enables the identification of disease biomarkers, elucidation of disease mechanisms, and evaluation of therapeutic interventions (Jacob et al., 2019). For instance, in diabetes research, metabolomics has been used for early disease detection and monitoring treatment

effectiveness (Adamski, 2016). More recently, the application of metabolomics in adipogenesis studies has also gained momentum, as previous research efforts by Airaksinen et al. (2018) and Candi et al. (2018) have demonstrated its potential in identifying metabolite changes associated with specific interventions during adipocyte differentiation. Miehle et al. (2020) also reported that as preadipocytes differentiate into mature adipocytes during adipogenesis, there is a substantial increase in the synthesis of various lipid classes, including triacylglycerols, diacylglycerols, phosphatidylcholines, and sphingomyelins.

Adipogenesis is the transformation of preadipocyte cells into fully developed adipocytes, also known as lipid cells. This process is a crucial and tightly controlled process that plays a key role in maintaining energy balance and regulating metabolism (Lee et al., 2019). The process involves two stages, which are commitment of mesenchymal stem cells to the preadipocyte lineage and then followed by terminal differentiation into functional adipocytes (Ambele et al., 2020). The differentiation process is influenced by a complex interaction of specific proteins that regulate gene expression. Notably, the peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs) are crucial in controlling the development of cells (Tolomelo & Grimaudo, 2020; Wu et al., 2020). When adipogenesis is disrupted, the ability of adipose tissue to effectively store excess fat decreases, which can lead to abnormal fat accumulation and metabolic complications (Hammarstedt et al., 2018). This dysfunction results in an accumulation of circulating free fatty acids that are deposited in non-adipose tissues such as the liver, muscle, and pancreas, contributing to lipotoxicity and metabolic dysregulation. Such abnormalities are closely related to hyperlipidemia, a condition characterised by elevated triglyceride and cholesterol levels, which will then lead to an increased risk of cardiovascular disease and metabolic syndrome. There are several pharmacological treatments available to manage hyperlipidemia caused by adipogenesis abnormalities (Abbasi et al., 2024). For example, as mentioned by Gesto et al. (2020), medications like statins, such as simvastatin, atorvastatin, and rosuvastatin, are a group of drugs that can successfully reduce cholesterol levels by blocking the activity of Hydroxymethylglutaryl-CoA (HMG-CoA) reductase, an essential enzyme in the production of cholesterol. Meanwhile, fibrates such as fenofibrate and gemfibrozil act as PPAR-α activators that effectively lower triglycerides and increase HDL cholesterol (Fernandes et al., 2021). Despite their widespread use for treatment purposes, these drugs have been linked to

various adverse effects like insomnia, muscle weakness, and liver issues. Additionally, they may also cause kidney problems, thyroid dysfunction, difficulty concentrating, and headaches, as noted in studies by Azemi et al. (2022) and Essa et al. (2025).

The limitations and potential adverse effects of conventional lipid-lowering drugs have stimulated increasing scientific interest in herbal formulations enriched with plant-derived bioactive compounds as alternative therapeutic strategies. Phytochemicals present in these formulations are particularly attractive because of their favourable safety profile, cost-effectiveness, and broad spectrum of biological activities (Mopuri & Islam, 2017). Recent research suggests that natural compounds from plants may offer an alternative to statins and fibrates. While these medications are known for their ability to reduce cholesterol levels effectively, they are also associated with side effects like muscle discomfort, liver issues, and an elevated chance of developing diabetes (Hajela, 2024). As mentioned by Saad et al. (2021), dietary phytochemicals have demonstrated promising anti-hyperlipidemic properties by targeting multiple stages of adipocyte development. These natural compounds can help inhibit the development of preadipocytes before they mature and also trigger the breakdown of lipids while causing the death of fully grown lipid cells, collectively leading to a decrease in adipose tissue mass. Among the various classes of phytochemicals, flavonoids such as quercetin and kaempferol have attracted significant attention for their lipid-lowering effects. Several plants have been identified as rich sources of anti-adipogenic phytochemicals, including Sargassum muticum (Lee et al., 2017), Caulerpa okamurae (Sharma et al., 2017), Dendropanax morbifera (Song et al., 2018), and Catharanthus roseus (Borah et al., 2019). Those compounds have been found to inhibit cholesterol synthesis while enhancing lipid metabolism and exerting anti-inflammatory effects (Al-Snafi, 2022). Along with flavonoids, phenolic acids like gallic acid and p-coumaric acid have shown promise in ameliorating metabolic disorders and dyslipidemia. For example, gallic acid has been proven to have effects in reducing lipid levels and protecting the liver of high-fat diet-induced metabolic syndrome in rats (Guo et al., 2017). Similarly, it has been discovered that p-coumaric acid can reduce lipid levels by increasing hepatic lipolysis and fatty acid oxidation through the activation of PPARα and PPARγ (Yuan et al., 2023). All of these findings highlight the potential of plant-based therapies in regulating lipid metabolism and addressing hyperlipidemia with minimal side effects.

In 2019, Azizul et al. (2019) proposed that the seed and pod of P. speciosa, commonly known as the stink bean, may be beneficial for addressing chronic health conditions such as inflammation, diabetes, atherosclerosis, and cancer. Other research studies conducted by Kamisah et al. (2017), Ghasemzadeh et al. (2018), Mustafa et al. (2018), Sonia et al. (2018), Gui et al. (2019), Fitrya et al. (2020), and Singhania et al. (2021) have also provided supportive evidence for the therapeutic properties of *P. speciosa*. However, despite the abundance of studies available on the subject matter, there is a lack of concrete scientific proof on the anti-adipogenic and anti-obesity effects of *P. speciosa* pod, as well as the underlying mechanisms of adipogenesis. The bioactive compounds in *P. speciosa* pods, such as polyphenols, have been reported to exhibit antioxidant, anti-inflammatory, and lipid-lowering properties (Chhikara et al., 2018). Considering that adipogenesis is a tightly regulated process controlled by factors like oxidative stress and inflammatory signals, these natural components could potentially impact important pathways involved in adipocyte differentiation and metabolism. Therefore, this study aims to investigate how *P. speciosa* pod extract (PSPE) may inhibit the buildup of lipids during adipogenesis and elucidate its effects on relevant metabolic pathways. A comprehensive understanding of the molecular mechanisms driving the promising effects of PSPE could position it as a potentially innovative functional product in the fight against obesity. While metabolomics has been extensively utilised in adipogenesis studies, there is an absence of investigations focused on the utilisation of *P. speciosa* species, particularly its pods. Thus, this study aims to unveil the anti-adipogenic activity of *P. speciosa* pod extract (PSPE) on 3T3-L1 adipocyte differentiation by profiling metabolites and metabolic pathways affected by PSPE intervention.

1.2 Problem Statement

Adipogenesis, a process by which preadipocytes differentiate into mature adipocytes, plays a critical role in lipid metabolism, and its dysregulation is closely associated with obesity and metabolic syndrome. The accumulation of excessive lipids in adipose tissue due to impaired adipogenesis can lead to systemic metabolic disturbances, contributing to hyperlipidemia. Hyperlipidemia has become a prevalent health concern in contemporary society, prompting heightened public awareness of its detrimental consequences and creating an expansive commercial market for anti-

hyperlipidemia treatments. However, therapeutic approaches involving synthetic medications can be costly and are often associated with significant side effects. Currently, conventional lipid-lowering medications, including fibrates, statins, and bile acid sequestrants, are employed for hyperlipidemia treatment, despite their known adverse effects (Azemi et al., 2022; Essa et al., 2025). For instance, although statins are generally well tolerated and widely prescribed, they have been associated with adverse effects, most commonly muscle-related symptoms, and less frequently hepatic or renal abnormalities (Wiggins et al., 2022). In addition, these treatments primarily target circulating lipid levels and may not fully address underlying metabolic disturbances such as adipocyte dysfunction. Collectively, these considerations have encouraged ongoing exploration of complementary strategies, including plant-derived bioactive compounds, as potential adjunct approaches for lipid metabolic regulation.

Among various natural alternatives, *Parkia speciosa* has garnered interest due to its reported medicinal properties, including anti-inflammatory, antioxidant, and antidiabetic activities (Kamisah et al., 2017; Mustafa et al., 2018; Sonia et al., 2018; Gui et al., 2019; Fitrya et al., 2020). However, studies elucidating the anti-hyperlipidemia activities of *P. speciosa* remains limited (Gao et al., 2021; Singhania et al., 2021). In addition, despite the extensive application of metabolomics in obesity-related research, there remains a significant gap in the investigation of *P. speciosa* pod and its role in modulating adipogenesis at the metabolic level. Thus, this study may help to bridge the current knowledge gap by employing metabolomic analysis to investigate the effects of PSPE on 3T3-L1 adipocyte differentiation.

1.3 Research Objectives

1.3.1 General Objective

To elucidate the mechanism(s) of anti-adipogenic activity of *Parkia speciosa* pod extract (PSPE) on 3T3-L1 adipocytes differentiation via metabolomics approach.

1.3.2 Specific Objectives

a) To assess the phytochemical contents and antioxidant activity of *Parkia speciosa* pod extract.

b) To evaluate the inhibitory effects of *Parkia speciosa* pod extract against adipogenesis in 3T3-L1 adipocytes.
c) To identify metabolite alterations, potential biomarkers, and key metabolic pathways associated with the effects of *Parkia speciosa* pod extract on 3T3-L1 adipocytes differentiation.

1.4 Scope of The Study

This *in vitro* research aimed to understand the metabolomic changes resulting from the suppression of 3T3-L1 cells differentiation when treated with *P. speciosa* pod extract (PSPE). The experiment was conducted using 3T3-L1 preadipocytes, a cell line derived from mouse 3T3 cells that are extensively utilised in adipose tissue research. This study involved observing changes in the matured adipocytes through oil red O staining and followed by an assessment of lipid pool size by measuring triglyceride content. A spectrophotometry-based metabolomics analysis using high-performance liquid chromatography coupled with tandem mass spectrometry (LC-QTOF-MS) was utilised to profile and evaluate the changes in metabolites and related metabolic pathways resulting from the differentiated 3T3-L1 cells after the treatment with PSPE.

1.5 Significance of Study

The outcomes of this project will contribute to a better understanding of the anti-hyperlipid effect of PSPE. This revelation will open the path for future research into developing new anti-lipemic agents based on natural resources. Most importantly, the findings of this investigation will help to establish the scientific evidence for the antioxidant and anti-adipogenic properties of *P. speciosa* pod. The results of this investigation will reveal changes in metabolites and their related metabolic pathways in adipocytes treated with PSPE. Thus, the information gathered will be used to guide future studies on the anti-adipogenesis properties of *P. speciosa* pod. Moreover, by extending the use of PSPE, it would help to create a local natural based anti-adipogenic product while at the same time providing new avenues for Malaysia's economic development.

CHAPTER 2
LITERATURE REVIEW

2.1 Obesity and Hyperlipidemia

2.1.1 Epidemiology of Obesity

Obesity has become one of the most serious and widespread public health threats of the 21st century, posing a significant challenge for both developed and developing countries (Balwan & Saba, 2025). The World Health Organization (WHO) defines obesity as the abnormal or excessive accumulation of fat that poses a risk to health, typically determined using the body mass index (BMI) as a standard metric (Rutter, 2018; Salam et al., 2023). The global prevalence of obesity has increased dramatically over the past few decades, with approximately 2 billion people currently overweight and one-third of them obese (Salam et al., 2023). Alarmingly, these figures continue to rise, driven by complex interactions between economic growth, urbanization, sedentary lifestyles, and dietary changes (Holt, 2019). The global prevalence of obesity is projected to increase significantly by 2030, with estimates suggesting that 38% of adults will be overweight and 20% obese (Ampofo & Boateng, 2020). This alarming trend has serious implications for the global burden of non-communicable diseases (NCDs), as obesity is strongly associated with type 2 diabetes, cardiovascular disease, hypertension, and certain cancers (Alfaris et al., 2023). In addition to its physical health impact, obesity also contributes to psychosocial distress (Kumari & Chopra, 2024), reduced quality of life (Jaison et al., 2024), and increased healthcare costs (Schneider et al. 2020), particularly in countries with limited public health infrastructure.

In Southeast Asia, the obesity epidemic is particularly remarkable in Malaysia, which now ranks among the highest in obesity prevalence within the region. The national prevalence of obesity in Malaysia increased by 280% between 1996 and 2007, reaching 11.7% among those aged 15 and above (Rampal et al., 2007). Recent studies highlight the growing prevalence of overweight and obesity among Malaysian adults. The National Health and Morbidity Survey (NHMS) 2019 revealed that 50.1% of adults were overweight or obese (Chong et al., 2023), with obesity alone affecting 20.1% of the population (Chong et al., 2022). This rising trend underscores significant shifts in

dietary patterns, characterised by increased consumption of processed foods, sugar-sweetened beverages, and high-fat diets, alongside reduced physical activity and a more sedentary lifestyle, particularly in urban populations (Popkin et al., 2012). What is even more concerning is that obesity is no longer confined to adults. The prevalence among Malaysian children and adolescents has also increased, pointing toward a generational shift in metabolic health risk (Mohamed et al., 2022). According to NHMS 2019, 14.8% of children aged 5 to 17 were classified as obese, a number that is expected to increase if preventive measures are not implemented at an early age (Lai et al., 2024). This growing burden places a tremendous strain on the healthcare system of Malaysia, both in terms of direct medical costs associated with obesity-related diseases (Wulandari & Kristina, 2018) and indirect costs resulting from reduced productivity and increased disability rates (Yusefzadeh et al., 2019).

Epidemiologically, the rise of obesity in Malaysia reflects not just changes in lifestyle but also cultural, economic, and infrastructural shifts that have altered the national health condition. Rapid urbanization has reduced opportunities for physical activity while promoting car-dependent commuting, extended screen time, and reliance on fast food, especially among the younger population (Lee et al., 2020). Moreover, lower-income communities frequently face food insecurity and have limited access to healthy food, further exacerbating the risk (Vivian et al., 2014). Public health campaigns, such as the Malaysian Healthy Plate (Suku-Suku Separuh), and government initiatives to curb sugar intake through taxation policies are steps in the right direction, but have yet to yield significant behavioural change at the population level (Sritharan et al., 2024). At the molecular level, obesity represents more than a simple imbalance between energy intake and expenditure. It is increasingly recognised as a complex metabolic disease involving inflammation, oxidative stress, hormonal imbalances, and adipose tissue dysfunction (Marseglia et al., 2014; Manna & Jain, 2015). This growing scientific understanding has sparked interest in investigating the cellular and biochemical mechanisms that drive adipose tissue expansion, particularly adipocyte differentiation, which directly contributes to fat mass accumulation.

2.1.2 Etiology of Obesity

The etiology of obesity is complex and multifactorial, shaped by an intricate interplay between genetic, physiological, behavioral, socioeconomic, and

environmental determinants (Spahić, 2020). Although excess caloric intake and physical inactivity are often regarded as the primary drivers of weight gain, obesity cannot be attributed solely to a simple energy imbalance (Vettor & Conci, 2019). Instead, it represents a multifaceted metabolic disorder influenced by both exogenous factors, such as diet composition, sleep patterns, and sedentary behaviour (McHill & Wright, 2017), as well as endogenous mechanisms, including hormonal regulation, adipocyte biology, inflammation, and genetic predisposition (Vettor & Conci, 2019; Bhattacharya et al., 2020). At the core of energy homeostasis lies the hypothalamic-pituitary axis, which integrates peripheral signals, such as leptin, ghrelin, insulin, and adiponectin, to modulate hunger, satiety, and metabolic rate (Valjevac, 2020). Dysregulation in this signalling network, whether through genetic mutations, chronic stress, inflammation, or nutrient overload, can lead to sustained hyperphagia, reduced energy expenditure, and impaired lipid and glucose metabolism, all of which contribute to adipose tissue expansion (Xiao et al., 2020). Moreover, emerging evidence suggests that early-life exposures, such as maternal obesity, infant feeding practices, and gut microbiota composition, may epigenetically influence adiposity later in life, indicating that obesity risk begins long before adulthood (Richmond et al., 2015; Li, 2018).

From a lifestyle perspective, the global rise in obesity closely tracks changes in dietary patterns, characterised by increased consumption of ultra-processed, energy-dense foods high in refined sugars, saturated fats, and sodium (Popkin et al., 2012; Zobel et al., 2016). These dietary shifts, combined with reductions in physical activity resulting from increasingly sedentary jobs, urban transportation systems, and screen-based leisure activities, have created an obesogenic environment that promotes a positive energy balance and fat accumulation (Hill, 2013; Economos et al., 2015). Compounding this are socioeconomic disparities that influence food choices and access to healthy lifestyles. In lower-income communities, including some segments of the Malaysian population, affordable and convenient food options are often calorie-rich but nutrient-poor, while opportunities for exercise are limited due to infrastructure and safety concerns (Azizan et al., 2018; Eng et al., 2022). Psychological factors, such as stress (Demori & Elena, 2016), sleep deprivation (Marks et al., 2016; Konttinen, 2020), and emotional eating (Konttinen, 2020), also play significant roles in weight gain by disrupting the hormonal regulation of appetite and metabolic control. Furthermore, pharmacological agents such as antipsychotics and corticosteroids (Anekwe et al., 2024) as well as endocrine disorders, including hypothyroidism, Cushing's syndrome,

and certain viral infections (Pomahacova, 2023) have been implicated as secondary contributors to obesity, although these cases are less common.

At the cellular level, obesity is fundamentally a disorder of adipose tissue expansion, where the body stores excess energy in the form of lipids within adipocytes. Adipose tissue grows either through hypertrophy (increased cell size) or hyperplasia (increased cell number via adipogenesis) (Rutkowski et al., 2015; Haczeyni et al., 2018), both of which are influenced by metabolic signals, oxidative stress, and hormonal cues, which leads to adipose tissue dysfunction. This dysfunctional state triggers the release of pro-inflammatory cytokines, such as TNF-α and IL-6, and increases the production of reactive oxygen species (ROS) (McArdle et al., 2013; Ahmed, 2021). This collective impairment of insulin signalling promotes a chronic, low-grade inflammatory state (Tam & Redman, 2013). These processes not only contribute to insulin resistance and type 2 diabetes but also reinforce further lipid accumulation and adipose tissue remodelling. According to Manna and Jain (2015) and Yang et al. (2024), understanding the root causes of obesity is crucial not only for prevention but also for developing effective therapeutic strategies that modulate adipose tissue metabolism. One of the earliest and most prominent metabolic disturbances observed in obesity is hyperlipidemia**,** a condition marked by elevated levels of triglycerides, free fatty acids, and cholesterol in circulation (Curley et al., 2020). These lipid abnormalities both reflect and reinforce adipose tissue dysfunction, establishing a feedback loop that accelerates metabolic deterioration.

2.1.3 Hyperlipidemia

One of the most critical metabolic disturbances closely associated with obesity is hyperlipidemia. It is a pathological condition characterised by elevated levels of circulating lipids, including triglycerides, total cholesterol, low-density lipoprotein cholesterol (LDL-C), and reduced levels of high-density lipoprotein cholesterol (HDL-C) (Vekić et al., 2019; Su et al., 2021). As adipose tissue expands in the setting of chronic energy surplus, particularly through hypertrophic growth, its lipid-handling capacity becomes compromised (Haczeyni et al., 2018). This dysfunction leads to an overspill of free fatty acids (FFAs) into the circulation, overwhelming peripheral tissues such as the liver and skeletal muscle, and initiating a cascade of lipid metabolic abnormalities (Tumova et al., 2016). Elevated FFAs stimulate hepatic very low-density

lipoprotein (VLDL) secretion and *de novo* lipogenesis, exacerbating triglyceride accumulation and fostering the development of atherogenic dyslipidemia (Heeren & Scheja, 2021). This lipid overload is further exacerbated by impaired lipoprotein lipase (LPL) activity and insulin resistance, both of which contribute to inefficient lipid clearance and abnormal lipid partitioning (Mthembu et al., 2022). Over time, this persistent state of dysregulated lipid metabolism promotes ectopic fat deposition in non-adipose tissues and fuels systemic inflammation, oxidative stress, and endothelial dysfunction, all of which are key contributors to the pathogenesis of type 2 diabetes, cardiovascular disease, and non-alcoholic fatty liver disease (Montgomery et al., 2019).

Notably, lipid imbalance is not only a consequence of obesity but also a contributor to its pathogenesis, as lipid metabolites act as bioactive signalling molecules that modulate cellular stress, inflammation, and metabolic function (Devito et al., 2022). Clinically, the burden of hyperlipidemia is substantial, particularly in populations with rising obesity rates. In Malaysia, for instance, hyperlipidemia is highly prevalent among adults and has been linked to increased cardiovascular risk and premature mortality (Rifin et al., 2018; Mohamed-Yassin et al., 2021; Chan et al., 2021). Rifin et al. (2018) and Mohamed-Yassin et al. (2023) reported that 38.1% to 53% of adults had elevated total cholesterol levels, reflecting the ongoing nutritional transition of the country, marked by increased intake of saturated fats, sugars, and ultra-processed foods.

According to Li and Spalding (2022), the presence of hyperlipidemia corresponds with notable structural and functional alterations in adipocytes. Excess saturated fatty acids and lipid intermediates, such as diacylglycerols and ceramides, can impair insulin signalling by disrupting the insulin receptor substrate (IRS) pathway and activating inflammatory mediators (Saadati et al., 2025). Moreover, the composition of fatty acids within adipocytes influences differentiation, lipid storage capacity, and mitochondrial oxidative efficiency (Abbasi et al., 2025). Polyunsaturated fatty acids, particularly those involved in the linoleic and arachidonic acid pathways, give rise to lipid mediators, such as eicosanoids and prostaglandins, which either promote or inhibit adipogenesis depending on receptor interactions and metabolic context (Dyall et al., 2022; Nartey et al., 2023). This lipid-derived signalling plays a central role in determining adipocyte fate, as lipids not only serve as substrates for triglyceride storage but also act as ligands for nuclear receptors, such as PPARγ, which orchestrate the transcriptional regulation of adipogenesis. Alterations in these lipid signalling

networks, therefore, have downstream effects on adipocyte proliferation, differentiation, and function (Qian et al., 2020).

Collectively, hyperlipidemia is a key link between systemic metabolic dysregulation and cellular dysfunction in adipose tissue. It not only reflects the metabolic overload seen in obesity but also actively contributes to impaired insulin signalling, inflammation, and altered adipogenesis (Chait et al., 2020). Since adipocytes serve as the primary site of lipid storage and are themselves responsive to lipid cues, understanding the mechanisms of lipid metabolism and dysregulation is essential in obesity-related research. This naturally leads to the exploration of adipocyte differentiation, the process through which preadipocytes develop into mature, lipid-storing adipocytes.

2.2 Adipogenesis and Adipocyte Differentiation

2.2.1 Adipose Tissue and Adipocyte

Adipose tissue is recognised as a complex metabolic and endocrine organ that plays a crucial role in energy homeostasis and systemic metabolism (Harvey et al., 2020). Beyond its traditional functions of energy storage and thermoregulation, adipose tissue secretes numerous bioactive factors, known as adipokines, that communicate with other organs and regulate various metabolic pathways (Luo & Liu, 2016). It is composed of various cell types, including mature adipocytes and stromal vascular cells. While adipocytes are the primary cells that store lipids, the stromal vascular fraction comprises preadipocytes, endothelial cells, fibroblasts, and immune cells, which collectively maintain the dynamic structure and function of the tissue. This cellular heterogeneity contributes to the multifaceted role of adipose tissue in energy storage, regulation of metabolism, and endocrine signalling (Müller et al., 2016). Under normal physiological conditions, adipose tissue stores excess energy in the form of triglycerides during periods of caloric surplus and mobilizes stored lipids via lipolysis during energy demand (Kirsh et al., 2020). This energy buffering capacity is critical for protecting non-adipose tissues, such as the liver and muscle, from lipotoxicity (Saporano et al., 2015).

There are two primary forms of adipose tissue in mammals: white adipose tissue (WAT) and brown adipose tissue (BAT) (Cedikova et al., 2016). WAT is primarily

responsible for long-term energy storage, while BAT is involved in non-shivering thermogenesis through the activity of uncoupling protein-1 (UCP1) in its densely packed mitochondria (Cedikova et al., 2016). A third category, known as beige or brite adipocytes, can emerge within WAT depots under specific stimuli, such as cold exposure or sympathetic activation, and exhibit thermogenic properties similar to brown adipocytes (Altinova, 2022). Figure 2.1 illustrates the morphologies of white, brown and beige adipocytes. The morphological differences between the three types of adipocytes are the number of lipid droplets and mitochondria. These distinct adipocyte types demonstrate the functional plasticity of adipose tissue in adapting to different physiological and environmental conditions.

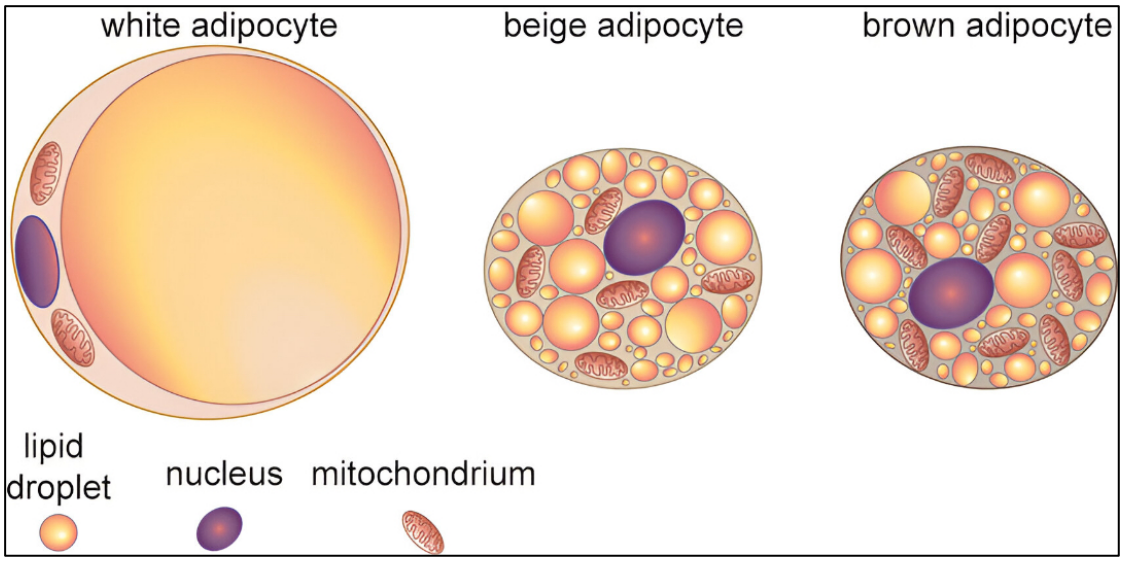

Figure 2.1 Different Types of Adipocytes. Adapted from Paul et al. (2017).

The health of adipose tissue is closely tied to the size and function of its constituent adipocytes. In lean individuals, adipocyte size remains relatively small, maintaining insulin sensitivity and metabolic flexibility (Sakaguchi, 2024). However, in the setting of chronic energy surplus, as seen in obesity, adipose tissue undergoes expansion primarily through adipocyte hypertrophy. This enlargement compromises cellular function, leading to local hypoxia, inflammation, and the infiltration of immune cells such as macrophages (Liu et al., 2020). These changes impair the ability of adipose tissue to safely store lipids and contribute to systemic metabolic disturbances, including insulin resistance and dyslipidemia (Zatterale et al., 2020). Notably, hypertrophic adipocytes display elevated basal lipolysis and reduced insulin responsiveness,

increasing the release of free fatty acids into circulation and further exacerbating lipid imbalance (Morigny et al., 2016).

In addition to their metabolic roles, adipocytes serve an important endocrine function by secreting adipokines, which are bioactive peptides that influence appetite regulation, energy expenditure, glucose metabolism, and immune function (Valjevac, 2020). Key adipokines include leptin, which signals satiety to the hypothalamus; adiponectin, which enhances insulin sensitivity; and pro-inflammatory cytokines such as TNF-α and IL-6, which are elevated in obesity and contribute to metabolic dysregulation (Pereira & Alvarez-Leite, 2014). The balance of these adipokines is crucial for maintaining metabolic health, and alterations in their expression are closely associated with the development of insulin resistance, cardiovascular disease, and other obesity-related complications (Fadaei, 2023).

Moreover, adipocytes are not static. They exhibit turnover throughout life, and the ability of adipose tissue to expand or contract depends partly on the recruitment and differentiation of preadipocytes into mature adipocytes (Scheidl et al., 2023). This process allows adipose tissue to adapt to energy demands, growth, and hormonal changes over time (White & Ravussin, 2018). However, when adipocyte formation is insufficient to meet storage needs, lipid spillover into non-adipose tissues may occur, leading to lipotoxicity and worsening metabolic outcomes (Fano et al., 2022). The capacity of adipose tissue to expand via hyperplasia rather than hypertrophy has therefore been proposed as a protective mechanism in obesity, emphasising the importance of understanding how adipocytes develop and function. Collectively, adipose tissue is a highly dynamic organ, and adipocytes lie at the core of its metabolic and endocrine functions. Their structural adaptability, lipid storage capabilities, and hormonal activity make them key players in both physiological and pathological energy regulation. Thus, understanding adipocyte development is essential for decoding the link between adipose dysfunction and metabolic disease.

2.2.2 Adipocyte Differentiation

Adipocyte differentiation, commonly referred to as adipogenesis, is a complex and highly regulated biological process in which undifferentiated precursor cells commit to the adipogenic lineage and mature into fully functional adipocytes. This transformation is fundamental to the development and expansion of adipose tissue,

particularly white adipose tissue, which plays a central role in energy storage and metabolic regulation (Dowker-Key et al., 2024). Adipogenesis, the formation of adipocytes from mesenchymal stem cells, occurs in two distinct phases: commitment and terminal differentiation (Ambele et al., 2020). During commitment, mesenchymal stem cells become preadipocytes, influenced by cell shape, extracellular matrix remodelling, and WNT signalling (Saidova & Varobjev, 2020). The terminal differentiation phase involves preadipocytes acquiring the characteristics of mature adipocytes, regulated by transcription factors such as PPARγ and C/EBP (Ambele et al., 2020). During this process, cells undergo dramatic changes in gene expression, metabolic activity, and cellular architecture, including the accumulation of intracellular lipid droplets and the expression of adipocyte-specific proteins involved in lipid metabolism, glucose uptake, and endocrine signalling (Mor-Yossef Moldovan et al., 2018; Pei et al., 2022; Klingelhuber et al., 2023).

This differentiation process is not only essential for normal adipose tissue development but also for maintaining systemic metabolic homeostasis. The ability of preadipocytes to differentiate and form new adipocytes is particularly important during states of energy surplus, as it allows for healthy adipose tissue expansion through hyperplasia rather than hypertrophy (Haczeyni et al., 2018). When this capacity is impaired or overwhelmed, as often occurs in obesity, existing adipocytes become hypertrophic and dysfunctional, contributing to insulin resistance, inflammation, and ectopic lipid accumulation in non-adipose tissues (Ahmed et al., 2021). Thus, the regulation of adipogenesis is closely tied to both protective and pathological processes in metabolic health and disease.

Moreover, adipocyte differentiation is not merely a switch between cell states. It involves a reprogramming of the entire cellular machinery to support lipid storage and metabolic flexibility. This includes increased expression of enzymes involved in lipogenesis, enhanced glucose uptake, and shifts in mitochondrial function to support the energetic demands of the maturing cell (Oates & Antoniewicz, 2022). The tight regulation of this process ensures that adipocytes can efficiently store energy and respond to hormonal cues such as insulin and catecholamines. However, when dysregulated, the adipogenic program can contribute to excessive fat mass accumulation and metabolic disturbances, underscoring the importance of understanding its underlying mechanisms.

Adipocyte differentiation has been extensively studied in vitro using preadipocyte cell lines such as 3T3-L1, which have become a standard model for dissecting the molecular and biochemical pathways underlying adipogenesis. Upon stimulation with a hormonal cocktail typically containing insulin, dexamethasone, and isobutylmethylxanthine (IBMX), these cells undergo a well-characterised progression toward terminal differentiation, making them highly suitable for evaluating external modulators of adipogenesis, including nutrients, pharmaceuticals, and natural bioactive compounds (Zhao et al., 2019). Despite its experimental simplicity, this model closely mirrors many of the cellular events occurring in vivo, including clonal expansion, transcription factor activation, lipid droplet formation, and adipokine expression.

2.2.3 Molecular Mechanisms Regulating Adipogenesis

The differentiation of preadipocytes into mature adipocytes is coordinated by a sophisticated network of transcriptional regulators and signalling pathways that ensure proper lineage commitment, lipid accumulation, and metabolic function (Ambele et al., 2020). Central to this regulatory network are two master transcription factors: peroxisome proliferator-activated receptor gamma (PPARγ) and members of the CCAAT/enhancer-binding protein (C/EBP) family, particularly C/EBPα, C/EBPβ, and C/EBPδ (Guo et al., 2015). These transcription factors not only initiate the adipogenic program but also sustain it through feedback mechanisms that reinforce adipocyte identity (Bahrami-Nejad et al., 2020). Upon adipogenic stimulation, early regulators such as C/EBPβ and C/EBPδ are rapidly upregulated, setting the stage for the activation of PPARγ and C/EBPα, which act synergistically to drive terminal differentiation. C/EBPδ plays a crucial role in mitotic clonal expansion and early gene expression during adipocyte differentiation (Lee et al., 2020). PPARγ is considered the master regulator of adipogenesis due to its essential role in activating genes involved in lipid uptake, triglyceride synthesis, insulin sensitivity, and adipokine production (Kim et al., 2024). Without PPARγ activity, adipocyte formation is severely impaired, as demonstrated in both in vitro and in vivo studies, underscoring its indispensable function.

Beyond transcriptional control, adipogenesis is influenced by a multitude of extracellular signals and intracellular cascades (Ahmad et al., 2020). Hormonal stimuli, such as insulin, glucocorticoids, and cyclic AMP-elevating agents, modulate the

expression of adipogenic genes through pathways including PI3K/AKT, MAPK, and cAMP/PKA (Tang & Lane, 2012). These signals contribute to mitotic clonal expansion, chromatin remodelling, and the establishment of a transcriptional landscape that favors adipocyte commitment (Auld et al., 2007). Additionally, members of the Wnt, Hedgehog, and TGF-β signalling families serve as potent inhibitors of adipogenesis by maintaining preadipocytes in an undifferentiated state and suppressing PPARγ expression (Prestwich & MacDougald, 2007). This delicate balance between pro-adipogenic and anti-adipogenic signals ensures that adipocyte differentiation is tightly regulated in response to physiological needs. Moreover, epigenetic modifications such as histone acetylation, DNA methylation, and microRNA regulation have emerged as critical modulators of gene accessibility and stability during adipogenesis, further adding complexity to the regulatory network (Ambele et al., 2020).

In addition to these well-characterised transcriptional events, adipogenesis is modulated by metabolic cues, redox status, and nutrient availability (Sánchez-Ramírez et al., 2022; Yu et al., 2023; Fu et al., 2025). For example, AMPK acts as a cellular energy sensor and can inhibit adipogenesis by suppressing lipogenic gene expression and interfering with PPARγ activity (Shen et al., 2019). Reactive oxygen species (ROS), which increase transiently during early differentiation, are also involved in modulating signalling pathways such as MAPK and NF-κB that influence adipogenic gene expression (de Villiers et al., 2017). These findings reflect the strong interplay between cellular metabolic status and differentiation signals, reinforcing the view of adipogenesis as a process highly sensitive to internal and external environments. Understanding these molecular mechanisms is not only critical for elucidating the biological underpinnings of adipose tissue formation but also for identifying therapeutic targets to combat obesity and related metabolic disorders. The precise coordination of these molecular events determines the quality and functionality of newly formed adipocytes, with significant implications for energy balance and lipid handling. As adipogenesis progresses, cells move through distinct developmental stages characterised by clonal expansion, lipid droplet formation, and functional maturation.

2.2.4 Stages of Adipocyte Development

The development of adipocytes from undifferentiated precursor cells involves a tightly orchestrated multistep process that spans from lineage commitment to full

cellular maturation, culminating in lipid accumulation and endocrine functionality. Following the molecular priming described in the previous section, adipogenesis begins with the commitment of mesenchymal stem cells to the adipocyte lineage, forming preadipocytes that are morphologically fibroblast-like yet transcriptionally distinct (Dowker-Key et al., 2024). Upon exposure to adipogenic stimuli such as insulin, glucocorticoids, and cAMP-elevating agents, these preadipocytes undergo a phase known as mitotic clonal expansion (MCE), wherein they re-enter the cell cycle and complete several rounds of division, a requirement for initiating the terminal differentiation phase (Zhao et al., 2020; Hayakawa et al., 2023). Figure 2.2 portrays the formation of adipocytes in three separate stages: (i) commitment stage; (ii) mitotic clonal expansion (MCE); and (iii) terminal differentiation. This early proliferative stage is essential for priming the adipogenic transcriptional machinery, including the sequential activation of C/EBPβ, C/EBPδ, and ultimately, the master regulator PPARγ, which governs the expression of genes involved in lipid metabolism, insulin sensitivity, and adipokine secretion (Guo et al., 2015; Merret et al., 2020).

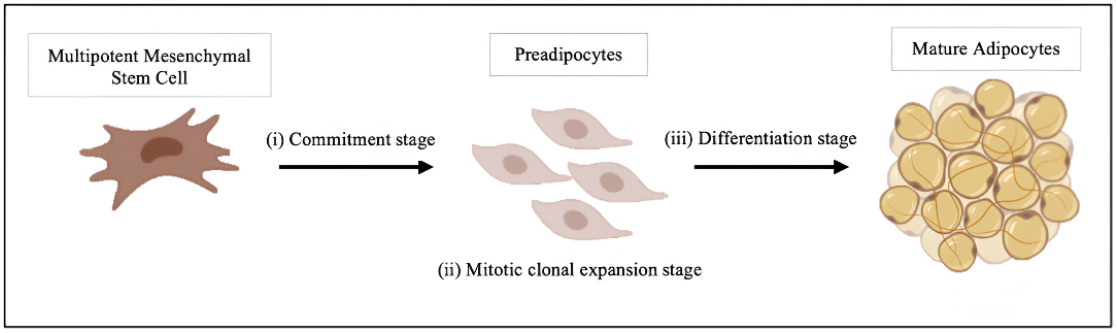

Figure 2.2 Stages of Adipocyte Formation. The Differentiation of Mesenchymal Stem Cells into Mature Adipocyte Involves A Commitment Stage, Mitotic Clonal Expansion Stage, and Terminal Differentiation Stage. Adapted from Giammona et al., (2024).

As differentiation progresses, adipocytes begin to acquire hallmark features of the mature phenotype, including the development of lipid droplet and increased expression of key metabolic enzymes such as fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and adipocyte-specific markers like adiponectin, leptin, and perilipin (Berger et al., 2015; Moseti et al., 2016). These changes reflect a broader shift in cellular metabolism, from a proliferative and glycolytic profile to one dominated by lipid synthesis, storage, and energy homeostasis (Oates & Antoniewicz, 2022). The accumulation of triglycerides within the lipid droplet is a defining feature of mature

white adipocytes and represents both a structural and functional endpoint of adipocyte differentiation (Barneda & Christian, 2017). This process is driven by the coordinated uptake of free fatty acids, de novo lipogenesis, and the esterification of fatty acids into triglycerides for long-term storage (Dinel et al., 2010). Notably, the expansion of the lipid droplet is not merely passive but is governed by specific lipid droplet-associated proteins, which regulate lipid turnover, droplet fusion, and interactions with organelles such as the endoplasmic reticulum and mitochondria (Olzmann & Carvalho, 2018).

Importantly, lipid accumulation is not only a marker of adipocyte maturation but also a determinant of adipose tissue function and metabolic health (Cho et al., 2023). While efficient lipid storage is protective in the context of nutrient excess, excessive hypertrophy of adipocytes can lead to cellular stress, hypoxia, and inflammation, all of which impair adipose tissue function and contribute to systemic metabolic disturbances (Liu et al., 2020). In contrast, a healthy balance between hyperplasia (the formation of new adipocytes) and moderate lipid accumulation is essential for maintaining metabolic flexibility and insulin sensitivity (Goodpaster & Sparks, 2017). The understanding of these cellular transitions provides a crucial framework for evaluating how nutritional or pharmacological interventions may modulate adipocyte development. In recent years, increasing attention has been paid to how bioactive compounds from natural sources can modulate each stage of adipogenesis, either by interrupting the signalling cascade, altering the expression of differentiation markers, or interfering with lipid metabolic enzymes (Valli et al., 2018; Murugan et al., 2021). Given the complex interplay between transcriptional regulation, metabolic rewiring, and structural remodelling during adipocyte maturation, in vitro systems, which is 3T3-L1 preadipocyte cell line, has become a gold standard in adipocyte biology due to its robust differentiation potential and reproducible response to adipogenic stimuli have become indispensable tools for dissecting these processes.

2.3 *In Vitro* Models for Studying Adipogenesis

2.3.1 Overview of Adipogenesis Experimental Models

Experimental models of adipogenesis play a pivotal role in advancing the understanding of the cellular and molecular mechanisms that govern adipocyte development, lipid metabolism, and their contributions to metabolic diseases such as

obesity and type 2 diabetes (Ruiz-Ojeda et al., 2016). Due to the complex nature of adipose tissue, which is influenced by hormonal, nutritional, and inflammatory cues, in vivo and in vitro models have been developed to replicate different aspects of adipogenesis under controlled conditions (Ruiz-Ojeda et al., 2016). In vivo models typically involve dietary or genetic manipulation in rodents to induce adipose tissue expansion, allowing researchers to investigate the systemic effects of obesity on metabolism, inflammation, and organ function (Martins et al., 2022). These models, including high-fat diet-induced obesity and genetically modified mice, have contributed significantly to identifying the physiological consequences of adipose tissue dysfunction. However, the heterogeneity of adipose depots, ethical concerns, and the complexity of systemic interactions in animal models often present challenges in isolating specific cellular events associated with adipocyte differentiation (Loureiro et al., 2021).

To address these limitations, in vitro models have become indispensable tools for dissecting the cellular and molecular events underlying adipogenesis (Ruiz-Ojeda et al., 2016; Vohra et al., 2020). Cell culture systems allow researchers to study adipocyte development in a simplified and highly reproducible manner, enabling the direct observation of differentiation, lipid accumulation, and gene expression in response to defined stimuli or treatment interventions (Kassotis et al., 2017). Primary preadipocytes isolated from adipose tissue and immortalized preadipocyte cell lines such as 3T3-L1 is the most commonly used in in vitro models (Poulos et al., 2010). These systems recapitulate the key stages of adipogenesis, including mitotic clonal expansion, transcription factor activation, and lipid droplet formation, providing a valuable platform for mechanistic studies and screening of anti-adipogenic compounds. Notably, the 3T3-L1 cell line has emerged as a gold standard due to its consistent differentiation potential and well-characterised adipogenic program, making it a widely accepted model in both academic and pharmaceutical research settings (Poulos et al., 2010). Thus, the 3T3-L1 preadipocyte cell line remains the most widely used due to its convenience, cost-effectiveness, and robustness in recapitulating adipocyte differentiation.

2.3.2 3T3-L1 Cell Line

The 3T3-L1 preadipocyte cell line has become an indispensable tool in adipogenesis research due to its well-documented capacity to undergo terminal differentiation into adipocyte-like cells. Originally established by Green and Kehinde from mouse embryonic fibroblasts, the 3T3-L1 cell line remains one of the most extensively characterised and widely adopted in vitro models for studying the molecular and cellular mechanisms of adipocyte differentiation. Its reproducibility, ease of culture, and clear phenotypic transition from fibroblast-like preadipocytes to lipid-laden adipocytes make it particularly suited for controlled experimental studies on adipogenesis (Cave & Crowther, 2018). Under standard culture conditions, these cells are induced to differentiate using a defined hormonal cocktail that typically includes insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX), which collectively trigger the adipogenic cascade (Xueyan Zhao et al., 2019). This hormonal stimulation initiates mitotic clonal expansion (MCE), a critical intermediate step in which growth-arrested cells re-enter the cell cycle prior to terminal differentiation and followed by a tightly regulated sequence of transcriptional events that govern adipocyte maturation (Sarjeant & Stephens, 2012).

As the cells progress through differentiation, there is a well-characterised induction of early adipogenic transcription factors such as C/EBPβ and C/EBPδ, followed by robust expression of the master regulators PPARγ and C/EBPα, which coordinate the activation of downstream genes involved in lipid metabolism, insulin sensitivity, and endocrine function (Guo et al., 2015). This includes genes encoding fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), adipocyte fatty acid-binding protein (aP2), and adiponectin (Moseti et al., 2016). Morphologically, differentiated 3T3-L1 cells exhibit a distinct shift from an elongated fibroblastic shape to a rounded cell filled with a central lipid droplet, which are an iconic hallmark of mature adipocytes (Alvarez, 1991). These structural and molecular changes render 3T3-L1 cells highly representative of in vivo white adipocytes, thereby making them a powerful model for dissecting the pathways involved in adipocyte biology.

One of the major strengths of the 3T3-L1 model is its high sensitivity to external factors, which makes it especially useful for testing pharmacological agents, dietary compounds, and natural product extracts for their effects on adipogenesis (Jang et al., 2016; Tung et al., 2016). It has been widely used in studies aimed at identifying anti-

adipogenic agents, investigating insulin signalling, and exploring oxidative stress and inflammatory responses within. Moreover, its well-defined and synchronized differentiation timeline allows researchers to interrogate specific stages of adipogenesis, from clonal expansion to late-stage lipid accumulation, making it suitable for temporal and mechanistic analyses (Schouwink et al., 2025). Various assays, including oil red O staining, triglyceride quantification, gene expression profiling, and immunoblotting, can be easily integrated into 3T3-L1 culture protocols, further enhancing its utility and flexibility (Kraus et al., 2016; Kassotis et al., 2017).

Despite being a murine cell line, 3T3-L1 cells exhibit many features that are conserved in human adipocyte biology, particularly in terms of gene expression and lipid metabolism. This cross-species relevance enhances their value in preclinical studies and high-throughput screening platforms. They also offer an ethical and cost-effective alternative to animal models, allowing researchers to generate large datasets under reproducible conditions. Their consistent differentiation efficiency, especially when maintained under standardized protocols, has made them a gold standard for in vitro adipogenesis research across academic and industry laboratories worldwide (Ahmed & Kim, 2019; Ahmed et al., 2020). However, while the advantages of the 3T3-L1 cell line are numerous, it is important to acknowledge that like all models, it is not without limitations (Guru et al., 2021). Nevertheless, when interpreted within context, findings from 3T3-L1 models can offer invaluable insights into the cellular dynamics of adipogenesis and the potential of various compounds to modulate adipocyte formation.

2.3.3 Advantages and Limitations of 3T3-L1 Model

The 3T3-L1 preadipocyte model has become a basis in adipogenesis research, offering a well-established, reproducible, and cost-effective platform for exploring the cellular and molecular events underlying adipocyte differentiation. One of its key strengths lies in its robust and synchronized differentiation process, which reliably mirrors many of the critical steps observed during in vivo adipogenesis (Cave & Crowther, 2019). When stimulated with a standard adipogenic cocktail, typically comprising insulin, dexamethasone, and IBMX, 3T3-L1 cells progress through mitotic clonal expansion, early and late transcriptional activation, and ultimately acquire a mature adipocyte phenotype characterised by intracellular lipid droplet formation and

expression of adipocyte-specific genes such as PPARγ and C/EBP (Tang et al., 2003). This controlled differentiation sequence enables researchers to monitor the impact of various treatments, including phytochemicals, drugs, and gene knockdowns, at defined stages of the adipogenic process. Additionally, 3T3-L1 cells respond predictably to insulin and other metabolic regulators, making them highly useful in studying insulin sensitivity, lipid accumulation, and energy homeostasis. The availability of standardized protocols for morphological observation, gene expression analysis, lipid staining, and triglyceride quantification has further solidified the 3T3-L1 model as a go-to system for investigating anti-adipogenic and pro-adipogenic effects under in vitro conditions (Cave & Crowther, 2018; Kassotis et al., 2021).

Despite these advantages, the 3T3-L1 model is not without its limitations, and its use must be interpreted within the boundaries of its biological relevance. As a murine-derived cell line, 3T3-L1 cells may not fully recapitulate the metabolic and transcriptional complexity of human adipose tissue. There are species-specific differences in the regulation of key pathways involved in lipid metabolism, inflammatory responses, and insulin signalling, which may limit the direct translatability of findings to human physiology (Börgeson et al., 2022; Sabogal-Guaqueta et al., 2023). Furthermore, 3T3-L1 adipocytes primarily model white adipose tissue and lack the heterogeneity observed in in vivo, such as the presence of brown and beige adipocytes, stromal vascular fraction cells, immune components, and vasculature. This monoculture system also does not reflect the dynamic interplay between adipocytes and their surrounding microenvironment, which plays a significant role in regulating adipose function, inflammation, and metabolic disease progression (Gijsen, 2018; Kahn et al., 2019; Dufau et al., 2021). In terms of differentiation potential, variability can arise between laboratories due to differences in cell passage number, culture conditions, and confluency at the time of induction, potentially affecting reproducibility if not rigorously controlled.

Nevertheless, when employed thoughtfully and interpreted within its limitations, the 3T3-L1 model remains an indispensable system for probing fundamental aspects of adipocyte biology. Its consistency, accessibility, and amenability to molecular manipulation make it especially suited for mechanistic and screening studies. Importantly, as adipogenesis is increasingly understood to be metabolically dynamic, researchers have turned to high-resolution analytical approaches such as metabolomics to capture the real-time biochemical changes

occurring throughout this differentiation process (Halama et al., 2016). The integration of metabolomics with 3T3-L1 adipocyte models has opened new avenues for exploring not just gene or protein expression, but the functional metabolic outputs that directly reflect cellular physiology.

2.4 Metabolomics and Its Application in Adipogenesis Study

2.4.1 Overview of Metabolomics

Metabolomics has emerged as a transformative discipline in systems biology, offering a powerful approach to quantitatively profile small-molecule metabolites within biological systems and uncover functional insights into cellular physiology and disease processes (Rinschen et al., 2019). Unlike genomics and proteomics, which describe potential or ongoing cellular functions, metabolomics provides a direct snapshot of the biochemical activities occurring within a cell at a given moment, thus reflecting the real-time physiological status of the system under investigation (Kim et al., 2016). As the final downstream product of gene and protein interactions, the metabolome is highly sensitive to both intrinsic signals and environmental stimuli, making metabolomic analysis particularly valuable in studying dynamic processes like cell differentiation, metabolic reprogramming, and drug response (Balcerczyk et al., 2020). Technological advances in high-throughput analytical platforms such as nuclear magnetic resonance (NMR) spectroscopy, gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (LC-MS) have greatly enhanced the resolution, sensitivity, and scope of metabolomic investigations, enabling researchers to map broad biochemical networks across diverse physiological conditions (Saleem & Chugh, 2020).

In the context of adipogenesis, metabolomics provides an integrative lens through which the complex metabolic shifts associated with preadipocyte commitment, lipid accumulation, and mature adipocyte function can be elucidated in unprecedented detail (Oates & Antoniewicz, 2022). This whole approach is particularly relevant in adipocyte biology, where differentiation involves tightly coordinated changes in energy metabolism, redox balance, and lipid synthesis pathways. The use of metabolomics in adipogenesis studies allows for the identification of pathway-specific biomarkers, such as amino acids, fatty acids, and carbohydrate intermediates, that not only reflect

metabolic adaptation but may also function as regulators of differentiation (Halama et al., 2016). Moreover, metabolomic profiling can reveal how pharmacological compounds or natural extracts modulate these pathways, offering insight into the molecular mechanisms underlying anti-adipogenic or pro-adipogenic effects (Mladenova et al., 2021). By integrating metabolomic data with transcriptomic or proteomic analyses, researchers can construct comprehensive metabolic maps that connect upstream signalling events with downstream metabolic consequences, allowing for a systems-level understanding of adipocyte development (Kumagai et al., 2023).

2.4.2 Metabolomic Approaches

Broadly, metabolomics can be divided into untargeted and targeted approaches, which differ in their scope, resolution, and underlying analytical philosophy (Braga & Adamec, 2019). These strategies are not mutually exclusive but rather complementary, often forming sequential steps in a comprehensive metabolomics workflow. Untargeted metabolomics is generally used for discovery-driven studies aiming to detect as many metabolites as possible without prior bias (Lelli et al., 2021). Targeted metabolomics, in contrast, focuses on predefined metabolites of interest, achieving high sensitivity and quantification accuracy (Braga & Adamec, 2019). Understanding the definition, principles, strengths, and limitations of each approach is crucial for designing robust experiments and accurately interpreting metabolic data.

2.4.2.1 Types of Metabolomics Approaches

i. Untargeted Metabolomics

Untargeted metabolomics is an exploratory strategy aimed at the comprehensive profiling of metabolites in a biological sample without prior knowledge of their identity or abundance (Gertsman & Barshop, 2018; Braga & Adamec, 2019). This approach is inherently hypothesis-generating, allowing researchers to capture a global snapshot of the metabolome and identify unexpected or novel metabolic features (Di Minno et al., 2021). In untargeted workflows, advanced analytical platforms such as high-resolution mass spectrometry (HRMS) or nuclear magnetic resonance (NMR) spectroscopy are used to detect a broad spectrum of metabolites across multiple chemical classes,

including lipids, amino acids, nucleotides, organic acids, and carbohydrates (Tebani et al., 2018). The principle rests on acquiring high-density spectral data followed by computational deconvolution, peak picking, alignment, and annotation against metabolite databases such as HMDB, METLIN, and GNPS (Ivanisevic & Want, 2019).

In a typical untargeted metabolomics pipeline, biological sample preparation aims to maximize metabolite coverage while minimizing degradation or transformation (Ivanisevic & Want, 2019; Jeppesen & Powers, 2023). Depending on the platform, metabolites may be separated via liquid chromatography (LC), gas chromatography (GC), or capillary electrophoresis (CE) prior to detection. Data are then processed through multivariate statistical analyses such as Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) to identify features that discriminate between experimental groups (Schrimpe-Rutledge et al., 2016). While annotation confidence varies, a tiered identification system (MSI levels 1–4) is often employed, ranging from confirmed identities with authentic standards to unknown features with only spectral characteristics (Kodra et al., 2021).

One of the key advantages of untargeted metabolomics is its unbiased nature, making it ideally suited for exploratory studies where the metabolic changes are unknown. While untargeted metabolomics offers broad coverage of the metabolome, it primarily provides information on relative differences in metabolite pool sizes, whereas stable isotope methods can reveal differences in reaction rates (Zamboni et al., 2015). The field has seen significant advancements in mass spectrometry technologies, bioinformatic tools, and software, allowing for rapid measurement of thousands of metabolites from minimal sample amounts (Patti et al., 2012). It is also highly comprehensive, capable of covering a vast chemical space when combined with multiple chromatographic and ionization methods. Furthermore, untargeted datasets can be mined retrospectively to address new research questions as improved identification tools and databases become available (Strutz et al., 2022).

However, the extensiveness of coverage in untargeted metabolomics comes at the expense of quantification accuracy and reproducibility. Ion suppression, matrix effects, and variable ionization efficiencies can make absolute quantification challenging without targeted follow-up (Alseekh et al., 2021). Feature annotation remains a major bottleneck, as many detected signals cannot be confidently assigned to known metabolites, leading to large proportions of “unknowns” in datasets (Chaleckis et al., 2019). Data processing pipelines are also computationally intensive and

susceptible to user-defined parameters, which can introduce variability between studies (Guo et al., 2022). Additionally, untargeted approaches are sensitive to batch effects and analytical drift, necessitating rigorous quality control procedures (Märtens et al., 2023).

ii. Targeted Metabolomics

Targeted metabolomics, on the other hand, is a quantitative approach focusing on predefined metabolites, often within specific pathways or compound classes (West et al., 2016; Begou et al., 2017). This method is more hypothesis-driven compared to untargeted approaches, aiming for absolute or relative quantification of selected analytes (Tebani et al., 2018). The metabolite list is established prior to analysis based on existing biological knowledge, preliminary untargeted findings, or specific diagnostic relevance. Analytical methods are optimised for sensitivity, selectivity, and quantification precision of the targeted analytes (West et al., 2016).

Targeted workflows in mass spectrometry-based metabolomics involve tailored sample preparation and optimised separation or detection parameters for specific analytes of interest. Automated sample preparation enhances throughput and reproducibility, crucial for processing large cohorts in biomarker validation studies (Fu et al., 2021). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has revolutionized biomarker analysis, offering reduced sample preparation and increased chemical coverage compared to traditional methods (Pramod et al., 2020). Multiple reaction monitoring (MRM) with isotopically labelled internal standards enables absolute quantification, providing robust data for clinical validation (Gaither et al., 2020). The use of isotopically labelled internal standards enables absolute quantification, providing robust data suitable for clinical validation or biomarker qualification (Koal & Deigner, 2010). Targeted metabolomics is frequently employed in validation phases following untargeted discovery, in nutritional studies, and in pharmacokinetic or toxicological research.

Several advantages of targeted metabolomics include high sensitivity, specificity, and quantitative accuracy, often reaching sub-nanomolar detection limits. This approach enables precise quantification of endogenous compounds, contributing to biomarker discovery and validation. The method provides reproducible data across laboratories, especially when standardized protocols and calibration curves are used

(Koal & Deigner, 2010). By focusing only on predefined metabolites, data complexity is greatly reduced, facilitating interpretation and statistical power. Targeted approaches are also well-suited for regulatory and clinical environments where robust quantification is essential (Anh et al., 2024).

Even though targeted metabolomics offers high sensitivity, specificity, and quantitative accuracy, the key limitation of targeted metabolomics is its restricted scope. Metabolites outside the predefined panel remain undetected, which may cause biologically relevant changes to be missed. Additionally, targeted methods require substantial prior knowledge of the system, potentially biasing the analysis towards well-characterised pathways. Method development can be time-consuming and costly, particularly when authentic standards are expensive or unavailable. Furthermore, targeted metabolomics cannot identify novel metabolites, limiting its utility for early-stage exploratory research (Amer et al., 2023).

2.4.2.2 Analytical Platforms

The successful application of metabolomics studies relies heavily on the choice of analytical platform. Metabolomics encompasses a diverse array of analytical techniques, each with distinct advantages, limitations, and suitability depending on the chemical nature of the metabolites under investigation (Segers et al., 2019). Broadly, the two most widely employed platforms are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS)-based techniques (Yousf & Chugh, 2020), with the latter often coupled to chromatographic separation methods such as gas chromatography (GC), liquid chromatography (LC), or capillary electrophoresis (CE) (Baidoo et al., 2012). These hyphenated techniques (GC-MS, LC-MS, CE-MS) are valuable tools for natural product identification, authentication, and quantification in various biological materials (Seger et al., 2013). NMR offers high reproducibility, easy sample preparation, and non-destructive analysis, while MS provides higher sensitivity and resolution (Yousf & Chugh, 2020). However, each technique has its strengths and limitations. For instance, GC-MS requires extensive sample preparation but offers high resolution, while LC-MS provides high throughput with minimal sample preparation.

NMR spectroscopy is a powerful tool for metabolomics research, offering high reproducibility, quantitative accuracy, and non-destructive analysis with minimal sample preparation (Hu, 2016; Emwas et al., 2019). Its ability to provide structural

elucidation makes it valuable for untargeted studies (Emwas et al., 2019). The strengths of NMR include easy quantification, simultaneous identification of multiple metabolites, and high experimental reproducibility (Hu, 2016; Yousf & Chugh, 2020). However, a major limitation of NMR is its lower sensitivity compared to mass spectrometry (MS) techniques (Yousf & Chugh, 2020). Despite this, NMR remains a preferred platform for large-scale clinical metabolomic studies due to its advantages in sample preparation, quantification, and non-destructive nature (Emwas et al., 2019). For biological samples such as adipocytes, where metabolite concentrations can span several orders of magnitude, NMR is often complemented by MS to capture low-abundance metabolites.

Gas chromatography-mass spectrometry (GC-MS) is a powerful technique for metabolite profiling, offering high reproducibility and sensitivity (De Souza, 2013). It excels in detecting a wide range of metabolites, particularly volatile and thermally stable compounds (Frisvad et al., 2020). While GC-MS is suitable for volatile lipids, it often requires derivatization for less volatile or thermally labile compounds (Rey-Stolle et al., 2022). Chemical derivatization combined with MS has emerged as a powerful strategy to enhance ionization efficiency, improve structure identification, and facilitate quantitative analysis in lipidomics (Zhao et al., 2020; Wang et al., 2023). This approach has been applied to various lipid categories, including fatty acids, glycerolipids, glycerophospholipids, and sterols (Wang et al., 2023). Derivatization techniques have been developed for different MS platforms, such as GC-MS, liquid chromatography-MS, and mass spectrometry imaging, addressing challenges related to chain lengths, saturation degrees, and conjugation groups of fatty acids and fatty acyls (Li et al., 2020). These advancements have significantly contributed to the progress of targeted lipidomics and related applications in biology, medicine, and pharmacy (Zhao et al., 2020).

LC-MS, in contrast, offers greater versatility in handling a broad range of polar and non-polar metabolites without the need for derivatization, making it particularly suitable for plant metabolomics and secondary metabolite studies (Allwood & Goodacre, 2010). For example, the use of high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC) prior to MS detection enables the separation of complex metabolite mixtures and improved quantitation. Ultra-performance liquid chromatography (UPLC) coupled with MS provides improved sensitivity, speed, and resolution compared to traditional HPLC methods (Churchwell

et al., 2005). UPLC-MS has been successfully applied to various biological samples, including cells, serum, and tissue, facilitating metabolic pathway screening, biomarker discovery, and drug development (Snyder et al., 2013). The combination of UPLC with high-resolution MS has expanded the coverage of detectable analytes and improved mass measurement accuracy, making it a valuable tool in the diagnosis of disease. When coupled with high-resolution mass analysers, such as quadrupole time-of-flight (QTOF) or Orbitrap instruments, LC-MS provides both high mass accuracy and excellent dynamic range, which are crucial for untargeted metabolomics (Glauser et al., 2013).

Untargeted metabolomics using ultra-high pressure liquid chromatography (UHPLC) coupled with high-resolution mass spectrometry has emerged as a powerful approach for applications that are of particular relevance to adipogenesis research. Both QTOF and Orbitrap mass analysers offer comparable performance in terms of sensitivity, mass accuracy, and ability to detect subtle differences between sample groups (Glauser et al., 2013). These techniques enable the detection of thousands of metabolic features in complex biological samples (Forcisi et al., 2013). The combination of chromatographic separation with high-resolution MS provides the sensitivity and selectivity needed to cover a wide range of metabolites in complex food and biological samples (Lacalle-Bergeron et al., 2021). Thus, this approach has great potential for identifying food biomarkers and investigating metabolic changes related to food quality, health, and disease. In the context of anti-adipogenic studies, these capabilities are especially advantageous. The complexity of cellular metabolomes, specifically in 3T3-L1 adipocyte models, necessitates the detection of diverse metabolite classes, ranging from small organic acids to complex triglycerides. UPLC-QTOF platforms can capture these chemical classes in both positive and negative ionization modes, providing a more comprehensive metabolic snapshot (Du et al., 2018; Liu et al., 2014). Furthermore, the high resolution and mass accuracy facilitate confident metabolite annotation using spectral databases such as HMDB, METLIN, and LIPID MAPS (Guijas et al., 2018).

In summary, while NMR, GC-MS, LC-MS, and CE-MS each hold value in metabolomics, the selection of UPLC-QTOF for the present study is justified by its high sensitivity, broad metabolite coverage, and structural elucidation capabilities. Its ability to resolve complex metabolite mixtures, detect subtle biochemical perturbations, and integrate plant phytochemical profiling with adipocyte metabolic analysis makes it an optimal platform for investigating the metabolomic effects on 3T3-L1 adipocyte

differentiation. This approach not only facilitates a comprehensive understanding of the metabolic alterations induced by the extract but also aligns with the growing emphasis on high-resolution, systems-level characterisation in modern metabolomics. Furthermore, the application of untargeted and targeted metabolomics to 3T3-L1 adipocyte models has enabled the discovery of novel metabolic checkpoints involved in adipogenic regulation, as well as the identification of therapeutic targets to combat metabolic disorders such as obesity and insulin resistance (Roberts et al., 2009; Mastrangelo et al., 2016). Given the metabolically active nature of adipocytes and their central role in energy homeostasis, inflammation, and endocrine signalling, the application of metabolomics to adipogenesis research is not only timely but essential. It enables researchers to go beyond conventional marker gene analysis and interrogate the functional metabolic output of adipocyte differentiation, thereby improving understanding of adipose tissue biology and its dysregulation in metabolic diseases.

2.4.3 Metabolic Pathways in Adipocyte Differentiation

Adipocyte differentiation is not merely a transcriptional event, but a metabolically intensive process governed by an orchestrated network of biochemical pathways that underpin the acquisition of the mature adipocyte phenotype. Throughout the course of adipogenesis, precursor cells undergo dramatic metabolic reprogramming to accommodate the increasing demands of lipid biosynthesis, energy storage, redox balance, and cellular remodelling (Oates & Antoniewicz, 2022). Among the most prominently altered pathways are those associated with lipid metabolism, including de novo lipogenesis, fatty acid elongation and desaturation, and triglyceride synthesis. These processes are tightly regulated by a shift in enzymatic activity and substrate availability, facilitating the accumulation of lipid droplets characteristic of mature adipocytes (Oates & Antoniewicz, 2022). Parallel to lipid metabolism, amino acid pathways, particularly those involving branched-chain amino acids, glutamine, and tryptophan, play critical roles in supporting cell growth, redox homeostasis, and signalling events that influence the progression of differentiation (Salinas-Rubio et al., 2018). Tryptophan metabolites, for example, can activate the aryl hydrocarbon receptor (AhR), which has been implicated in modulating adipogenic transcriptional programs, either promoting or inhibiting adipocyte formation depending on the cellular context (Jin et al., 2014).

Carbohydrate metabolism also undergoes substantial remodelling during adipogenesis, with increased flux through glycolysis and the pentose phosphate pathway to meet biosynthetic and energetic requirements (Oates & Antoniewicz, 2022). The enhanced glycolytic activity not only supplies ATP but also provides essential intermediates for glycerol backbone synthesis and NADPH for reductive biosynthesis of fatty acids (Xue et al., 2017). Meanwhile, nucleotide metabolism supports heightened transcriptional and translational activity, contributing to the synthesis of nucleic acids and coenzymes such as NAD^+ and FAD, which are critical for mitochondrial function and oxidative metabolism (Heikal, 2010). Additionally, the metabolism of vitamins and cofactors such as biotin, pantothenic acid, and riboflavin becomes increasingly important as these nutrients serve as essential cofactors for carboxylases, acyl transferases, and redox enzymes involved in lipid and energy metabolism (Velázquez-Arellano & Hernandez-Vazquez, 2020). Together, these interdependent pathways form a dynamic metabolic landscape that both reflects and drives adipocyte differentiation. The capacity of metabolomics to map these interconnected networks provides a systems-level understanding of adipogenesis that surpasses what transcriptomic or proteomic data alone can capture. By detecting subtle shifts in metabolite concentrations and pathway fluxes, researchers can identify key metabolic inflection points that govern the transition from preadipocyte to adipocyte. Such insights are invaluable for uncovering novel regulatory nodes, understanding adipocyte plasticity, and assessing the impact of therapeutic agents.

2.4.4 Metabolomics in Assessing Anti-Adipogenic Interventions

The integration of metabolomics into adipogenesis research has developed the way anti-adipogenic interventions are evaluated, offering an astonishing opening into the functional metabolic changes that emphasise phenotypic transitions in adipocytes. Traditional approaches to studying adipocyte differentiation have primarily relied on transcriptomic or proteomic analyses, oil red O staining, and triglyceride quantification to assess lipid accumulation and adipogenic marker expression (Kraus et al., 2016). While these methods provide valuable structural and gene-level insights, they often fail to capture the full complexity of metabolic reprogramming occurring during adipogenesis, particularly when evaluating the subtle, multi-targeted effects of natural compounds or pharmacological inhibitors. Metabolomics, by contrast, directly

quantifies the end products of cellular activity, which are small-molecule metabolites, thereby enabling the identification of key metabolic nodes and fluxes that are modulated during the course of adipocyte development or its inhibition (Xu & Liu, 2021).

When applied to 3T3-L1 preadipocytes and their differentiated counterparts, metabolomic profiling has consistently revealed that interventions with anti-adipogenic potential often exert their effects through multiple, interconnected pathways. For instance, treatments that reduce lipid accumulation are frequently associated with the downregulation of metabolites in the linoleic acid and arachidonic acid pathways, attenuation of glycolytic intermediates, and perturbations in amino acid metabolism, particularly tryptophan, phenylalanine, and branched-chain amino acids (BCAA) (Du et al., 2018). These changes indicate that anti-adipogenic agents may suppress the biosynthetic demand for lipid storage while enhancing oxidative or catabolic pathways, thus redirecting cellular metabolism away from adipocyte maturation. Furthermore, certain metabolites such as indoleacetic acid or N-acetyl-L-phenylalanine, which are involved in tryptophan and phenylalanine metabolism respectively, have been shown to act as signalling modulators through pathways like the aryl hydrocarbon receptor (AhR), which negatively regulates the expression of PPARγ and other adipogenic transcription factors (Ghiboub et al., 2020).

The strength of metabolomics lies not only in its ability to identify individual metabolite alterations but also in its power to map entire biochemical networks that reflect the state of the cell in response to intervention (Perez de Souza et al., 2020). This systems-level approach is particularly advantageous when evaluating botanical extracts or phytocompounds, which often act through polypharmacology by modulating several pathways simultaneously rather than targeting a single receptor or enzyme (Sharma & Yadav, 2022). Metabolomic data can be coupled with multivariate statistical tools such as principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and variable importance in projection (VIP) scores to identify key metabolites that discriminate treatment groups from controls with statistical confidence (Worley & Powers, 2012; Bylesjö, 2015). Moreover, receiver operating characteristic (ROC) curve analysis can help evaluate the diagnostic potential of these metabolites, providing insight into their value as biomarkers for anti-adipogenic efficacy (Søreide et al., 2011).

In addition, metabolomics provides a valuable means of detecting early biochemical perturbations before macroscopic or transcriptional changes become

apparent. This early detection potential is critical for screening anti-adipogenic compounds, especially in time-sensitive experiments or dose-response studies. It also allows researchers to differentiate between compounds that merely delay lipid accumulation and those that fundamentally alter the trajectory of adipocyte differentiation. Studies have demonstrated that metabolomic shifts often precede morphological changes such as lipid droplet formation, underscoring the utility of this approach in preclinical evaluation of therapeutic agents (Sillé & Hartung, 2024). Importantly, this holistic metabolomic perspective aligns with the growing recognition that metabolic disease, such as obesity, is not driven by isolated molecular defects but by system-wide dysregulation of metabolic networks (Wishart, 2019). By focusing on real-time biochemical outputs, metabolomics facilitates a deeper understanding of how anti-adipogenic treatments affect factors between molecular biology and phenotype, providing a mechanistic context that supports or refines hypotheses generated from genomics or proteomics. As such, the role of metabolomics in anti-adipogenic research extends beyond mere observation; it is an indispensable tool for validating therapeutic strategies, identifying predictive biomarkers, and ultimately guiding the development of more targeted and effective interventions (Zhang et al., 2017; Xu & Liu, 2021). This metabolomics-driven framework becomes even more relevant in the exploration of medicinal plants as therapeutic agents for obesity and related metabolic disorders. Given the complex phytochemical composition of medicinal plants, metabolomics offers a sensitive and high-resolution approach to trace how these bioactive compounds influence key metabolic pathways during adipogenesis.

2.5 Medicinal Plants as Therapeutic Agents

2.5.1 Role of Medicinal Plants in Metabolic Health

The application of metabolomics in adipogenesis research has not only deepened the understanding of the dynamic changes in cellular metabolism during adipocyte differentiation but also provided valuable insights into how external interventions, such as phytochemical treatments, can influence these processes at a systems level (Oates & Antoniewicz, 2022). As researchers continue to unravel the biochemical pathways involved in adipocyte development, medicinal plants have emerged as promising modulators of metabolic health. Their complex mixtures of

bioactive compounds can elicit wide-ranging effects across key metabolic networks, including those governing lipid metabolism, redox regulation, inflammation, and energy homeostasis (Graf et al., 2010). These properties make medicinal plants attractive candidates for countering metabolic syndromes like obesity, especially in light of the limitations associated with conventional pharmacotherapies.

Pharmaceutical agents commonly prescribed for obesity and hyperlipidemia, including statins and fibrates, are effective in specific contexts but are frequently accompanied by adverse effects such as hepatic dysfunction, myopathy, and rhabdomyolysis, particularly when used in combination (Newman, 2022). GLP-1 receptor agonists, which were also used for obesity and diabetes, have raised concerns about pancreatitis and thyroid cancer (Filippatos et al., 2014). Orlistat, another anti-obesity drug, can cause gastrointestinal side effects (Khalil et al., 2020). Moreover, their single-target mechanisms often fall short in addressing the multifactorial nature of metabolic disorders. In contrast, medicinal plants offer a polypharmacological approach, targeting multiple biochemical and signalling pathways simultaneously. This integrative action is particularly advantageous in obesity, which involves a complex interplay between adipose tissue expansion, hormonal dysregulation, oxidative stress, and low-grade chronic inflammation (Saglam & Şekerler, 2024). Consequently, growing interest has turned toward natural products as potential therapeutic agents capable of exerting both preventive and corrective metabolic effects.

Numerous studies have demonstrated that plant-derived compounds can inhibit adipogenesis, promote lipolysis, and enhance insulin sensitivity in adipocyte models, including 3T3-L1 preadipocytes (Shobha et al., 2017). These effects are often mediated through modulation of key adipogenic regulators such as PPARγ, C/EBPα, AMPK, and SREBP-1c, as well as upstream pathways like MAPK and PI3K/Akt signalling (Ha et al., 2016; Wang et al., 2020; Chung & Hyun, 2021). Additionally, many phytochemicals possess strong antioxidant capacity, which enables them to reduce intracellular reactive oxygen species (ROS) and restore redox sensitive signalling pathways that influence adipocyte fate (Wu et al., 2020). Oxidative stress is a known driver of adipocyte hypertrophy and insulin resistance; therefore, attenuation of ROS via antioxidant-rich plant extracts can contribute to improved metabolic profiles. This dual action, modulating both signalling and oxidative stress, sets medicinal plants apart as metabolic regulators.

From a metabolomics perspective, the effects of medicinal plants on adipocytes can now be traced with remarkable detail. Untargeted and targeted metabolomics analyses in 3T3-L1 cells have shown that treatment with plant-derived compounds leads to measurable alterations in lipid intermediates, amino acids, organic acids, and cofactor metabolites that reflect suppressed adipogenesis and enhanced energy metabolism (Du et al., 2018). For example, suppression of lipid biosynthetic metabolites such as linoleic acid, palmitate, or triglyceride precursors may indicate inhibited lipogenesis, while the elevation of fatty acid oxidation products and TCA cycle intermediates suggests increased mitochondrial activity. Similarly, changes in amino acid metabolism, particularly tryptophan, phenylalanine, and glutamine pathways, have been associated with anti-inflammatory and anti-adipogenic responses (Ye et al., 2020). These metabolic shifts offer mechanistic explanations that complement phenotypic outcomes, such as reduced lipid droplet formation or triglyceride content in treated adipocytes.

Moreover, the utility of medicinal plants in metabolic health extends beyond fat cells alone. Their impact on systemic metabolism has been reported in animal and clinical studies, where improvements in glucose homeostasis, lipid profiles, and inflammatory markers have been observed. These outcomes are typically attributed to the synergistic action of multiple phytoconstituents working across tissues, including adipose tissue, liver, muscle, and pancreas, underscoring the systemic therapeutic potential of botanicals (Nyakudya et al., 2020; Gauttam et al., 2024). In summary, medicinal plants represent a rich source of therapeutic agents that can modulate metabolic pathways relevant to obesity and adipocyte biology. Their ability to influence lipid synthesis, mitochondrial function, oxidative balance, and signalling cascades makes them valuable tools in the fight against adipose-related disorders.

2.5.2 Bioactive Phytochemicals in Medicinal Plants

The remarkable therapeutic potential of medicinal plants in modulating metabolic health lies largely in their complex reservoir of bioactive phytochemicals, which often work in concert to exert anti-adipogenic, antioxidant, and anti - inflammatory effects (Saad et al., 2021; Gauttam et al., 2024). These naturally occurring compounds, including polyphenols, flavonoids, alkaloids, terpenoids, and tannins, have been extensively studied for their ability to interfere with adipogenesis and regulate oxidative stress, both of which are central to the pathophysiology of obesity and

metabolic syndrome. According to Liu et al. (2022), there is an increasing number of reports on phytochemicals that have beneficial effects in preventing or fighting hyperlipidemia. Myrica nagi Thunb. (Prashar & Patel, 2020), Ramulus mori (Park et al., 2020), Ginkgo vinegar (Hosoda et al., 2020), Hordeum vulgare L (Thatiparthi et al., 2019), Liupao tea (Wu et al., 2021), as well as Polygonum multiflorum Thunb. (Choi et al., 2018) are some of the examples of plants that were documented to have a lipid-lowering ability due to the presence of phytochemicals such as rutin, catechin, taxifolin, astragalin, flavonoids, saponins, terpenoids, alkaloids, glycosides, steroids, amino acids, carbohydrates as well as tannins. Numerous studies also have reported that specific phytochemicals such as quercetin, genistein, kaempferol, catechin, and chlorogenic acid can inhibit adipocyte differentiation by targeting key regulatory transcription factors including PPARγ, C/EBPα, and SREBP-1c, thereby reducing lipid accumulation in 3T3-L1 preadipocytes (Chien et al., 2020; Liu et al., 2020; Lin et al., 2023). These compounds are also known to activate AMPK and modulate upstream insulin and MAPK signalling pathways, suggesting that they not only block lipid synthesis but also shift cellular energy metabolism towards enhanced fatty acid oxidation and mitochondrial respiration. The inhibition of adipogenic gene expression by phytochemicals is frequently accompanied by phenotypic changes such as smaller lipid droplets, decreased triglyceride content, and altered metabolic enzyme activity (Chang et al., 2018).

In parallel, many of these phytochemicals exhibit strong antioxidant activity, a property that complements their anti-adipogenic functions. Oxidative stress has long been implicated in adipocyte hypertrophy, insulin resistance, and chronic inflammation, hallmarks of dysfunctional adipose tissue (Murugan et al., 2021; Pérez-Torres et al., 2021). Antioxidant-rich plant compounds scavenge reactive oxygen species (ROS), thereby preventing ROS-induced activation of redox-sensitive transcription factors that promote adipocyte differentiation. This redox-modulating property also helps restore mitochondrial balance and improve adipocyte metabolic flexibility, making antioxidant phytochemicals particularly valuable in cellular environments (Pérez-Torres et al., 2021).

What sets phytochemicals apart from many synthetic drugs is their polypharmacological behaviour, wherein a single compound can target multiple signalling pathways, enzymes, and gene networks simultaneously (Bhutadiya & Mistry, 2021; Shuvalov et al., 2023). This broad-spectrum activity is especially beneficial in

addressing the complex etiology of obesity, which involves lipid accumulation, inflammation, redox imbalance, and metabolic inflexibility (Poulios et al., 2023). Furthermore, recent advancements in metabolomics have allowed for high-resolution tracking of cellular responses to phytochemical treatments. These studies reveal that bioactive compounds not only suppress adipogenic transcription but also cause measurable shifts in lipid intermediates, redox cofactors, bile acid precursors, and amino acid metabolites, which collectively reflecting a rewiring of intracellular metabolism toward an anti-adipogenic profile (Du et al., 2018).

Overall, the growing body of evidence supports the role of phytochemicals as effective modulators of adipocyte metabolism through their dual action in inhibiting adipogenesis and reducing oxidative stress (Murugan et al., 2021). These properties make them promising candidates for the development of nutraceuticals or adjunctive therapies for metabolic syndrome, particularly in populations at risk of obesity-related complications. As interest in plant-derived therapeutics continues to grow, it becomes increasingly important to examine examples of medicinal plants with robust evidence for their metabolic benefits, as well as to explore the specific bioactivities of their constituent phytochemicals.

2.5.3 Medicinal Plants and Their Health Benefits

Across diverse cultural and geographical contexts, medicinal plants have long been used to prevent and manage metabolic disorders, and in recent decades, a growing body of scientific evidence has validated many of these traditional applications (Nyakudya et al., 2020). These plants often contain complex mixtures of bioactive compounds that work synergistically to modulate metabolic pathways relevant to obesity, hyperlipidemia, insulin resistance, and associated complications (Mohanraj, 2021). Several species have been studied in both in vitro and in vivo models, as well as in human clinical trials, demonstrating significant improvements in lipid metabolism, glucose regulation, and inflammatory status (Džamić & Matejić, 2021).

One of the most extensively studied examples is Camellia sinensis (green tea), which is rich in catechins such as epigallocatechin gallate (EGCG). Green tea catechins have been shown to reduce body weight gain, suppress lipogenesis, and enhance fatty acid oxidation through activation of AMPK and modulation of PPARγ activity (Li et al., 2018). Clinical studies have reported improvements in plasma lipid profiles and

reductions in visceral fat among individuals consuming green tea extracts (Macêdo et al., 2022). Another notable species is Curcuma longa (turmeric), whose principal polyphenol, curcumin, exhibits anti-inflammatory and antioxidant properties with potential benefits for obesity and related metabolic disorders. Studies have shown that curcumin can inhibit the SREBP pathway, reducing cholesterol and fatty acid biosynthesis, as well as ameliorating high-fat diet-induced obesity and insulin resistance in mice (Ding et al., 2016). Curcumin also has been reported to downregulate adipogenic transcription factors, suppress NF-κB activation, and improve insulin sensitivity in high-fat diet-induced obese animal models (Aggarwal, 2010; Septembre-Malaterre et al., 2016).

Garcinia cambogia, containing the active compound hydroxycitric acid (HCA), has been widely investigated for its appetite-suppressing and lipogenesis-inhibiting effects (Jena et al., 2002). Although human trials have yielded mixed results, some studies indicate modest reductions in body weight and fat mass when used alongside dietary interventions (Tomar et al., 2019). Similarly, Panax ginseng, rich in ginsenosides, has demonstrated significant effects on glucose and lipid metabolism in various studies. A systematic review and meta-analysis found that P. ginseng supplementation reduced glucose levels, insulin area under the curve, body fat percentage, blood pressure, and lipid profiles (Park et al., 2021). Clinical trials have shown that ginseng extract decreases total cholesterol, triglycerides, and LDL while increasing HDL and antioxidant activity (Hamzah & Jawad, 2022). In diabetic rat models, P. ginseng improved glucose metabolism by increasing AMPK, insulin receptor A, and GLUT-2 expression in the liver (Abdelazim et al., 2018). A randomized controlled trial of ginseng berry extract revealed significant reductions in fasting glucose, postprandial glucose, and glucose area under the curve in participants with fasting glucose levels ≥110 mg/dL (Choi et al., 2017). Furthermore, Nigella sativa (black seed) and its bioactive compound thymoquinone have shown promising effects in managing metabolic syndrome and diabetes. Studies indicate that N. sativa and thymoquinone can improve insulin sensitivity, reduce oxidative stress, and attenuate inflammation (Maideen, 2021). Thymoquinone enhances glucose-stimulated insulin secretion from pancreatic β-cells by regulating NAD(P)H/NAD(P)+ ratios and normalizing malonyl-CoA accumulation under glucose overload conditions (Gray et al., 2016). Clinical trials have demonstrated the efficacy of N. sativa in reducing fasting

blood glucose, postprandial blood glucose, HbA1c, and improving insulin resistance markers (Mahomoodally et al., 2022).

Several traditional Asian medicinal plants have also gained attention for their anti-adipogenic potential. Morus alba (white mulberry) leaves, containing flavonoids and alkaloids, has shown promising anti-adipogenic effects in several studies. Extracts from M. alba leaves inhibited lipid accumulation in 3T3-L1 adipocytes, with some compounds demonstrating significant anti-adipogenic activity (Li et al., 2018). The ethyl acetate-soluble fraction of M. alba fruits decreased intracellular lipid accumulation by 38.5% at 100 μg/mL, suppressing mRNA levels of PPARγ and C/EBPα (Park et al., 2013). Similarly, mulberry leaf ethanol extract reduced fat accumulation and protein levels of adipogenesis-related factors, including PPARγ, C/EBPα, and FAS, without cellular toxicity (Yang et al., 2014). The potential of M. alba for weight management extends beyond adipogenesis inhibition, encompassing digestive enzyme inhibition, improvements in glucose and lipid metabolism, and attenuation of adiposity. These effects have been demonstrated in vitro, in vivo, and in clinical investigations, suggesting the potential of M. alba as a safe and efficient supplement for healthy weight management (Ntalouka & Tsirivakou, 2024). Salacia reticulata, used in Ayurvedic medicine, contains unique compounds like salacinol and kotalanol that exhibit potent anti-diabetic and anti-obesity effects. These compounds are powerful inhibitors of intestinal α-glucosidase and pancreatic lipase, with de-O-sulfonated kotalanol being the most potent inhibitor of maltase-glucoamylase reported (Sim et al., 2010). According to Stohs & Ray (2015), Salacia extracts modulate multiple targets affecting carbohydrate and lipid metabolism, including α-glucosidase, aldose reductase, and peroxisomal proliferator-activated receptor-α. This human studies have shown that Salacia extracts decrease plasma glucose, insulin, and HbA1c levels, and modulate serum lipids without adverse effects. In addition, animal studies demonstrated similar results, along with weight loss and decreased weight gain. The multi-target action of Salacia roots, attributed to compounds like mangiferin, salacinol, and kotalanol, contributes to their effectiveness in preventing and treating diabetes and obesity (Li et al., 2008).

In addition to these well-known plants, recent studies have highlighted the potential of Orthosiphon stamineus (misai kucing), a Southeast Asian plant, in regulating adipogenesis and lipid metabolism. Ethanolic extracts of O. stamineus demonstrated significant anti-obesity and lipid-lowering effects in high-fat diet-induced

obese mice, reducing body weight gain and serum lipid levels while enhancing antioxidant activity (Seyedan et al., 2016). In vitro studies using 3T3-L1 adipocytes showed that O. stamineus extracts increased adipogenesis and glucose uptake, suggesting insulin-mimicking effects (Azahari et al., 2015). Additionally, O. stamineus extracts exhibited potential for managing diabetes by influencing glucose metabolism in diabetic cell lines (Adnan et al., 2024). These findings align with broader research on Asian medicinal plants, which have shown promise in regulating obesity-related mechanisms such as adipogenesis, lipogenesis, and lipolysis through various molecular pathways, including AMPK activation (Chung et al., 2021). Morover, Centella asiatica and its triterpenes have demonstrated potential therapeutic effects across various diseases, including cardiovascular and neurological conditions (Razali et al., 2019; Sun et al., 2020). The plant exhibits anti-inflammatory, antioxidant, and anti-apoptotic properties (Sun et al., 2020). Recent studies have also highlighted the anti-obesity potential of C. asiatica. A hot-water extract of C. asiatica (CHE) showed significant antioxidant activity and inhibition of lipase activity. CHE suppressed the expression of adipogenesis-related genes in 3T3-L1 preadipocytes, including PPAR-γ, CEBP-α, FAS, and SCD1, suggesting an anti-obesity effect through inhibition of adipocyte development and lipid accumulation (Hong et al., 2023).

Collectively, these examples illustrate the diversity of medicinal plants with potential benefits for metabolic health. While many have undergone substantial pharmacological and clinical evaluation, others, particularly those from underexplored tropical regions, remain to be studied in depth. The varied mechanisms of action, ranging from transcriptional regulation of adipogenic pathways to modulation of oxidative stress and gut microbiota, highlight the breadth of therapeutic strategies offered by botanical interventions.

2.5.4 Phytochemicals and Their Biological Activities

The therapeutic properties of medicinal plants derive largely from their phytochemical constituents, which encompass a vast array of structurally diverse compounds with distinct yet often overlapping biological activities (Nyamai et al., 2016). These bioactive compounds, including polyphenols, alkaloids, and terpenoids, exhibit antioxidant, antimicrobial, and anticancer properties (Nyamai et al., 2016). Understanding the relationship between specific phytochemicals and their bioactivities

is crucial for elucidating the mechanisms by which medicinal plants exert their effects on metabolic health.

Polyphenols are among the most extensively studied phytochemical classes in the context of obesity and metabolic disorders. Flavonoids have emerged as potential therapeutic agents for obesity and metabolic disorders. Studies suggest that these compounds modulate adipogenesis, enhance fatty acid oxidation, and improve metabolic health through various mechanisms (Saikia et al., 2025). Flavonoids such as quercetin, catechins, and resveratrol have been shown to activate AMP-activated protein kinase (AMPK), a key regulator of energy metabolism and lipid homeostasis (Tanabe et al., 2017; Saikia et al., 2025). This activation leads to increased glucose uptake, reduced lipid synthesis, and attenuated inflammation (Saikia et al., 2025). Meanwhile, phenolic acids have also shown promising effects in ameliorating metabolic disorders and diabetes-related complications. Studies demonstrate that these compounds can alleviate features of metabolic syndrome, including hyperlipidemia, glucose intolerance, and oxidative stress (Guo et al., 2017). Caffeic, ferulic, gallic, and protocatechuic acids have been found to reverse insulin resistance, hyperglycemia, dyslipidemia, inflammation, and oxidative stress in high-fructose diet-induced metabolic syndrome rats (Ibitoye & Ajiboye, 2018). These phenolic acids exhibit strong antioxidant properties, enhancing the activities of antioxidant enzymes and reducing oxidative stress biomarkers (Ibitoye & Ajiboye, 2018; Kiokias et al., 2020). Additionally, they modulate carbohydrate and lipid metabolism, improve adipose tissue metabolism, and attenuate inflammatory processes. The diverse effects of different phenolic acids suggest potential for developing combinations or formulas with comprehensive benefits for metabolic syndrome management (Guo et al., 2017).

Terpenoids represent another important class, with triterpenes such as ursolic acid (UA) and oleanolic acid (OA) exhibiting promising anti-inflammatory and anti-obesity effects. These compounds inhibit adipogenesis by downregulating key transcription factors like PPARγ, C/EBPα, and SREBP-1c. UA activates the LKB1/AMPK pathway, leading to increased fatty acid oxidation and decreased lipogenesis in adipocytes (He et al., 2013). Both UA and OA exhibit hepatoprotective properties and can alleviate chemically induced liver injury. Their anti-inflammatory effects are mediated through the inactivation of NFκB, STAT3/6, and Akt/mTOR pathways (Kashyap et al., 2016). These triterpenes also show potential in managing metabolic syndrome by regulating various factors involved in adipogenesis, lipolysis,

insulin resistance, and inflammation (Sharma et al., 2018). Saponins, another bioactive compound found in plants like ginseng and fenugreek, have also been shown to modulate lipid metabolism through various mechanisms. They inhibit pancreatic lipase, reducing dietary fat absorption and leading to weight gain reduction in mice (Karu et al., 2007). Hydrolysed saponins from fenugreek and quinoa demonstrate improved pancreatic lipase inhibition and hypocholesterolemic effects (Navarro del Hierro et al., 2021). Saponins regulate lipid metabolism by suppressing appetite, activating the AMPK signalling pathway, and modulating gut-liver interactions through effects on gut microbes and their metabolites (Cao et al., 2024). These plant-derived compounds offer potential alternatives to conventional pancreatic lipase inhibitors like orlistat for combating obesity.

Furthermore, alkaloids like berberine and capsaicin have shown promising effects on metabolic regulation and insulin resistance. Berberine, found in plants such as Coptis chinensis, demonstrates antidiabetic and anti-obesity properties by enhancing insulin signalling, reducing adiposity, and improving lipid metabolism (Ntakirutimana, 2025). It undergoes extensive metabolism, with active metabolites contributing to its pharmacological effects, including hypoglycemic and hypolipidemic activities (Kun et al., 2017). Berberine activates AMPK, improves glucose uptake, and benefits cardiovascular health (Affuso et al., 2010). Capsaicin, along with other alkaloids like piperine and trigonelline, has been shown to increase insulin sensitivity and reduce insulin resistance in preclinical and clinical studies. These alkaloids improve various markers of insulin sensitivity, including the Matsuda index, HOMA-IR, and HOMA-β, while also reducing fasting blood glucose, insulin, and HbA1c levels (Eddouks & Amssayef, 2023). Meanwhile, ligans, found abundantly in flaxseed and sesame, exhibit various cardiometabolic benefits. These compounds can improve lipid profiles, reduce body fat, and enhance insulin sensitivity (Biasiotto et al., 2014). Flaxseed lignans, particularly secoisolariciresinol diglucoside (SDG), are converted to enterodiol and enterolactone in the colon, potentially inhibiting tumor growth (Toure & Xu, 2010). Sesame lignans, including sesamin and sesamolin, modulate fatty acid metabolism, inhibit cholesterol absorption, and display antioxidant properties (Kamal-Eldin et al., 2011). Flaxseed supplementation also has been shown to improve various cardiometabolic risk factors, including body weight, blood pressure, lipid levels, and markers of inflammation and oxidative stress.

Recent metabolomics studies have revealed insights into the cellular effects of phytochemicals. For instance, quercetin treatment of hypertrophied adipocytes led to decreased intracellular reactive oxygen species (ROS), correlating with quercetin metabolite levels (Herranz-López et al., 2017). Quercetin also modulates mitochondrial processes, including biogenesis, membrane potential, and ATP production (de Oliveira et al., 2016). Curcumin treatment of breast cancer cells showed biphasic effects on glutathione metabolism, increasing levels at low doses but decreasing at high doses (Bayet-Robert & Morvan, 2013). It also altered lipid metabolism, causing accumulation of free fatty acids at high doses. Similarly, xanthohumol treatment in obese rats decreased products of dysfunctional fatty acid oxidation and ROS, while increasing uncoupled respiration and activating glutathione recycling in myocytes (Kirkwood et al., 2013). These studies demonstrate how metabolomics can elucidate the complex cellular responses to phytochemicals, revealing their potential therapeutic mechanisms. Notably, phytochemical interactions in whole foods and plant extracts can produce synergistic effects that enhance their biological activities. These interactions can amplify antioxidant, anti-inflammatory, and anti-carcinogenic properties beyond what individual compounds achieve alone (Phan et al., 2018; Chen et al., 2021). Synergistic effects are particularly relevant in managing hyperlipidemia, where combinations of phytochemicals can regulate multiple metabolic pathways simultaneously, potentially offering more effective treatment than single-target approaches (Liu et al., 2022). Taken together, as interest in plant-derived therapeutics continues to grow, it becomes increasingly important to examine specific botanical sources rich in such compounds. Thus, in this study, the plant of interest is *Parkia speciosa*, a traditional medicinal species widely used in Southeast Asia. The following section provides an in-depth look at the botanical and ethnopharmacological profile of *Parkia speciosa*, laying the groundwork for its relevance in the context of metabolic health interventions.

2.6 *Parkia speciosa* Hassk.

2.6.1 Overview of *Parkia speciosa*

Parkia speciosa Hassk., commonly referred to as “stink bean” or locally known as “petai” in Malaysia and Indonesia, is a leguminous plant widely distributed throughout Southeast Asia, particularly in Malaysia, Thailand, Indonesia, and the

Philippines (Kamisah et al., 2013). Belonging to the Fabaceae family (formerly Leguminosae or Mimosaceae), *P. speciosa* is a perennial tropical tree recognised for its elongated, flat pods containing bright green, almond-shaped seeds with a distinct aroma and flavor, often described as earthy or reminiscent of shiitake mushrooms (Figure 2.3). These seeds are commonly consumed in traditional Southeast Asian cuisines, either raw, blanched, or cooked in various local dishes. While the seeds are typically the most consumed part, the pods, which constitute over 60% of the biomass, are frequently discarded despite holding potential as a valuable source of phytochemicals (Azizul et al., 2019).

Figure 2.3 *Parkia speciosa* Fruit (left). *P. speciosa* Fruit Showing the Bean and Pod (right).

Ethnobotanically, *P. speciosa* holds a longstanding place in traditional medicine systems across the region. Indigenous communities in Peninsular Malaysia have historically employed the seeds, pods, and roots of this plant in managing chronic ailments, most notably diabetes, hypertension, and infections. The therapeutic reputation of *P. speciosa* is strongly supported by emerging scientific findings, which have highlighted its diverse pharmacological properties. Notably, several studies have reported its anti-inflammatory (Mustafa et al., 2018; Gui et al., 2019), antidiabetic (Sonia et al., 2018), antioxidant (Ghasemzadeh et al., 2018), antihypertensive (Kamisah et al., 2017), antibacterial, and immunomodulatory effects (Fitrya et al., 2020). These biological effects are largely attributed to the rich profile of secondary metabolites from the plant, including phenolic acids, flavonoids, carotenoids, glucosinolates, isothiocyanates, and folates (Singhania et al., 2021).

Its multifaceted bioactivity positions *P. speciosa* as a promising candidate in the development of functional foods or plant-based therapeutics, especially in the context of metabolic disorders such as obesity and type 2 diabetes, where inflammation and oxidative stress are prominent contributors to disease progression (Kamisah et al., 2013; Azemi et al., 2022). Moreover, the traditional use of *P. speciosa* aligns with the broader trend of re-evaluating local ethnobotanical knowledge through a scientific lens, particularly in the quest for safer, more holistic alternatives to synthetic drugs that often carry undesirable side effects. The resurgence of interest in underutilised tropical species like *P. speciosa,* specifically its pods, underscores the need for in-depth biochemical and pharmacological studies to validate and harness their therapeutic potential. Thus, the exploration of *P. speciosa* pods*,* in the context of adipogenesis not only contributes to understanding its traditional medicinal value but also opens new possibilities for utilising plant-based interventions in obesity-related research.

2.6.2 *Parkia speciosa* Pods

While *Parkia speciosa* seeds have traditionally gained more attention in culinary and medicinal contexts, growing interest has recently turned toward its pods, an abundant but often overlooked byproduct, as a potential reservoir of bioactive phytochemicals. Comprising more than 60% of the total biomass of the plant, these empty pods are typically discarded following seed extraction and have long been regarded as agricultural waste. However, emerging phytochemical analyses suggest that the pods contain a rich array of secondary metabolites, many of which exhibit pharmacological properties relevant to metabolic health (Saleh et al., 2021). Ko et al. (2014) reported that ethanolic extracts of *P. speciosa* pods contain several notable polyphenols and flavonoids, including catechin, ellagic acid, kaempferol, p-coumaric acid, gallic acid, and quercetin, compounds widely known for their antioxidant, anti-inflammatory, and metabolic regulatory functions. The presence of these constituents aligns with the broader phytochemical trends observed in many leguminous species, particularly those within the Fabaceae family, which are increasingly recognised for their therapeutic relevance in non-communicable diseases such as obesity, diabetes, and cardiovascular disorders.

Each of these bioactive compounds contributes uniquely to the suppression of adipogenesis and modulation of lipid metabolism. Rutin and betanin, demonstrated anti-

adipogenic effects by attenuating the expression of PPARγ, C/EBPα, and SREBP-1c (Chyau et al., 2018). Similarly, kaempferol, a naturally occurring flavonol, exerts its anti-adipogenic effects by modulating pathways such as AMPK and Wnt/β-catenin signalling, both of which are crucial in the regulation of adipocyte fate (Lee et al., 2015; Xu et al., 2024). Ellagic acid, another polyphenolic compound detected in the pods, has been associated with enhanced lipolysis and decreased triglyceride accumulation (Woo et al., 2015; Okla et al., 2015). Furthermore, p-coumaric acid and gallic acid are phenolic acids known to attenuate inflammation and oxidative stress, two hallmarks of dysfunctional adipose tissue, by modulating NF-κB signalling and scavenging reactive oxygen species (Abdel-Moneim et al., 2018). Quercetin, one of the most studied plant flavonoids, exerts a multitude of biological effects, including the inhibition of lipid accumulation, promotion of mitochondrial biogenesis, and restoration of insulin sensitivity in hypertrophied adipocytes (de Oliveira et al., 2016; Akan et al., 2024).

Despite this promising phytochemical profile, the functional characterisation of *P. speciosa* pod extract in the context of adipocyte biology remains limited. Most of the existing studies have focused on either isolated compounds or whole seed extracts, with relatively few investigating the pod-derived bioactives as a complex mixture (Gao et al., 2021; Nurdyansyah & Widyastuti, 2024). This gap is particularly noteworthy considering that the synergistic interactions among phytochemicals often yield more pronounced biological effects than those observed from individual compounds alone. Moreover, while several in vitro and in vivo studies have hinted at the potential of pod-derived compounds to modulate lipid metabolism and inflammatory pathways, the exact mechanisms by which these compounds interact with intracellular targets involved in adipogenesis are still not fully understood (Xinran Ma et al., 2018).

Furthermore, the metabolic influence of *P. speciosa* pod constituents on broader signalling networks remains underexplored. The lack of metabolomics-based data also limits the understanding of how these bioactives shift the metabolic landscape within adipocytes, particularly regarding lipid intermediates, amino acid utilisation, and cofactor biosynthesis (Garcia et al., 2024). Considering the global rise in obesity and metabolic syndrome, exploring the adipose-modulating potential of natural plant materials, such as *P. speciosa* pods, is not only scientifically relevant but also economically and environmentally sustainable. Given this context, the present study aims to advance current knowledge by investigating the metabolomic changes associated with the intervention of *Parkia speciosa* pod extract (PSPE) in a well-

established adipocyte model. By profiling the metabolic shifts that occur upon treatment, this research aims to clarify the biofunctional role of pod-derived phytochemicals and elucidate their potential mechanisms in inhibiting adipogenesis.

2.6.3 Potential of *Parkia speciosa* Pods in Inhibition of Adipogenesis, Hyperlipidemia and Obesity

In recent years, *Parkia speciosa* pods have gained increasing attention not only for their phytochemical richness but also for their potential in modulating metabolic disorders such as adipogenesis, hyperlipidemia, and obesity (Chhikara et al., 2018; Saleh et al., 2021; Azemi et al., 2022). While much of the traditional and scientific interest has centered on the seeds, emerging research suggests that the pods contain a reservoir of bioactive compounds with promising therapeutic relevance. As discussed in previous sections, the pods are rich in flavonoids, phenolic acids, and other phytochemicals known for their antioxidant and anti-inflammatory properties. Several of these compounds, such as catechin (Furuyashiki et al., 2004), kaempferol (Mosqueda-Solís et al., 2017), ellagic acid, and p-coumaric acid (Aranaz et al., 2019), have been individually demonstrated in other studies to interfere with the adipogenic process by downregulating key transcription factors such as PPARγ and C/EBPα, suppressing lipid accumulation, and promoting lipolytic pathways. These mechanisms directly address the cellular events behind adipocyte differentiation, making pod-derived bioactives particularly appealing as anti-obesity agents. Moreover, the antioxidant-rich profile of *P. speciosa* pods plays a crucial role in mitigating oxidative stress, a known driver of adipocyte hypertrophy and metabolic dysfunction (Azemi et al., 2022).

Beyond the cellular level, the impact of *P. speciosa* pods may extend to systemic lipid metabolism (Nurdyansyah & Widyastuti, 2024). Hyperlipidemia, a common comorbidity in obesity, is characterised by elevated levels of triglycerides, LDL cholesterol, and total lipids (Klop et al., 2013; Mumthaj et al., 2021). The presence of bioactive compounds in *P. speciosa* pods, such as quercetin, gallic acid, and kaempferol, are widely recognised for their hypolipidemic effects, suggesting potential in improving lipid profiles through multiple pathways. These compounds have been shown to exert statin-like effects without the associated side effects such as hepatotoxicity or myopathy, making them promising alternatives for long-term

management of dyslipidemia (Azemi et al., 2022; Cheong et al., 2024). Additionally, the fiber content and tannins in *P. speciosa* pods could contribute to reduced intestinal fat absorption and improved lipid excretion, further supporting their anti-hyperlipidemic potential. Although studies specifically evaluating the anti-obesity effects of the pods are limited, the convergence of antioxidant, anti-adipogenic, and lipid-lowering properties observed in preliminary *in vitro* and phytochemical studies supports their potential as a functional food or nutraceutical (Chhikara et al., 2018; Azemi et al., 2022).

Notably, the impact of the pods on metabolic health aligns with a growing scientific interest in using plant-based strategies to counteract diet-induced obesity. This is particularly relevant in regions like Southeast Asia, where *P. speciosa* is both culturally significant and readily available, offering a sustainable approach to combat obesity-related diseases. As obesity and hyperlipidemia continue to pose public health challenges, especially in Malaysia where prevalence rates are among the highest in Asia (Mohamed-Yassin et al., 2021), the exploration of *P. speciosa* pods as an anti-adipogenic agent is not only scientifically promising but also socioeconomically relevant. Nevertheless, the underlying mechanisms through which these phytochemicals exert their effects, especially within the context of cellular metabolic pathways, remain underexplored. Given the paucity of research focusing specifically on the pods and their interaction with adipocyte metabolism, further studies, including metabolomics-based approaches, are warranted to comprehensively map their effects. This study, therefore, aims to fill that gap by employing 3T3-L1 adipocytes as a model system to investigate the intervention effects of *Parkia speciosa* pod extract (PSPE) on adipogenesis, offering new insights into the biochemical and functional significance of an underutilised plant resource. Understanding the potential of *P. speciosa* pods in combating obesity requires not only identifying their phytochemical composition but also elucidating the molecular mechanisms by which they modulate adipocyte biology.

2.7 Extraction and Preparation of Plant Extract

2.7.1 Extraction of Plant Bioactives

Extraction is a critical step in plant-based experimental research, serving as the primary means of obtaining bioactive compounds for subsequent biological evaluation.

In the context of *in vitro* and *in vivo* studies, including investigations on adipogenesis, lipid metabolism, and related cellular pathways, the efficiency and accuracy of the extraction process directly determine the quality, reproducibility, and interpretability of experimental outcomes (Mosić et al., 2020). The purpose of extraction in such contexts is not merely to obtain a crude mixture of plant constituents, but rather to maximize the recovery of target phytochemicals, such as phenolics, flavonoids, alkaloids, terpenoids, and saponins, while preserving their structural integrity and bioactivity (Bitwell et al., 2023). These phytochemicals often serve as functional agents in modulating biological processes, whether through antioxidant, anti-inflammatory, or metabolic regulatory pathways (Tan et al., 2011; Spagnuolo et al., 2023).

Unlike extraction for analytical profiling, which focuses on comprehensive metabolite identification, extraction for intervention purposes prioritizes reproducibility in compound yield, chemical stability, and suitability for biological assays. This requires a careful balance between exhaustive recovery and minimal degradation of thermolabile or oxidation-sensitive constituents (Mushtaq et al., 2014). The selection of an extraction method is therefore dictated by factors such as the polarity of target compounds, solvent safety, especially for downstream cell culture work, extraction efficiency, scalability, and compliance with laboratory safety and environmental standards (Jibhkate et al., 2023). For example, aqueous ethanol or methanol mixtures are commonly chosen for polyphenol-rich extracts due to their ability to dissolve a broad polarity range of metabolites, while also being relatively safe for handling and compatible with cell-based assays after solvent removal (Mohammedi & Atik, 2011).

Furthermore, the quality of the plant extract is inherently linked to the chemical nature of its constituents. Many bioactive molecules are prone to degradation when exposed to excessive heat, light, oxygen, or unsuitable pH conditions (Nishad, 2022). Therefore, the extraction process must be designed to minimize exposure to degradative factors, both during and after the extraction phase (Jones & Kinghorn, 2012). The importance of extraction extends beyond simple yield. Suboptimal methods can lead to the loss of key compounds, the presence of unwanted degradation products, or variability between batches, all of which can confound biological results (Duportet et al., 2012). Given that downstream biological assays, including those involving 3T3-L1 adipocytes, rely on consistent and well-characterised plant extracts, the implementation of rigorous extraction protocols forms the foundation of experimental validity.

2.7.2 Sample Collection and Pre-Treatment

The preparation of a plant extract begins well before the actual extraction step, as the collection, handling, and pre-treatment of plant material significantly influence the final chemical composition and bioactivity of the extract (Jones & Kinghorn, 2012). Factors such as plant part selection, maturity stage, seasonal timing, and geographic origin contribute to the inherent variability of phytochemical content (Ramesh et al., 2024). For example, flavonoid concentration may be higher in certain developmental stages or specific plant parts, meaning that careful selection is crucial when aiming to study bioactivity linked to these compounds (Julkunen-Tiitto et al., 2015). In the context of experimental reproducibility, standardizing the source material is essential, ideally by collecting from the same location, at a consistent maturity stage, and under similar environmental conditions.

Once harvested, plant materials are typically subjected to cleaning and drying to prevent microbial growth and enzymatic degradation (Krakowska-Sieprawska et al., 2022). Drying methods are chosen based on the chemical sensitivity of target compounds. Oven drying at controlled temperatures, generally below 50 to 60°C, can be efficient but may lead to partial degradation of heat-sensitive molecules, such as certain polyphenols and volatile terpenes (Elgamal et al., 2023). Shade drying and freeze-drying (lyophilization) are often preferred for preserving thermolabile phytochemicals, with freeze-drying offering the added benefit of removing moisture under low temperature and vacuum, thereby minimizing oxidative reactions (Pinela et al., 2011; Ma et al., 2022). Shade drying, although slower, is more cost-effective and less equipment-intensive, making it a common method in resource-limited settings (Babiker et al., 2010).

Grinding or milling of dried plant material is a key pre-treatment step aimed at increasing surface area and facilitating solvent penetration during extraction (Alsaud & Farid, 2020; Sagili et al., 2023). The particle size should be optimised to allow efficient solvent contact without generating excessive heat from mechanical action, which could promote degradation (Krakowska-Sieprawska et al., 2022). The ground material should then be stored under conditions that minimize exposure to light, oxygen, and moisture, typically in airtight, opaque containers at low temperatures (Milay et al., 2020). Even short-term storage can lead to measurable losses in sensitive compounds, so rapid progression from grinding to extraction is generally advisable. Importantly, for extracts

intended for cell culture work, consideration must also be given to potential contaminants introduced during collection and pre-treatment. Soil residues, environmental pollutants, and microbial contaminants can interfere with biological assays or introduce confounding variables (Nims & Price, 2017). Therefore, standardized cleaning procedures, validated drying protocols, and hygienic handling are as critical as the extraction itself in ensuring the scientific integrity of plant-based intervention studies.

2.7.3 Solvent-Based Extraction Techniques

Solvent-based extraction remains the most widely used approach for obtaining plant bioactives, primarily due to its versatility, relative simplicity, and ability to target compounds across a broad polarity spectrum (Jibhkate et al., 2023). The choice of solvent is arguably the most important parameter, as it dictates both the yield and chemical profile of the extract. For polyphenols, flavonoids, and other polar to semi-polar metabolites, aqueous solutions of ethanol or methanol are generally preferred (Ng et al., 2020). These solvents offer strong solubilizing capacity while also being relatively safe to handle and easy to remove under reduced pressure. For non-polar constituents, such as lipids, terpenoids, and certain alkaloids, solvents like hexane, chloroform, or dichloromethane are employed, although their use in bioassay-directed studies may be limited due to toxicity concerns (Prache et al., 2016; Funari et al., 2023).

Among conventional solvent-based techniques, maceration is one of the oldest and simplest methods. It involves soaking plant material in the chosen solvent at room temperature or slightly elevated temperatures for extended periods, with occasional agitation to improve mass transfer (Blicharski & Oniszczuk, 2017). While maceration is gentle and preserves thermolabile compounds, it is time-consuming and often less efficient in extracting the full complement of bioactives (Jha & Sit, 2021). Soxhlet extraction, in contrast, provides continuous solvent cycling over the plant material, ensuring exhaustive extraction within a shorter time frame. However, it typically requires elevated temperatures and prolonged heating, which may not be suitable for all phytochemicals (Danlami et al., 2014).

Modern adaptations of solvent extraction aim to improve efficiency, reduce solvent consumption, and preserve compound integrity. Deep eutectic solvents (DESs) have emerged as promising alternatives to traditional toxic organic solvents, especially

when combined with techniques like ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) (Fu et al., 2025). Ultrasound-assisted extraction (UAE) is an efficient method for obtaining food additives, nutraceuticals, and oils from various biosources (Syahir et al., 2020; Laurora et al., 2021). UAE utilises acoustic cavitation to enhance extraction efficiency by fragmenting raw materials, improving solvent penetration, and increasing mass transfer of intracellular compounds (Laurora et al., 2021). This technique offers several advantages over conventional methods, including reduced extraction time, lower solvent consumption, and the ability to operate at lower temperatures, making it suitable for heat-sensitive compounds (Syahir et al., 2020). Microwave-assisted extraction (MAE) is another innovative technique that uses microwave energy to rapidly heat solvents and plant matrices, accelerating extraction kinetics through improved mass and heat transfer (Veggi et al., 2012). While MAE is efficient, care must be taken to avoid overheating and consequent degradation of sensitive compounds.

The choice between these methods is typically guided by the chemical nature of the target compounds, available equipment, and the intended downstream application of the extract. For cell culture experiments, extraction parameters should also be optimised to ensure that residual solvent levels remain well below cytotoxic thresholds after concentration and drying (Zheng Ser et al., 2015). In many studies, the crude extract is subsequently reconstituted in a biocompatible solvent such as dimethyl sulfoxide (DMSO) or phosphate-buffered saline (PBS) prior to dosing cells (Nguyen et al., 2020), underscoring the importance of solvent compatibility at both extraction and application stages.

2.7.4 Non-Solvent-Based Extraction Techniques

In recent years, growing environmental awareness and safety considerations have driven the adoption of non-solvent and ‘green’ extraction technologies for plant bioactives. These methods aim to minimize or eliminate the use of hazardous organic solvents, reduce energy consumption, and improve selectivity for target compounds (Soquetta et al., 2018). One prominent example is supercritical fluid extraction (SFE), most commonly performed with carbon dioxide (CO_2) as the extraction medium. Supercritical CO_2 behaves as both a gas and a liquid, allowing it to penetrate plant tissues and dissolve non-polar to moderately polar compounds effectively (Sapkale et

al., 2010). By adjusting temperature and pressure, the solvating power of CO_2 can be fine-tuned, and the addition of small amounts of co-solvents such as ethanol can broaden its polarity range (Wrona et al., 2017). SFE is particularly valued for its eco-friendliness, short processing times, ability to produce residue-free extracts, and suitability for high value bioactives, although its equipment costs can be prohibitive for routine laboratory use (Bimakr & Ganjloo, 2016; Herzyk et al., 2024).

Pressurized liquid extraction (PLE) is an advanced technique that uses elevated temperatures and pressures to enhance extraction efficiency from various matrices (Raut et al., 2015). This method offers several advantages over conventional extraction, including reduced solvent usage, shorter extraction times, and automation (Raut et al., 2015). PLE is particularly effective for extracting bioactive compounds such as carotenoids, phenols, and lipids from food and herbal sources (Mustafa et al., 2012). The technique's efficiency can be optimised by adjusting parameters like extraction time, temperature, and solvent choice (Mustafa et al., 2012). PLE's versatility extends to the extraction of food contaminants, including environmental pollutants, pesticides, and mycotoxins (Barp et al., 2023). Other emerging techniques include enzyme-assisted extraction (EAE) and pulsed electric field (PEF) extraction are innovative techniques for recovering valuable compounds from plants and microorganisms. EAE uses hydrolytic enzymes to degrade cell walls, facilitating solvent penetration and metabolite elution (Łubek-Nguyen et al., 2022). PEF applies high-voltage pulses to disrupt cell membranes, enabling selective release of intracellular compounds without significant heating (Martínez et al., 2020; Vorobiev & Lebovka, 2022). Both methods offer advantages over conventional extraction techniques, including improved yields, reduced solvent use, and shorter processing times (Ranjha et al., 2021). These methods are particularly attractive for maintaining the bioactivity of thermolabile compounds, though their adoption in phytochemical research remains limited by equipment availability and cost.

From a cell-based research perspective, the primary advantage of these alternative methods lies in their ability to produce extracts with minimal solvent residues and reduced degradation of active compounds, thereby increasing the reproducibility and biological relevance of experimental findings. However, scalability, cost, and method standardization remain practical challenges. Regardless of the chosen extraction technique, rigorous documentation of parameters such as solvent type, ratio,

extraction time, temperature, and post-processing conditions is essential to ensure reproducibility and facilitate comparison across studies.

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Overall Workflow

The methodology employed throughout this study was specifically structured into three main phases, each meant to fulfill the specific research objectives. Figure 3.1 depicts a schematic representation of the experimental procedure used to investigate the effects of *Parkia speciosa* pod extract (PSPE) intervention in 3T3-L1 adipocytes differentiation.

To achieve the first research objective, the study began with the preparation of PSPE. During the initial phase, extraction and freeze-drying procedures were utilised to obtain a well-characterised plant extract for further investigation. Following that, the phytochemical composition and antioxidant activities of PSPE were examined using a total phenolic content (TPC) and total flavonoid content (TFC), as well as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and ferric reducing antioxidant power (FRAP) assay, respectively. High-Performance Liquid Chromatography (HPLC) was used to identify and quantify phytochemical constituents, and antioxidant assays was utilised to determine the antioxidant capacity of the plant extract.

The second phase of the study was designed to address the second research objective, which involved evaluating the effects of PSPE on lipid accumulation in 3T3-L1 adipocytes. This was achieved through a sequence of in vitro tests, which started with the cultivation and differentiation of 3T3-L1 preadipocytes into mature adipocytes, followed by treatment with PSPE and a commercial lipid-lowering drug for comparative analysis. In this phase, MTT assay was first performed to evaluate the viability of the treated cells. Based on the MTT assay results, several concentrations of PSPE were chosen and proceeded with oil red O staining assay to determine one optimum concentration for the treatment study. Following that, the accumulation of lipids was evaluated using oil red O staining and a triglyceride assay, revealing the anti-adipogenic effects of PSPE.

The final stage of the methodology focused on a comprehensive metabolomic analysis to assess the impact of PSPE on the metabolic profile of 3T3-L1 adipocytes differentiation. This was accomplished using Liquid Chromatography-Mass

Spectrometry Quadrupole Time-of-Flight (LCMS-Q-TOF), a high-resolution analytical approach that allowed for the detection of metabolite changes and metabolic pathways related caused by PSPE treatment.

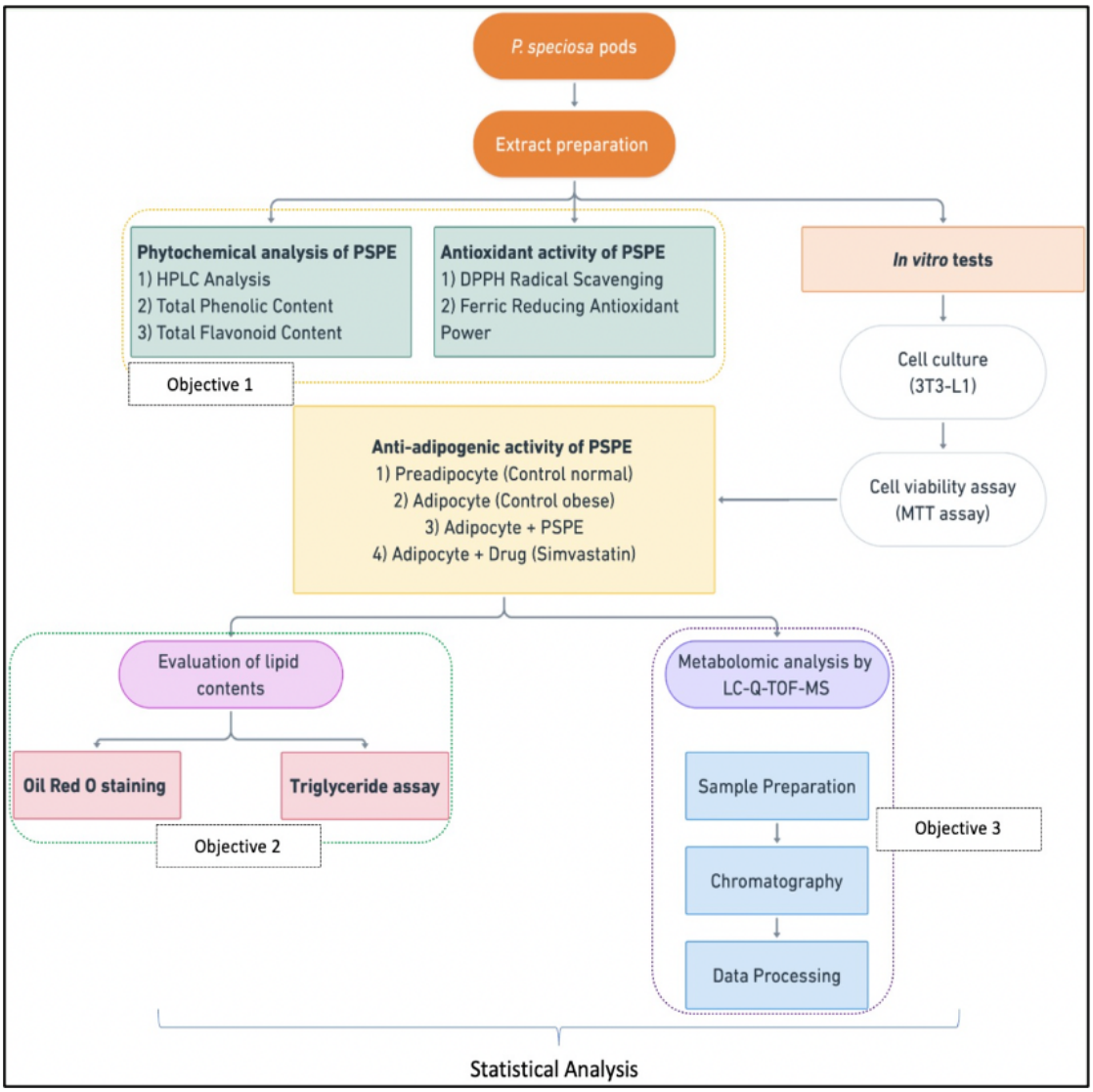

Figure 3.1 Flowchart of the Study on the Intervention Effect of *P. speciosa* Pod Extract (PSPE) on 3T3-L1 Adipocytes Differentiation.

3.1.1 Plant Material

Parkia speciosa fruits were obtained in March 2022 from a local market in Kuala Krai, Kelantan, Malaysia. The fruits were selected at a mature stage, as evidenced by the presence of fully developed elongated pods, a dark green to greenish-brown pod colour, firm texture, and completely formed seeds, which are characteristic features of mature *P. speciosa* fruits. The plant material was subsequently authenticated, and a

voucher specimen (KM 0051/23) was deposited at the Universiti Putra Malaysia (UPM) Herbarium for future reference.

3.1.2 3T3-L1 Cells

The 3T3-L1 mouse embryonic fibroblast cells (ATCC CL-173) were obtained from the American Type Culture Collection (ATCC, USA).

3.1.3 Chemicals and Reagents

Table 3.1
List of the Main Chemicals and Reagents Used in the Study.

Chemicals and Reagents	Manufacturer
Extraction	
Absolute ethanol	QREC Asia, Rawang, Malaysia
Phytochemical Analysis of PSPE	
Quercetin	MedChem Express, New Jersey, USA
Gallic acid	MedChem Express, New Jersey, USA
p-Coumaric acid	MedChem Express, New Jersey, USA
Catechin	MedChem Express, New Jersey, USA
Ellagic acid	MedChem Express, New Jersey, USA
Acetonitrile	ChromAR, New Jersey, USA
Cell Culture	
Dulbecco's Modified Eagle's Medium (DMEM)	Nacalai Tesque, Kyoto, Japan
Calf bovine serum (CBS)	ATCC, Virginia, USA
Fetal bovine serum (FBS)	Capricorn Scientific, Frankfurt, Germany
Insulin	Nacalai Tesque, Kyoto, Japan
Antibiotics (100 units/mL penicillin, 100 µg/mL streptomycin)	Nacalai Tesque, Kyoto, Japan
Isobutylmethylxanthine (IBMX)	Merck KGaA, Darmstadt, Germany
Dexamethasone	MedChem Express, New Jersey, USA
TrypLE™ Express Enzyme	Thermo Fisher, Massachusetts, USA
Phosphate buffered saline (PBS)	Invitrogen, California, USA
Oil Red O Assay	
Oil red O	R&M, Semenyih, Malaysia
Isopropyl alcohol	R&M, Semenyih, Malaysia
Triglyceride Assay	
Triglyceride Colorimetric Assay Kit	Elabscience, Wuhan, China

Metabolomics Analysis	
Methanol	Merck KGaA, Darmstadt, Germany

3.2 Preparation of *P. speciosa* Pod Extract (PSPE)

3.2.1 Preparation of *P. speciosa* Pod Powder

The beans of *P. speciosa* were carefully separated from the fruit, and the pods were collected for further processing. Approximately 2 kg of *P. speciosa* pods were then chopped into smaller pieces, thoroughly cleaned by using distilled water to remove any surface contaminants, before dried in an oven at 40°C for three days to ensure complete moisture removal. After drying, the pods were ground finely with an electric grinder to obtain a homogenous powder. The resulting powder was stored in sealed containers at room temperature to ensure stability and prevent degradation.

3.2.2 *P. speciosa* Pod Extraction

The *P. speciosa* ground pods were exhaustively extracted following the method described by Fithri et al. (2019) with minor adjustments and followed by filtration which was executed using Whatman filter paper No.1. Briefly, the ground pods were soaked with 70% ethanol at room temperature, with periodic solvent replacement every 3-4 days, continuing until the solution attained a colourless state. The pooled extract solution was evaporated with a rotary evaporator under reduced pressure at 45°C, removing ethanol. The pooled extracts were concentrated by using a vacuum concentrator for 2 hours to further eliminate excess liquid. Lastly, the concentrated extract went through freeze-drying and was kept at -20°C for subsequent utilisation. The percent yield of the *P. speciosa* pod extract was calculated using the following formula:

$$\text{Yield (\%)} = \frac{\text{Final extract weight } (g)}{\text{Initial material weight } (g)} \text{ X } 100 \qquad (3.1)$$

3.3 Phytochemical Analysis of *P. speciosa* Pod Extract (PSPE)

3.3.1 HPLC Analysis

Reverse-phase High-Performance Liquid Chromatography (HPLC) analysis was performed using the Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan) to characterise the phytochemical composition of PSPE, namely, gallic acid, p-coumaric acid, catechin, and kaempferol. This system was equipped with an LC-20AT pump, a DGU-20A5 degasser, an SPD-20A UV/VIS detector, a SIL-20A autosampler, a CTO-10AS VP column oven, and controlled using LC Solution software. The analytical methodology was adapted from Mustafa et al. (2018), with slight modifications to optimize detection and quantification parameters.

An Eclipse Plus C18 column (4.6 × 250 mm, 5 µm particle size) was employed for chromatographic separation as the stationary phase. The column temperature was maintained at 40°C, and the pressure limit was set at 4000 psi to ensure optimal resolution and peak separation. The mobile phase consisted of 0.1% acetic acid in water (solvent A) and acetonitrile (solvent B). A gradient elution program was implemented, starting at 10% solvent B and progressively increasing to 60% over a 25-minute runtime. The autosampler was set to inject 10 µL of each sample, and the flow rate was adjusted to 1.0 mL/min. The detection wavelength was set at 280 nm to ensure the accurate identification of phenolic and flavonoid compounds.

The identification and quantification of phytochemicals in PSPE (10 mg/mL) were carried out by comparing the retention times and spectral data of detected peaks with gallic acid, p-coumaric acid, catechin, and kaempferol standard compounds. Each standard was analysed in triplicate across five different concentrations (20 – 100 µg/mL) to construct calibration curves. The area under the curve (AUC) for each compound was determined, and corresponding calibration equations were generated. These fitted equations were subsequently used to calculate the concentrations of the detected compounds in PSPE, ensuring precise quantification and reproducibility of the results.

3.3.2 Determination of Total Phenolic Content

The total phenolic content of PSPE was determined spectrophotometrically using the Folin-Ciocalteu colorimetric method according to Azizan et al. (2020). Briefly, the reaction mixture was prepared by mixing 20 µL of PSPE (31.25 – 1000 µg/mL) and 100 µL of Folin-Ciocalteu's reagent. After 5 minutes of incubation, 80 µL of 0.75% sodium carbonate was added and the mixture was further incubated in the dark for another 20 minutes at room temperature. A blank mixture was prepared in the same manner, except that 20 µL of PSPE was replaced with distilled water. The absorbance was read spectrophotometrically at 765 nm by using microplate reader. The same procedure was repeated for the standard solution of gallic acid (3.125 – 100 µg/mL) and subsequently, the standard curve was constructed. The content of phenol in the extract was expressed in terms of gallic acid equivalent (GAE) (µg GAE/mL of extract).

3.3.3 Determination of Total Flavonoid Content

The determination of total flavonoid content in PSPE was done by using spectrophotometric method according to Manan et al. (2019). A mixture of 100 µL of diluted PSPE (31.25 – 1000 µg/mL) and 2% aluminium chloride (2g of $AlCl_3$ diluted in 100 mL of distilled water) was prepared and left to incubate for 15 minutes at room temperature. Utilising a microplate reader, the absorbance was determined spectrophotometrically at 415 nm. The calibration curve was created by repeating the same procedure with the quercetin standard solution (3.125 – 100 µg/mL). The flavonoid concentration in PSPE was quantified and expressed in terms of quercetin equivalents (QE) (µg QE/ mL of extract).

3.4 Antioxidant Activities of *P. speciosa* Pod Extract (PSPE)

3.4.1 DPPH Free Radical Scavenging Assay

With some minor modifications, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was used to evaluate antioxidant activity following the methodology described by Bobo-García et al. (2015). Briefly, a 96-well microplate was employed, wherein 20 µL of the diluted sample (31.25 - 1000 µg/mL) was combined

with 180 µL of a DPPH solution (150 µmol/L) in a methanol-water mixture (80:20, v/v). The resulting mixture was subjected to agitation for 60 seconds. After allowing the contents of the microplate to react for 40 minutes at room temperature in the dark, the absorbance was measured at 515 nm using a microplate reader (Tecan Infinite M200) against a blank. Ascorbic acid was utilised as a positive control within a concentration range of 3.125 - 100 µg/mL and all measurements were conducted in triplicate to ensure accuracy and reliability. The percentage of DPPH quenched was calculated as:

$$\text{Scavenging effect (\%)} = \left[1 - \frac{(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})}{\text{Abs}_{\text{control}}}\right] \times 100\% \quad (3.2)$$

where $\text{Abs}_{\text{sample}}$ is the absorbance at 515 nm of 20 µL extract or positive control with 180µL of DPPH solution after 40 min; $\text{Abs}_{\text{blank}}$ is the absorbance at 515 nm of 20µL water with 180µL of methanol-water (80:20, v/v) after 40 min; and $\text{Abs}_{\text{control}}$ is the absorbance at 515 nm of 20 µL water with 180 µL DPPH solution after 40 min. The IC_{50} value, which indicates the effective concentration at which 50% of DPPH radicals were scavenged, was used to express the scavenging activity of the samples. The relationship curve between scavenging activities (%) and concentrations of each sample was developed to calculate the IC_{50} values.

3.4.2 Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing antioxidant power (FRAP) of the PSPE was determined by adopting the methodology proposed by Manan et al. (2019), with slight modifications. A freshly prepared working FRAP reagent which composed of acetate buffer (300 mM, pH 3.6), a 10 mM solution of 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) in 40 mM hydrochloric acid (HCl), and 20 mM ferric chloride hexahydrate ($FeCl_3.6H_2O$). Prior to use, 100 mL of acetate buffer, 10 mL of TPTZ solution, and 10 mL of ferric solution ($FeCl_3.6H_2O$) were combined in a ratio of 10:1:1 to create the FRAP reagent. Next, 125 µL of the FRAP reagent and 25 µL of appropriately diluted samples were mixed and incubated for 30 minutes at 37°C with gentle agitation in a 96-well microplate. The reduction of the colourless ferric complex (Fe^{3+}-tripyridyltriazine) into the blue ferrous

complex (Fe^{2+}-tripyridyltriazine) was measured at 593 nm using a microplate reader. Ferrous sulphate ($FeSO_4$) at different concentrations (3.125–1000 µg/mL) was used to construct a calibration curve, and the results were expressed in µg $FeSO_4$ equivalent per mL of extract.

3.5 *In Vitro* Anti-adipogenic Activities of *P. speciosa* Pod Extract (PSPE)

3.5.1 Cultivation and Maintenance of 3T3-L1 Preadipocytes

The 3T3-L1 mouse embryonic fibroblast cells (ATCC CL-173) were obtained from the American Type Culture Collection (ATCC, USA). The complete growth media (M1) used to culture the undifferentiated 3T3-L1 fibroblasts included Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% NCS, 100 units/mL of penicillin, and 100 µg/mL of streptomycin. These cell cultures were maintained in T75 flasks within a humidified 5% CO_2 incubator at 37°C until they attained 70–80% confluency. To sustain a sub-confluent state, the cells underwent regular media changes every 2-3 days. Upon reaching 70-80% confluency, trypsinization using TrypLE™ Express Enzyme (Thermo Fisher, USA) was employed, and the cells were subsequently cultured back in the incubator for further experimental procedures. Phase-contrast microscopy (CKX 53, Olympus, Japan) was used to examine the morphological features of 3T3-L1 cells.

For cell cryopreservation, the trypsinized cells were mixed at a 1:10 ratio with a freezing medium containing 7% dimethylsulfoxide (DMSO) in M1. The mixture was placed in a cryotube and stored overnight at -80°C. It was then moved to a liquid nitrogen tank and kept at -150°C until further usage. For cell revival, the frozen cells were thawed in a water bath set at 37°C. Following thawing, the cells were cultured in a T25 flask containing 5 mL of M1 and incubated overnight at 37°C with 5% CO_2. Media exchange was carried out the following day to remove any remaining DMSO from the cell culture. This cryopreservation and thawing protocol employed in this study adhered to established procedures for maintaining cell viability and functionality.

3.5.2 Differentiation of 3T3-L1 Preadipocytes

3T3-L1 cells were cultured in growth medium (M1) until reaching full confluence (100%). Preadipocytes were induced for differentiation (Day 0) upon reaching confluence by replacing the M1 medium with the differentiation medium (M2). Cells were cultured in the M2 for three days (Day 3), supplemented with 10% FBS, 0.5 mM IBMX, 10 µg/mL insulin, 0.25 µM dexamethasone, 100 units/mL penicillin, and 100 µg/mL streptomycin. Following the initial differentiation phase, the medium was replaced with maintenance media (M3), consisting of DMEM supplemented with 10% FBS, 10 µg/mL insulin, 100 units/mL of penicillin, and 100 µg/mL of streptomycin. The M3 medium was replaced every three days until the attainment of mature adipocytes on day 15. Full differentiation of preadipocyte cells occurred within the timeframe of 7 to 15 days following the application of the differentiation medium. The detailed formulation of the adipocyte differentiation medium is outlined in Table 3.2.

Table 3.2
Formulation for Culture and Differentiation Medium.

Medium	Formulation
M1	DMEM and 10% NCS
M2	DMEM, 10% FBS, 0.5mM IBMX, 10µg/mL insulin, 0.25 µM dexamethasone, 100 units/mL penicillin, and 100 µg/mL streptomycin
M3	DMEM supplemented with 10% FBS, 10 µg/mL insulin, 100 units/mL of penicillin, and 100 µg/mL of streptomycin

3.5.3 *P. speciosa* Pod Extract (PSPE) Dose Optimisation

3.5.3.1 Cell Viability Assay

The effects of PSPE on mature adipocyte viability were evaluated through MTT assay according to the method described by Oruganti et al. (2023). In this study, the cell viability assay serves as a preliminary analysis before examining the functional effects of the extract. Firstly, 3T3-L1 preadipocytes were cultured and differentiated in 96-well plates, with a seeding density of 1.0 x 10^4 cells per well. Prior to the MTT assay, the differentiated 3T3-L1 adipocytes cells were incubated with freshly prepared PSPE (31.25 - 1000 µg/mL), over 72 hours at 37 °C in a humidified 5% CO_2 incubator.

Control groups were treated solely with culture medium. MTT assay was performed briefly by adding 20 µL of 5 mg/mL MTT reagent to each well, followed by a 4-hour incubation at 37°C under identical culture conditions. Subsequently, the MTT-formazan complex was dissolved by replacing the culture media with 200 µL of dimethyl sulfoxide (DMSO). Formazan concentration was determined by measuring optical density at 590 nm using a microplate reader. The absorbance values were indicative of live cell numbers, and cell viability was expressed as a percentage relative to the control cells.

3.5.3.2 Oil Red O Staining

Dose optimisation for PSPE was further conducted using the oil red O staining assay in mature adipocytes, with treatments administered for 24, 48, and 72 hours. The objective was to identify the optimal concentration for the extract to effectively reduce lipid content without causing cellular damage. Lipid accumulation assessment was conducted using the oil red O staining method, following the protocol outlined by Hwang et al. (2021) with minor adjustments. To establish the optimal PSPE concentration for this study, a dose optimisation was performed across a range of concentrations (31.25 – 125 µg/mL) determined from prior viability tests of 3T3-L1 cells treated with PSPE.

In brief, 3T3-L1 preadipocytes were seeded in 12-well plates, differentiated and subjected to PSPE treatment (31.25 – 125 µg/mL), for durations of 24, 48, and 72 hours. Undifferentiated cells were also prepared for comparative analysis. Following the respective treatment periods, oil red O staining was performed, and the cells were examined under a light microscope. Quantification of eluted stains was carried out using a microplate reader set at 490 nm and the obtained results were compared to the differentiated control cells. Subsequently, a concentration of 62.5 µg/mL was selected based on its observed efficacy in mitigating lipid accumulation through the oil red O staining assay.

3.5.4 Treatment of 3T3-L1 Cells

3.5.4.1 PSPE and Simvastatin Preparation

A stock solution of PSPE was prepared at a concentration of 2 mg/mL by dissolving the extract in 1% dimethyl sulfoxide (DMSO). To ensure sterility and remove any potential particulate matter, the solution was filtered using a 0.45 µm membrane filter before further use. Similarly, a 2 mg/mL stock solution of simvastatin was prepared by dissolving a 20 mg SIMVOR 20 (Ranbaxy) tablet in 10 mL of 20% DMSO. This solution was also subjected to filtration through a 0.45 µm membrane filter to ensure purity and consistency in subsequent experimental procedures.

Following the preparation of stock solutions, both PSPE and simvastatin solutions were further diluted in cell culture medium to achieve their respective final working concentrations. PSPE was diluted to a final concentration of 62.5 µg/mL, while simvastatin was diluted to a final concentration of 4.19 µg/ml (10 µM). These prepared solutions were then used for treating the cultured 3T3-L1 adipocytes in subsequent experiments. The use of DMSO as a solvent was carefully controlled to ensure that its final concentration in the treatment medium remained within a non-toxic range for cell viability.

3.5.4.2 Treatment with PSPE and Simvastatin

3T3-L1 preadipocytes were seeded into 6-well plates at a density of 1.0×10^5 cells per well and maintained in a humidified incubator set at 37°C with 5% CO_2 to facilitate optimal cell growth and viability. To ensure clarity in experimental conditions, each well was designated for a specific treatment group as follows: Group 1 – Undifferentiated, Group 2 – Differentiated adipocytes (negative control), Group 3 – Treatment with 4.19 µg/ml Simvastatin (positive control), and Group 4 – Treatment with 62.5 µg/mL PSPE.

Once the cells reached 100% confluency (designated as Day 0), the differentiation process was initiated for Group 2, 3, and 4 by replacing the M1 medium with M2 medium, while Group 1 continued to receive M1 medium to serve as the undifferentiated control. On Day 3, the M2 medium was replaced with M3 medium

which was subsequently refreshed every three days to support adipocyte development. This process continued until Day 15, marking the completion of adipocyte maturation.

On Day 15, treatment with the test compounds was initiated. The culture medium in Group 3 was replaced with M3 medium supplemented with 4.19 µg/ml Simvastatin, while Group 4 received M3 medium containing 62.5 µg/mL PSPE. Both treatments were co-cultured for 48 hours to evaluate their respective effects on mature adipocytes.

3.5.5 Oil Red O Staining Assay

3.5.5.1 Preparation of Oil Red O Stain

To prepare the oil red O stock solution, oil red O powder was dissolved in 100% isopropanol and stirred overnight. The resultant solution was filtered using a 0.22 µm membrane filter and stored at 24°C until further use. This filtered solution was denoted as the oil red O stock solution. The oil red O working solution was freshly constituted by blending three parts of the stock solution with two parts of distilled water. The mixture stood at room temperature for 20 minutes and underwent additional filtration with a 0.22 µm membrane filter before utilisation.

3.5.5.2 Oil Red O Staining of 3T3-L1 Cells

The 3T3-L1 cells were seeded in 12-well plates and induced to undergo differentiation, following the procedures outlined in Section 3.5.2. Concurrently, undifferentiated cells were prepared. Upon complete differentiation, the media from both differentiated and undifferentiated plates were removed, and the cells were gently rinsed with PBS. Then, the cells were fixed with 1 mL of 10% formalin for 5 minutes at 24°C. After removal of the formalin, fresh formalin was applied, and the plates were wrapped in aluminum foil, undergoing overnight incubation at room temperature.

On the following day, the formalin was discarded, and the cells were washed with 60% isopropanol and air-dried completely. Lipid droplet staining was performed by adding 1 mL of oil red O working solution to each well for 20 minutes at 24°C. The solution was then removed, and the cells were washed three times with distilled water prior to observation under a light microscope.

Following image capture, 100% isopropanol was added to each well for 10 minutes at 24°C to elute the stain and assess lipid accumulation. The resulting solution was transferred to a 96-well plate and read using a microplate reader at 490 nm. Results were compared to those of differentiated control cells.

3.5.6 Triglyceride Assay

Triglyceride content in the treated 3T3-L1 cells was evaluated using Triglyceride Colorimetric Assay Kit (Elabscience, Wuhan, China). In this assay, the culture medium was used to assess the triglyceride content. In summary, 2.5 µL of culture medium from treatment and control, were dispensed into a 96-well microplate, with distilled water serving as the blank. Subsequently, 250 µL of lipase was introduced to each well, and the mixture was thoroughly mixed, followed by an incubation period at 37°C for 10 minutes. Absorbance readings were recorded at 510 nm using a microplate reader. All assessments were conducted in triplicate, and triglyceride concentrations were determined employing the formula provided by the kit manufacturer:

$$\text{Triglyceride (mmol/L)} = \left[\frac{(A_{sample} - A_{blank})}{A_{standard} - A_{blank}}\right] \times c \times f \qquad (3.3)$$

where A_{sample} is the absorbance of the culture medium, A_{blank} is the absorbance of distilled water, $A_{standard}$ is the absorbance of the standard, c is the concentration of the standard and f is the dilution factor of the samples before test.

3.6 Statistical Analysis

For all the phytochemical, antioxidant, and i*n vitro* anti-adipogenic activities of PSPE including MTT assay, oil red O staining assay and triglycerides assay, the results were presented as the mean ± standard error mean (SEM) from the three independent replicates. All statistical analyses were conducted using GraphPad Prism Version 9.5.1. To compare the results between groups, a one-way ANOVA was performed, followed by Tukey's *post hoc* test for multiple comparisons. The statistical significance level was set at $p < 0.05$.

3.7 Metabolomic Analysis of PSPE Intervention by LCMS-Q-TOF

3T3-L1 preadipocytes were cultured until reaching full confluence and subsequently subjected to differentiation in 12-well plates, following the procedures outlined in Section 3.5.2. Approximately, a total of 1.0 x 10^5 cells were seeded in each well. Upon attaining full differentiation, the 3T3-L1 cells were exposed to PSPE (62.5 µg/mL) and simvastatin (4.19 µg/ml). Control groups consisted of wells containing untreated adipocytes. Following a 48-hour treatment period, cellular lysates were collected and preserved at -80°C for subsequent analyses.

3.7.1 Sample Preparation

A mixture of methanol and deionised water with a ratio of 4:1 (v/v) was used as an extraction solvent. In brief, cells within the 12-well plates underwent three washes with 1 mL PBS, and following the removal of PBS, the plate was placed on ice. Quenching of cells was achieved by adding 1 mL of extraction solvent to each well, and subsequent gentle swirling ensued, followed by a 3-minute incubation to ensure comprehensive metabolite extraction. A cell scraper was utilised to scrape the well contents, and the cell lysates were transferred to an Eppendorf tube. Subsequently, the cell lysates underwent centrifugation at 13000 rpm for 30 minutes, and the resulting supernatant was transferred to a new Eppendorf tube. The supernatant was then dried by using a vacuum concentrator for 3 hours and the resultant tubes were stored at -80℃ until further analysis.

Next, the dried sample was reconstituted with 50 µL of mobile phase (50% dH_2O: 50% ACN) followed by 30 seconds of vortex mixing and then centrifuged at 13,000 rpm for 15 minutes at 4℃. A 40 µL of the supernatant was transferred into vial insert and then injected into the LCMS/QTOF system for analysis.

3.7.2 LC/MS-QTOF Analysis

Chromatographic analysis was conducted using an Agilent 1200 Rapid Resolution Series liquid chromatography system (Agilent Technologies, Santa Clara, CA, USA), featuring a binary pump, degasser, well plate autosampler with thermostat, thermostat column compartment, and coupled with a 6520 QTOF mass spectrometer

equipped with a dual electrospray ionization (ESI) source. Samples were analysed using a customized method developed in-house. Separation was achieved on a Zorbax Eclipse Plus C18 column (1.8 μm particle size, 2.1 x 100 mm dimensions, Agilent Technologies, Santa Clara, CA, USA) with the column temperature maintained at 40°C throughout the analysis.

For positive mode, the mobile phase (A) consisted of 0.1% formic acid (Supelco, Inc) in ultrapure water (18 MΩ), while mobile phase (B) comprised 0.1% formic acid in acetonitrile (LiChrosolv, Darmstadt, Germany). In negative mode, mobile phase (A) was composed of 0.1% ammonium formate in ultrapure water (18 MΩ), and mobile phase (B) was 100% acetonitrile.

Samples were analysed in batches, each containing 6 samples (4 experimental samples, 1 quality control sample comprising pooled samples, and 1 blank). The total analysis runtime for each batch was set at 30 minutes, with three replicates performed for each sample. The chromatographic gradient was designed based on the proportion of solvent B over time. Specifically, a linear gradient was employed over 18 minutes, starting from 5% to 95% mobile phase B, followed by a 5-minute wash phase and a 7-minute column equilibration phase. The flow rate was maintained at 0.25 mL/min, and each sample was injected at a volume of 2 μL. The specific mass spectrometry conditions used are outlined in Table 3.3.

Quality control (QC) samples were prepared by pooling a portion of each individual sample. For each batch of samples, one QC sample was analysed alongside four randomly selected samples, following the protocol recommended by Godzien et al. (2015). Each batch, comprising the QC and selected samples, was analysed continuously over a 24-hour period. To ensure system stability and performance throughout each batch, QC samples were injected at the beginning, middle, and end of the analytical run. The chromatograms of the QC samples were examined using MassHunter Workstation Software Qualitative Analysis Version B.05.00 2011 (Agilent Technologies, Santa Clara, CA, USA) to assess the quality of each batch. To evaluate the performance of QC samples, the relative standard deviation (RSD) of the several metabolites that were constantly present in 80% of the QC samples were calculated. The data were collected and computed according to the following formula:

$$\text{Relative standard deviation (\%)} = \frac{\text{Standard deviation}}{\text{Mean}} \text{ X } 100 \qquad (3.4)$$

3.7.3 Data Pre-processing

Given the extensive nature of metabolomics datasets, it is essential to employ robust data treatment and high-throughput analysis techniques to avoid systematic biases and facilitate meaningful biological interpretation. Several stages of data processing, encompassing data conversion, feature extraction and filtering, as well as differential analysis were performed. Various software tools were utilised throughout the data processing, including MassHunter Qual and Mass Profiler Professional, to execute these analytical tasks efficiently and accurately.

3.7.3.1 LCMS Q-TOF Data Analysis and Multivariate Data Analysis

Untargeted data acquisition was conducted using Agilent MassHunterTM B.05.00 software on the LCMS Q-TOF system (Agilent Technologies, Santa Clara, CA, USA). The acquired data were processed using Agilent MassHunterTM Qualitative Analysis B.05.00 (MassHunterTMQual), with the Molecular Feature Extractor (MFE), an untargeted data-mining algorithm designed to identify groups of co-variant ions corresponding to unique features in chromatograms. The mass range and detection window were configured as m/z 50 to 1000 for positive mode and m/z 50 to 1100 for negative mode. Peak filtering for profile and centroid spectra was set at 200 counts above the noise threshold. Molecular features extracted from each sample were exported in Compound Exchange Format (*.cef) for subsequent statistical analysis using Mass Profiler Professional (MPP) (version B.12.01, Agilent). Both multivariate and univariate analyses were employed to interpret the data effectively.

3.7.4 Differential Analysis and Compounds Identification

Agilent Mass Profiler Professional (MPP) B.02.00 software was used to process the Compound Exchange Format (*.cef) data files for the initial data treatment. The primary data underwent alignment, filtration, and normalisation procedures.

3.7.4.1 Filtering

Based on relevant variables, samples were appropriately labelled. In order to prepare the dataset for further analysis, low-intensity data had to be eliminated, or the data range had to be narrowed using filters include Filter by Frequency, Abundance, Variability, Flags, and Annotation.

3.7.4.2 Normalisation

To ensure comparability across different experimental conditions, all processed data underwent normalisation using quantile normalisation, which effectively removes unwanted systemic variation in human LC-MS metabolomics datasets, highlighting underlying biological differences (Lee et al., 2012).

Following filtration and normalisation, the data were submitted to The Human Metabolome Database (HMDB) (https://hmdb.ca) for compound identification. HMDB serves as a comprehensive and freely accessible online repository of small molecule metabolites, aiding in the identification of metabolites through mass analysis. HMDB is interconnected with and cross-referenced against other databases including Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/), LIPID MAPS (http://dev.lipidmapd.org:25424/), and PUBCHEM (http://pubchem.ncbi.nlm.nih.gov/) for comprehensive metabolite comparison and annotation.

3.7.4.3 Recursive Analysis

Lists of identified compound entities were subjected to recursion analysis to confirm the presence of positive data points. Recursion analysis scrutinizes the dataset to validate the presence of identified entities. During this recursive analysis, the identified compounds were exported in *.cef format from Mass Profiler Professional (MPP) software to MassHunter Qualitative software. Instead of utilising the Molecular Feature Extractor (MFE) tool, the Find Compound by Formula function was employed. The resulting .cef files were subsequently imported back into MPP for further differential analysis and exploration of compound variations across experimental conditions.

3.7.5 Clustering Analysis

3.7.5.1 Principal Component Analysis (PCA)

The collected data underwent multivariate analysis to effectively reduce dimensionality and visualise patterns (Engel et al., 2012). Principal component analysis (PCA), an unsupervised multivariate analysis method, was employed to identify trends, patterns, and sample groupings (Jollife & Cadima, 2016). Additionally, the web-based platform MetaboAnalyst (http://www.metaboanalyst.ca) was utilised for analysis using Partial Least Squares Discriminant Analysis (PLS-DA), Biplot analysis, and two-way Hierarchical Cluster Analysis (HCA). These analyses facilitated the visualisation of correlations between metabolites (in both positive and negative modes) and samples within the dataset.

3.7.5.2 Statistical Analysis and Fold Change Analysis

To assess differences in metabolites between comparable subjects, a differential analysis was conducted using Welch's t-test with unequal variances. This statistical method aimed to identify significantly different compounds. To control the false discovery rate (FDR) in multiple comparisons, the Benjamin-Hochberg procedure was applied. Subsequently, significant findings were subjected to fold change analysis using a threshold of >=2 to further characterise the magnitude of differences observed.

3.7.6 Network Pathway Analysis

3.7.6.1 Metabolomics Pathway Analysis (MetPA)

In order to better understand the complex metabolic interactions present in both the control and PSPE-treated groups, a detailed pathway analysis was conducted using the Metabolic Pathway Analysis (MetPA) tool. MetPA (http://www.metaboanalyst.ca) is a freely accessible, web-based platform that integrates pathway enrichment analysis with pathway topology analysis to identify key metabolic pathways that are significantly impacted by experimental conditions. The metabolic pathways were obtained from the KEGG database and presented as a network of chemical compounds.

The statistical significance of each pathway was determined using enrichment analysis, with *p*-values calculated to assess the degree of overrepresentation of specific metabolites within a given pathway. The results were described in a pathway impact diagram and ranked in a table for each metabolic pathway involved in the study accordingly based on the degree of significance for the metabolic pathways.

3.7.7 Biomarker Identification

3.7.7.1 Area Under Receiving Operator Characteristic (AUROC)

The dataset was further analysed using Receiver Operating Characteristic (ROC) curve analysis, which assesses the performance of a binary classifier system. This analysis was conducted using the web-based application at http://www.metaboanalyst.ca. The ROC curve is a graphical representation that combines the true positive rate (sensitivity) on the y-axis and the false positive rate (1 - specificity) on the x-axis. ROC curves generated at various cutoff points were used to evaluate the prediction model, specifically Partial Least Squares Discriminant Analysis (PLS-DA). An optimal cutoff point is characterised by a ROC curve that rapidly ascends towards the upper left corner of the graph, indicating strong discrimination capability.

3.7.7.2 Variable Importance in Projection (VIP)

The significant metabolites were ranked based on their Variable Importance in Projection (VIP) score, which is calculated as a weighted sum of squares of the Partial Least Squares (PLS) loadings. This ranking is presented as a plot showing the metabolites along the X-axis with their respective VIP scores, representing their relative importance. A higher VIP value indicates a greater contribution of the metabolite in distinguishing between treated and control groups.

CHAPTER 4
RESULTS

4.1 Extraction Yield

The extraction of *P. speciosa* pods using 70% ethanol yielded 37.83% of crude extract, with a total of 227 g of freeze-dried extract obtained from 600 g of dried pod powder. The extracted material initially appeared as a sticky, dark-colored residue with a mild herbal odor following solvent removal via rotary evaporation (Figure 4.1). Subsequent freeze-drying produced a fine, dark brown powder, indicating the successful recovery of soluble compounds (Figure 4.2). The extraction process, which involved repeated solvent replacement until colorless, ensured the thorough extraction of bioactive constituents.

Figure 4.1 Crude Form of PSPE After Removal of Extraction Solvent.

Figure 4.2 Freeze-Dried PSPE Powder.

4.2 Phytochemical Analysis of *P. speciosa* Pod Extract (PSPE)

4.2.1 HPLC Analysis

The phytochemical profile of PSPE (10 mg/mL) was analysed using reverse-phase HPLC, which revealed multiple peaks in the chromatogram as shown in Figure 4.3 (a), indicating the presence of various compounds. Three concentrations of PSPE (1000, 5000, and 10,000 µg/mL) were tested, with the 10,000 µg/mL concentration yielding the clearest chromatographic peaks suitable for compound identification. Comparison with standard compounds (Figure 4.3b) enabled the identification of two peaks corresponding to gallic acid (peak 1) and p-coumaric acid (peak 3). However, catechin (peak 2) and kaempferol (peak 4) could not be conclusively identified. Table 4.1 shows that the retention times of gallic acid and p-coumaric acid in the extract closely matched those of the reference standards. Using their respective calibration curves, the concentrations of these compounds in PSPE were quantified. The concentration of gallic acid in the ethanolic extract of *P. speciosa* pods was determined to be 53.97 ± 0.76 µg/mL. Meanwhile, the detected p-coumaric acid was below the detection value.

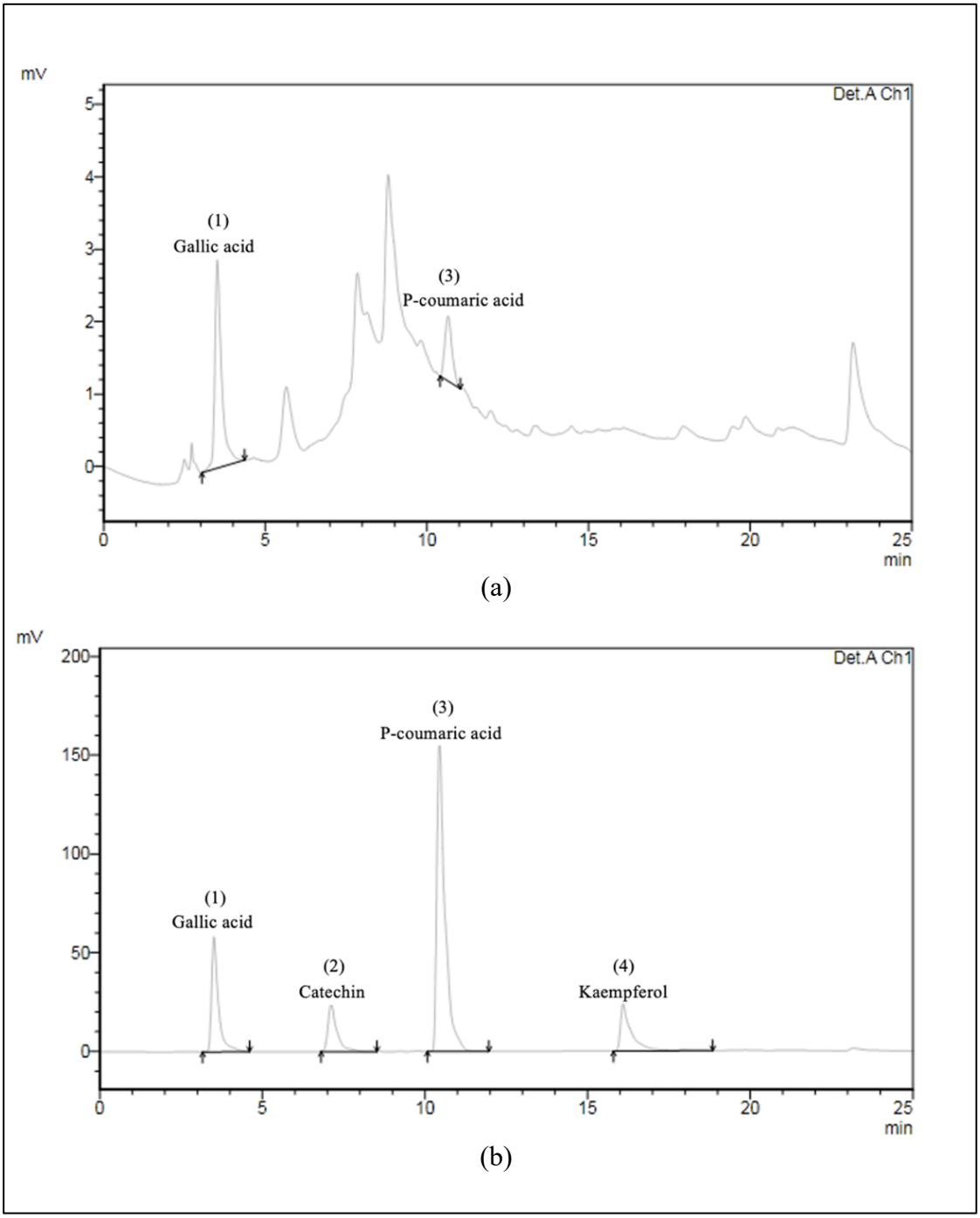

Figure 4.3 High-Performance Liquid Chromatography Chromatograms of (a) *P. speciosa* Pod Extract (10 mg/mL) and (b) Standards (100 µg/mL).

Table 4.1
Retention Time (tR) and Concentration of Gallic Acid and p-Coumaric Acid in 10mg/mL PSPE Compared to Standard Compounds and Reported as Mean ± SEM, n=3.

	Retention time (t_R) (min)		**Concentration of compounds in PSPE**
	Standard	**PSPE**	**(µg/mL)**
Gallic acid	3.506 ± 0.006	3.495 ± 0.005	53.97 ± 0.76
p-Coumaric acid	10.442 ± 0.03	10.442 ± 0.07	Below detection value

4.2.2 Determination of Total Phenolic Content

The results for total phenolic content (TPC) in PSPE ranged from 8.567 ± 0.12 to 242.8 ± 11.62 µg GAE/mL of extract. Detailed TPC values for varying concentrations of PSPE (31.25 – 1000 µg/mL) are shown in Table 4.2. These findings indicate the presence of phenolic compounds in PSPE.

Table 4.2
Total Phenolic Content of PSPE Reported as Mean ± SEM, n=3.

Concentration of PSPE (µg/mL)	Total Phenolic Compound (µg GAE/mL)
31.25	8.567 ± 0.12
62.5	18.38 ± 3.16
125	42.63 ± 4.20
250	82.88 ± 7.18
500	150.1 ± 9.59
1000	242.8 ± 11.62

4.2.3 Determination of Total Flavonoid Content

PSPE had a total flavonoid content (TFC) ranging from 0.191± 0.19 to 30.53 ± 0.52 µg QE/mL of extract. Table 4.3 provides a detailed breakdown of the TFC at various concentrations of PSPE (62.5 – 1000 µg/mL). These findings indicate the presence of flavonoid compounds in PSPE.

Table 4.3
Total Flavonoid Content of PSPE Reported as Mean ± SEM, n=3.

Concentration of PSPE (µg/mL)	Total Flavonoid Compound (µg QE/mL)
62.5	0.191 ± 0.19
125	2.463 ± 0.43
250	5.745 ± 0.56
500	13.53 ± 0.59
1000	30.53 ± 0.52

4.3 Antioxidant Activities of *P. speciosa* Pod Extract (PSPE)

4.3.1 DPPH Radical Scavenging Assay

The DPPH assay evaluates the ability of PSPE to scavenge DPPH free radicals. In this study, the antioxidant potential of PSPE was assessed across a concentration range of 31.25 to 1000 µg/mL, with the percentage inhibition values calculated for each concentration as shown in Figure 4.4. The concentrations of PSPE and ascorbic acid (positive control) required to achieve a 50% reduction in DPPH free radicals (IC_{50}) were determined to be 57.05 ± 0.22 µg/mL and 23.13 ± 0.33 µg/mL, respectively (Table 4.4). These findings indicate that PSPE exhibited approximately 0.4 times the potency of ascorbic acid. It is noteworthy that the IC_{50} value of an antioxidant compound is inversely proportional to its antioxidant activity, signifying that a lower IC_{50} value corresponds to higher antioxidant capacity.

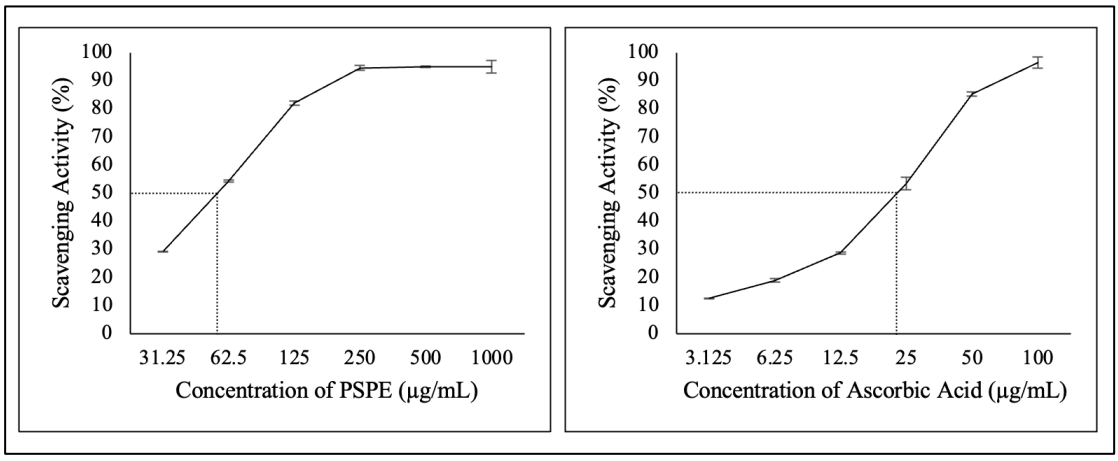

Figure 4.4 The DPPH Scavenging Activity of the Ethanolic Extract of PSPE and Ascorbic Acid.

Table 4.4
DPPH Scavenging Activity of PSPE and Ascorbic Acid Reported as Mean ± SEM, n=3.

Sample	DPPH scavenging activity (IC_{50} value (µg/mL))
PSPE	57.05 ± 0.22
Ascorbic acid	23.13 ± 0.33

4.3.2 FRAP Assay

The ferric reducing antioxidant power (FRAP) assay was performed to evaluate the reducing capacity and antioxidant potential of PSPE across a concentration range of 31.25 to 1000 µg/mL. The FRAP values of PSPE obtained ranged from 47.64 ± 2.35 to

325.3 ± 4.85 µg $FeSO_4$/mL, indicating a concentration-dependent increase in reducing ferric-tripyridyltriazine complex to ferrous in the presence of antioxidants. The FRAP values for PSPE and ascorbic acid (positive control) are detailed in Table 4.5. PSPE displayed its highest FRAP value at 325.3 ± 4.85 µg FeSO4/mL, while ascorbic acid exhibited a higher value at 377.5 ± 2.58 µg FeSO4/mL. These findings show that, across all concentrations, PSPE demonstrated a slightly lower FRAP value compared to ascorbic acid.

Table 4.5
FRAP Value of PSPE and Ascorbic Acid Reported as Mean ± SEM, n=3.

Concentration (µg/mL)	**FRAP value of PSPE (µg $FeSO_4$/mL)**	**FRAP value of ascorbic acid (µg $FeSO_4$/mL)**
31.25	47.64 ± 2.35	96.75 ± 2.47
62.5	92.46 ± 2.87	179.3 ± 0.94
125	150.1 ± 2.94	270.8 ± 5.12
250	230.9 ± 5.71	335.4 ± 1.01
500	294.9 ± 1.34	369.7 ± 0.39
1000	325.3 ± 4.85	377.5 ± 2.58

4.4 *In Vitro* Anti-adipogenic Activities of *P. speciosa* Pod Extract (PSPE)

4.4.1 *P. speciosa* Pod Extract (PSPE) Dose Optimisation

4.4.1.1 Cell Viability Assay

The MTT assay was employed to evaluate the toxicity effect of PSPE on the viability and survival of mature 3T3-L1 adipocytes. In this study, the cell viability assay serves as a preliminary analysis before examining the functional effects of the extract. Mature 3T3-L1 adipocytes were incubated with various concentrations of PSPE (31.25 - 1000 µg/mL) for 72-hours and percentages of cell viability were calculated (Table 4.6). The highest PSPE concentration (1000 µg/mL) resulted in a 59.62 ± 14.48% cell viability, while the lowest PSPE concentration (31.25 µg/mL) and simvastatin (4.19 µg/ml) exhibited viabilities of 99.86 ± 5.13% and 99.59 ± 5.89%, respectively.

Notably, cells treated with PSPE concentrations ranging from 31.25 to 125 µg/mL for 72 hours maintained over 90% cell viability compared to control cells, with a significant reduction observed at 1000 µg/mL, which is the highest concentration of extract (Figure 4.5). These findings suggest a concentration-dependent decrease in the

viability of mature 3T3-L1 adipocytes with increasing PSPE concentrations. The observed cell that exceeded 90% cell viability indicated that the tested PSPE concentrations were non-cytotoxic. Based on this, PSPE concentrations ranging from 31.25 to 125 µg/mL were considered safe and selected for further experimentation. These concentrations were subsequently used in oil red O staining assay.

Table 4.6
Percentages of Cell Viability Relative to the Differentiated Control Cells After 72 Hours of Cell Treatment and Reported as Mean ± SEM, n=3.

Concentration (µg/mL)	**Percentage of cell viability (%)**
Simvastatin (4.19 µg/ml)	99.59 ± 5.89
31.25	99.86 ± 5.13
62.5	99.91 ± 10.28
125	93.10 ± 4.35
250	82.15 ± 8.33
500	70.77 ± 9.17
1000	59.62 ± 14.48

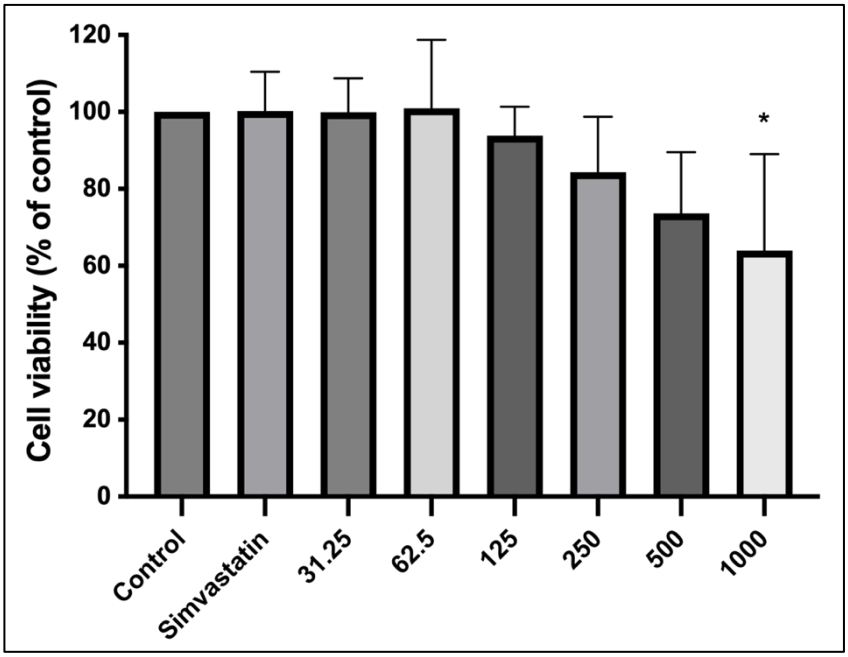

Figure 4.5 Effect of PSPE on 3T3-L1 Cell Viability. Cell Viability Was Significantly Reduced When Treated with PSPE at Concentration 1000 µg/mL. Results Were Expressed as Mean ±S.E.M (n = 3). *Indicate Significant Differences when Compared to Control ($p < 0.05$).

4.4.1.2 Oil Red O Staining Assay

Dose optimisation of PSPE was further conducted using the oil red O staining assay to evaluate its anti-adipogenic effect in differentiated adipocytes. Treatments with PSPE (31.25 – 125 µg/mL) and simvastatin (4.19 µg/ml) were administered at various incubation periods (24, 48, and 72 hours) with the aim of determining the optimal concentration capable of reducing intracellular lipid accumulation without inducing cytotoxicity. Post-treatment, stained cells were examined microscopically at 100X magnification, and representative images of lipid droplets were documented (Figure 4.6). At 24 hours of treatment, microscopic observations revealed no apparent differences in lipid droplet accumulation among the experimental groups. However, at 48 and 72 hours, cells treated with PSPE at concentrations of 62.5 and 125 µg/mL, as well as those treated with simvastatin (positive control), demonstrated a modest reduction in lipid droplets relative to the untreated control group. Following microscopic evaluation, the stained lipids were eluted and quantified at an absorbance of 490 nm using a microplate reader. While qualitative observations at 24 hours did not show marked differences, quantitative analysis revealed significant reductions in lipid content in some treatment groups. At 48 and 72 hours, both qualitative (microscopic) and quantitative (spectrophotometric) assessments confirmed a significant decrease in lipid accumulation in adipocytes treated with 62.5 and 125 µg/mL PSPE, as well as simvastatin, when compared to the control (Figure 4.7). Meanwhile, there is no significant reduced were observed in 31.25 µg/mL PSPE within all incubation periods.

These results indicate that the anti-adipogenic effects of PSPE become evident after 48 hours of treatment, particularly at concentrations of 62.5 and 125 µg/mL. Notably, no significant enhancement was observed when extending treatment to 72 hours. Therefore, a concentration of 62.5 µg/mL administered for 48 hours was selected as the optimal condition for subsequent investigations of PSPE's anti-adipogenic properties.

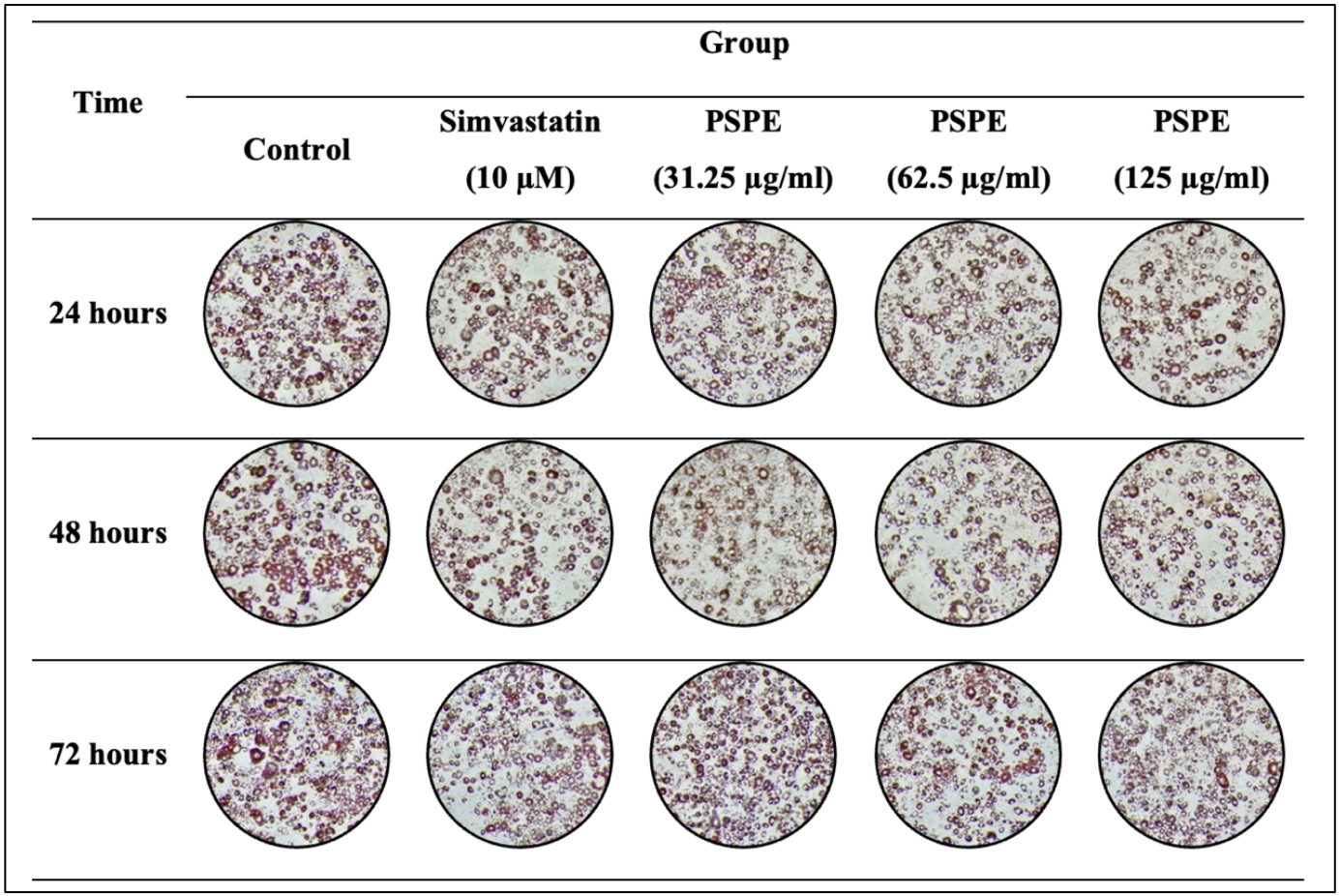

Figure 4.6 Images of Lipid Droplets in Control, Simvastatin- (4.19 µg/ml), and PSPE- (31.25 - 125 µg/mL) Treated Group Acquired at 100X Microscopic Field Magnification for 24 Hours, 48 Hours, and 72 Hours.

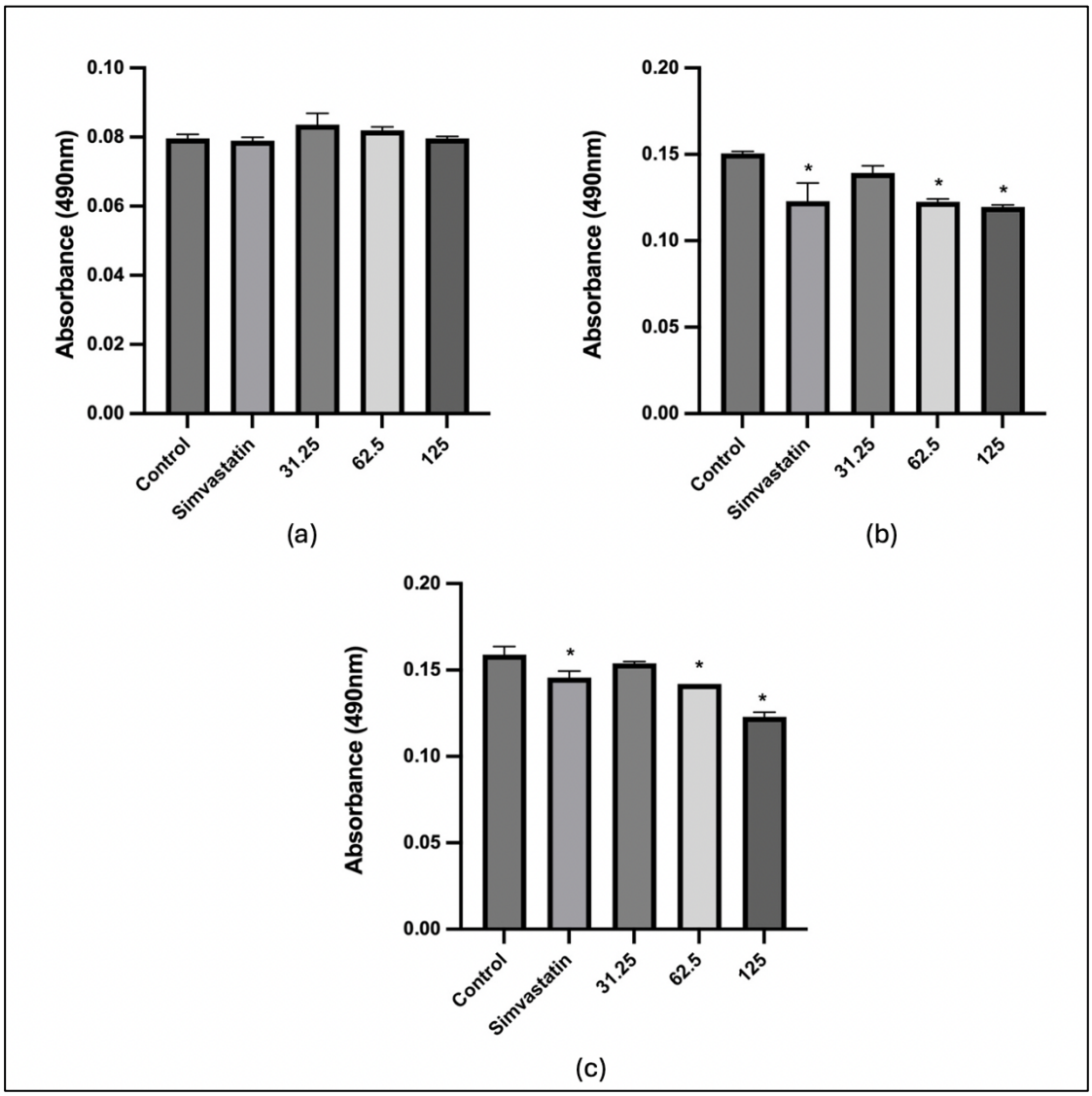

Figure 4.7 Lipid Accumulation in 3T3-L1 Cells After Treated with Simvastatin (4.19 µg/ml) and PSPE (31.25 - 125 µg/mL) for (a) 24 Hours, (b) 48 Hours and (c) 72 Hours. Data Are Expressed as the Mean ± SEM of Three Independent Experiments (n = 3). *Indicate Significant Differences When Compared to Control ($p < 0.05$).

4.4.2 Oil Red O Staining Assay

The effect of PSPE on lipid accumulation in mature 3T3-L1 adipocytes was assessed via the oil red O staining assay using 62.5 µg/mL as the optimum treatment concentration in this study. Validation of staining patterns and morphological characteristics was conducted under the guidance of experienced personnel to ensure consistency and accuracy. After a two-day treatment period, stained cells were visually

examined, and representative images of lipid droplets were captured at 100X microscopic field magnification (Figure 4.8). While the images from microscopic observations did not distinctly reveal differences in lipid accumulation, a subtle reduction was noted in the PSPE- and simvastatin-treated groups compared to the control differentiated group.

Table 4.7 shows the percentages of lipid reduction for the PSPE- and simvastatin-treated group relative to the differentiated control cells after 48 hours of cell treatment. These findings suggest that a PSPE concentration of 62.5 µg/mL exhibits potential anti-adipogenic effects comparable to a commercial drug used for obesity treatment. Following microscopic examinations, eluted cells were quantified at 490 nm using a microplate reader. The results indicated that, although the lipid accumulation in PSPE- and simvastatin-treated groups did not decrease to the level of the undifferentiated group (50.4%), there was a significant reduction in PSPE-treated cells (17.1%) and simvastatin-treated cells (19.4%) compared to the control differentiated group (Figure 4.9). Additionally, no significant difference was observed between the PSPE and simvastatin-treated groups.

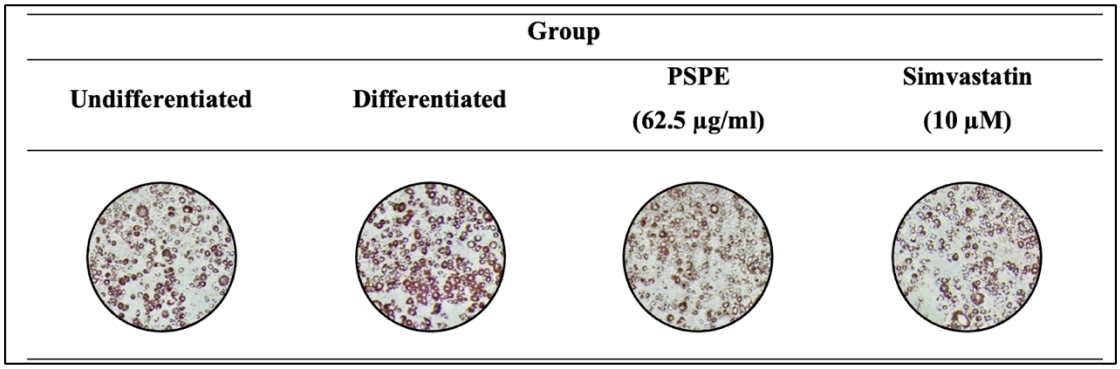

Figure 4.8 Images of Lipid Droplets in Undifferentiated, Differentiated, PSPE- (62.5 µg/mL), and Simvastatin- (4.19 µg/ml) Treated Group Acquired at 100X Microscopic Field Magnification for 48 Hours.

Table 4.7
Percentages of Lipid Reduction Relative to the Differentiated Control Cells After 48 Hours of Cell Treatment.

Group	Percentage of lipid reduction (%)
Undifferentiated	50.4
PSPE	17.1
Simvastatin	19.4

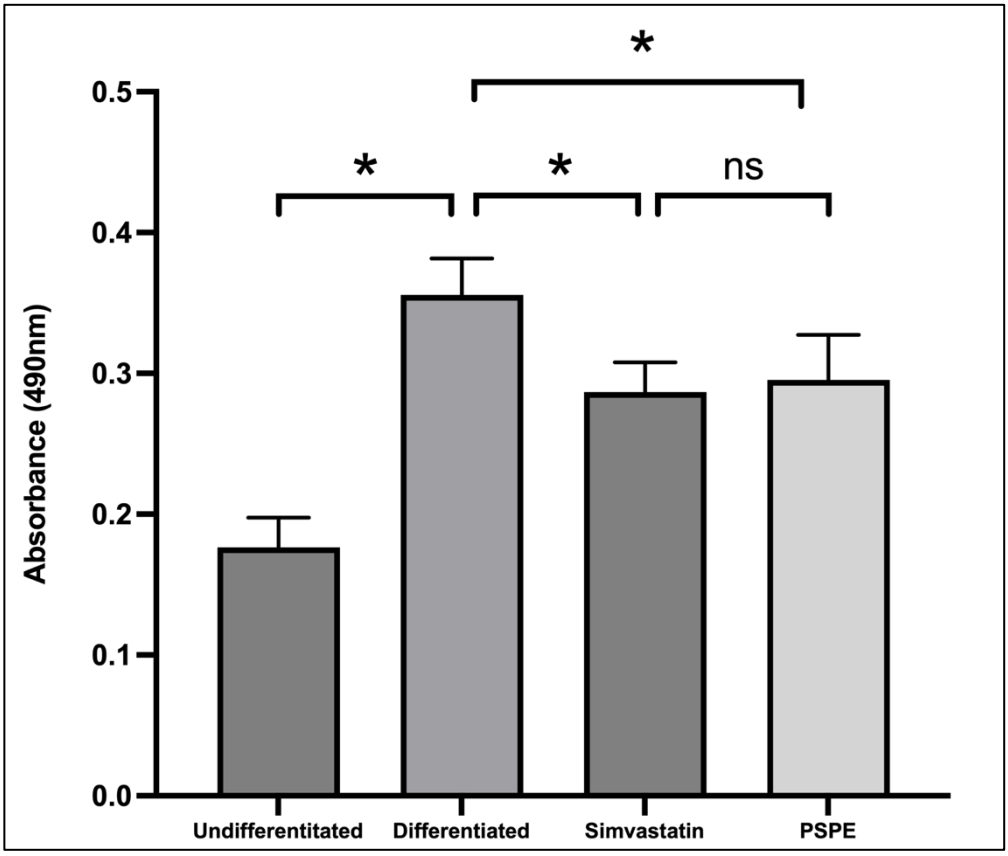

Figure 4.9 Lipid Accumulation in 3T3-L1 From All Groups for 48 Hours. The Lipid Accumulation of the PSPE and Simvastatin-Treated Cells Were Significantly Decreased. Data Are Expressed as the Mean ± SEM of Three Independent Experiments (n = 3). *Indicates Significant Differences Compared to Differentiated Group ($p < 0.05$), while ns Indicates No Significant Difference.

4.4.3 Triglyceride Assay

The impact of PSPE on lipid accumulation in mature 3T3-L1 adipocytes was further validated through the triglyceride assay. The triglyceride levels in the culture medium across different experimental groups were quantified and shown in Figure 4.10. As expected, the undifferentiated group exhibited minimal triglyceride deposition, measuring 0.10 ± 0.005mmol/L, whereas the untreated differentiated group showed a significantly higher triglyceride level of 1.39 ± 0.01 mmol/L. In comparison with the differentiated group, cells treated with PSPE (62.5 µg/mL) displayed a notable reduction in triglyceride concentration, reaching 0.90 ± 0.06 mmol/L, indicating its potential lipid-lowering effects. Similarly, treatment with 4.19 µg/ml simvastatin also resulted in a significantly reduced triglyceride level of 0.84 ± 0.08 mmol/L. Comparatively, differentiated cells exhibited a notable increase in triglyceride content

by 92.8% compared to undifferentiated cells ($p < 0.05$). Furthermore, when triglyceride levels were assessed in the presence of PSPE (62.5 µg/mL) and simvastatin (4.19 µg/ml), reductions of 34.8% and 39.6%, respectively, were observed relative to levels in differentiated 3T3-L1 adipocytes.

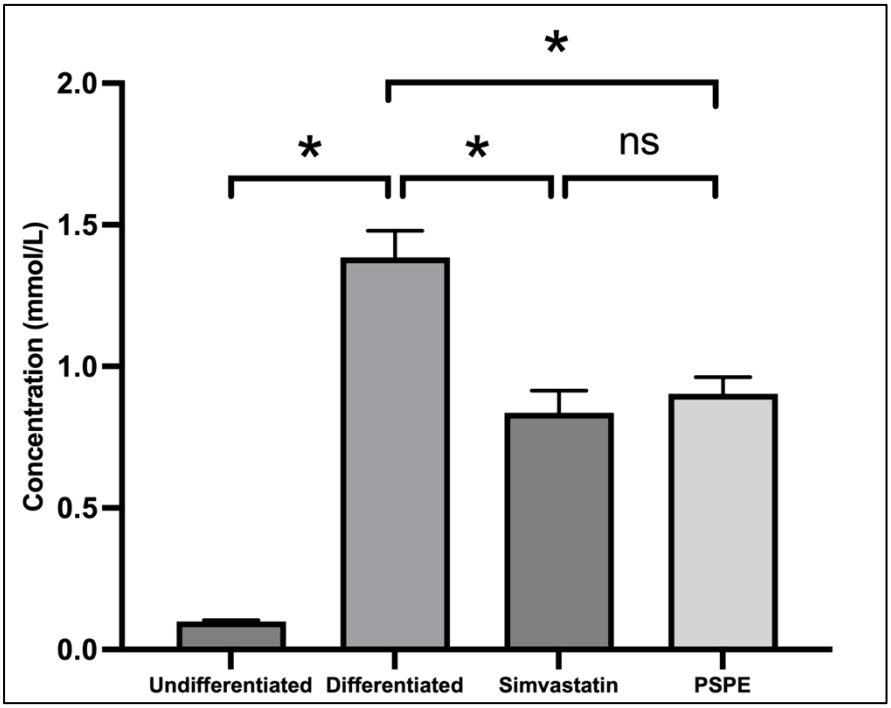

Figure 4.10 Triglycerides Content in 3T3-L1 Adipocytes in Four Different Groups. The Concentration of Triglycerides in the Culture Medium of the PSPE and Simvastatin-Treated Cells Were Significantly Decreased. Data Are Expressed as the Mean ± SEM of Three Independent Experiments (n = 3). *Indicates Significant Differences Compared to Differentiated Group ($p < 0.05$), While ns Indicates No Significant Difference.

4.5 Relative Standard Deviation (RSD)

Metabolomics datasets involve measurements of large numbers of metabolites using specific analytical tools such as NMR and LCMS. Technical factor (analytical) will induce huge variation between these measurements if not properly controlled (Parsons et al., 2009). Spectrum-wide relative standard deviations (RSD) can be used to evaluate the measurement variability. RSD should be calculated in order to verify the reproducibility of metabolomics datasets (Parsons et al., 2009).

Relative standard deviation is the absolute value of the coefficient of variation. It is often expressed as percentage. The relative standard deviation is widely used in analytical chemistry to express the precision and repeatability of an assay. Table 4.8

shows RSD values of the selected compounds present in the pooled samples. The RSD value of below 20% is acceptable (Nishiumi et al., 2012). This quality control was used both in the model and intervention studies.

Table 4.8
Relative Standard Deviation (RSD) Value of Selected Metabolites Present in Pooled Sample

No	Mass different (ppm)			Retention time different (min)			Peak area		
	Mean	S.D	RSD (%)	Mean	S.D	RSD (%)	Mean	S.D	RSD (%)
1	131.100	0.0003	0.0002	1.274	0.0100	0.7849	340533	30386	8.9231
2	219.100	0.0001	0.0001	1.879	0.0186	0.9899	136188	6703	4.9219
3	164.100	0.0001	0.0001	8.947	0.1010	1.1289	130800	2504	1.9144
4	375.300	0.0010	0.0002	15.580	0.1036	0.6649	937108	174256	18.5951
5	362.300	0.0010	0.0003	23.190	0.1434	0.6184	267380	22024	8.2369

4.6 Metabolic Responses in 3T3-L1 Adipocyte Differentiation via Metabolomics Approaches

4.6.1 Metabolites Profiling in 3T3-L1 Adipocyte Differentiation

In this study, a total of 14 metabolites were identified as significant in 3T3-L1 differentiated adipocyte cells, when compared to 3T3-L1 undifferentiated adipocyte cells (Table 4.9). These metabolites were mapped to 9 sub-pathways, which are associated with key metabolic pathways, including lipid metabolism, amino acid metabolism, carbohydrate metabolism, nucleotide metabolism, and glycan metabolism. Notably, among these pathways, the lipid metabolism pathway was particularly prominent, with a high number of significantly altered compounds.

The identified metabolites exhibit diverse physicochemical properties and were selected based on strict criteria, which include passing multivariate significance analysis with a corrected p-value of less than 0.05 and a fold change cut-off of ≥ 2.0. These criteria ensured the robustness and reliability of the findings. Detailed information on these 14 metabolites, including their associated pathways, sub-pathways, mass, and KEGG ID, is provided in Table 4.9.

Table 4.9
Altered Metabolites in Differentiated 3T3-L1 Adipocyte Cells.

Pathway	Sub-pathway	Metabolites	Mass	KEGG ID
Lipid metabolism	Sphingolipid metabolism	Phosphosphingolipids	780.6072	C00550
		Sphinganine	301.2982	C00836
	Linoleic acid metabolism	gamma-Linolenic acid	278.2246	C06426
		9,10-Epoxyoctadecenoic acid	296.235	C14825
		Linoleic acid	280.2414	C01595
		Arachidonic acid	304.2415	C00219
Amino acid metabolism	D-amino acid metabolism	Pyrrolines	129.0426	C04282
	Glutathione metabolism	Pyroglutamic acid	129.0426	C01879
	Phenylalanine metabolism	Phenylalanine	165.0791	C00079
	Citrate cycle	Fumaric acid	116.0112	C00122
Carbohydrate metabolism	Pentose and glucuronate interconversions	Uridine diphosphate glucose	566.0565	C00029
		Palmitoyl glucuronide	418.2895	C03033
Nucleotide metabolism	Pantothenate and CoA biosynthesis	Pantothenic acid	219.1106	C00864
Glycan metabolism	N-Glycan biosynthesis	Dolichyl b-D-glucosyl phosphate	466.2306	C01246

4.6.2 Cluster Analysis

4.6.2.1 Principle Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA)

The Principal Component Analysis (PCA) is an unsupervised method which does not require class labels and helps reveal natural grouping or trends in the data based on metabolite profiles. In this study, the PCA score plots classified all samples into two small clusters corresponding to the differentiated and undifferentiated 3T3-L1 cells (Figure 4.11a). Despite this classification, the PCA of the detected metabolites showed no clear separation between the differentiated and undifferentiated groups.

However, the supervised component analysis using Partial Least Squares Discriminant Analysis (PLS-DA) provided a more distinct separation, successfully distinguishing two distinct groups between the differentiated and undifferentiated 3T3-

L1 cells (Figure 4.11b). This enhanced separation by PLS-DA underscores its effectiveness in highlighting the differences in metabolic profiles that PCA alone could not fully capture.

To further elucidate the main contributors to this separation, a biplot was generated (Figure 4.11c and Figure 4.11d). The biplot analysis revealed that metabolites from lipid metabolism, including gamma-Linolenic acid, arachidonic acid, and 9,10-Epoxyoctadecenoic acid, played a significant role in differentiating the differentiated from the undifferentiated 3T3-L1 cells. Additionally, metabolites from glycan metabolism, such as dolichyl b-D-glucosyl phosphate, were also identified as key contributors to the separation.

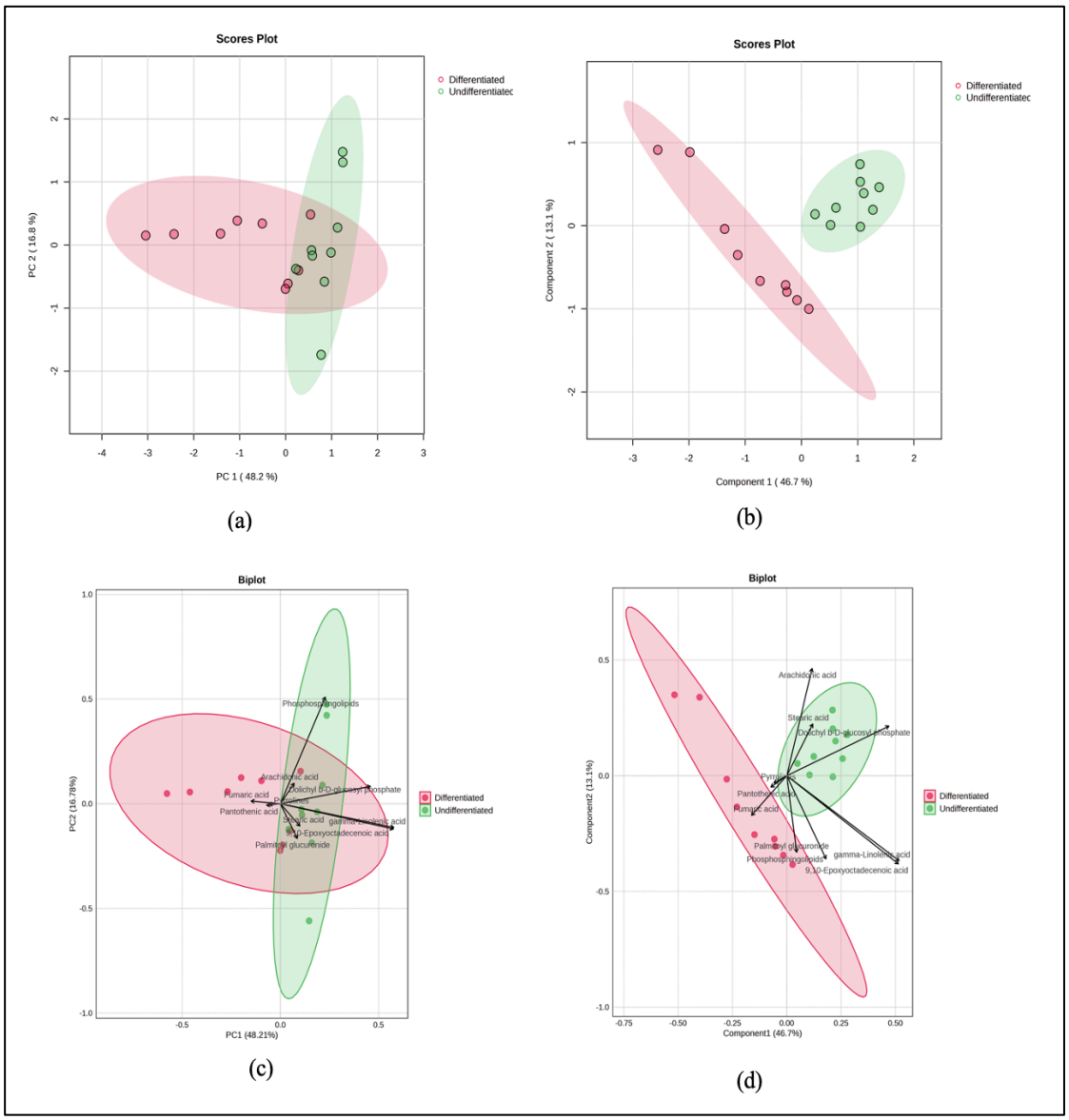

Figure 4.11 Cluster Analysis of Differentiated and Undifferentiated 3T3-L1 Adipocyte Cells Utilising Metaboanalyst's Data Annotation Tools. a) PCA of Detected Metabolites. b) PLS-DA of Detected Metabolites. c) Significant Changes of the Metabolites Illustrated in the PCA Biplot. d) Significant Changes of the Metabolites Illustrated in the PLS-DA Biplot.

4.6.2.2 Hierarchical Clustering

Figure 4.12 demonstrates the quantitative variation of identified metabolites in differentiated and undifferentiated 3T3-L1 cells, visualised as a heat map with hierarchical agglomerative clustering. The tree structure at the top of the heat map illustrates the clustering relationships among the samples, providing insights into how the metabolic profiles of the cells are grouped.

The hierarchical clustering analysis revealed distinct clustering of samples from the differentiated and undifferentiated groups based on their metabolite profiles. This separation aligns with the patterns observed in the Partial Least Squares Discriminant Analysis (PLS-DA) score plots (Figure 4.11b), which also demonstrated clear grouping between the two cell states.

Further examination of the heat map indicated that specific metabolites were differentially expressed between the differentiated and undifferentiated cells. In the differentiated group, there was a notable upregulation of metabolites such as uridine diphosphate glucose, pyrrolines, phenylalanine, pantothenic acid, sphinganine, and fumaric acid. In contrast, the undifferentiated group exhibited high expression levels of dolichyl b-D-glucosyl phosphate.

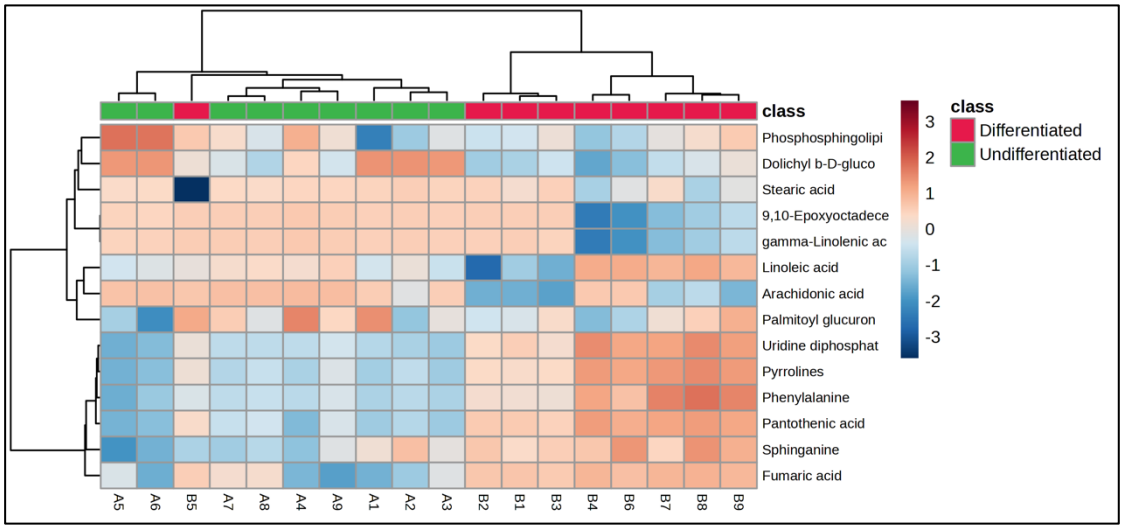

Figure 4.12 Heat Map of the Detected Metabolites in Differentiated and Undifferentiated 3T3-L1 Adipocyte Cells. The Columns Represent Samples, and the Rows Represent Detected Metabolites. Color Key Indicates Metabolite Expression Value, Blue: Lowest, Red: Highest.

4.6.3 Potential Metabolic Biomarkers in 3T3-L1 Adipocyte Differentiation by Variable Importance in Projection (VIP) and Area Under the ROC Curve (AUROC)

The differential expression of metabolites between differentiated and undifferentiated 3T3-L1 cells was assessed using Variable Importance in Projection (VIP) values obtained from the PLS-DA model. Metabolites with VIP values greater than one were selected as potential biomarkers due to their significant contribution to

the segregation between differentiated and undifferentiated 3T3-L1 groups. To further evaluate the discriminative power of these selected biomarkers, Receiver Operating Characteristic (ROC) curves were generated.

ROC analysis provides a comprehensive measure of the diagnostic ability of biomarkers, with the results often summarized by the Area Under the Curve (AUC) metric. The AUC offers a single value representing the overall performance of a biomarker: an AUC of 0.9-1.0 is considered excellent, 0.8-0.9 is good, 0.7-0.8 is fair, 0.6-0.7 is poor, and 0.5-0.6 is a failure (Xia et al., 2013).

Figure 4.13 displays the VIP scores for the metabolites detected in the study. The metabolites identified as potential biomarkers, with VIP values exceeding one (VIP > 1), include dolichyl b-D-glucosyl phosphate, gamma-linolenic acid, 9,10-epoxyoctadecenoic acid, and arachidonic acid. These metabolites exhibited significant differences in expression between the differentiated and undifferentiated groups, indicating their potential role in the differentiation process. Table 4.10 provides a detailed summary of the VIP values, AUC values, p-values, and fold changes for these identified potential biomarkers.

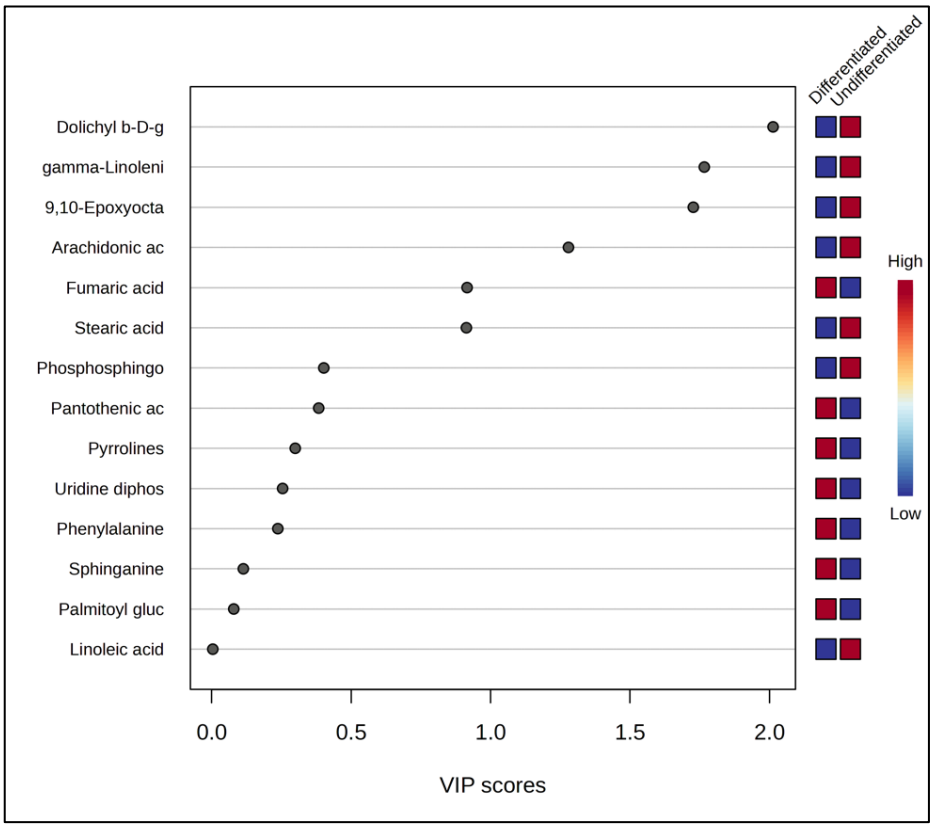

Figure 4.13 VIP Scores for Detected Metabolites in Differentiated 3T3-L1 Adipocyte Cells.

Table 4.10
VIP value, AUC value, p-value and Fold Change Analysis of Five Metabolites Identified Using VIP and AUROC. Green Indicates Increased Fold Change in Differentiated; Red Indicates Reduced Fold Change in Differentiated.

Metabolite	VIP value	AUC value	p-value	Fold Change (Differentiated vs Undifferentiated)
Dolichyl b-D-glucosyl phosphate	2.0128	0.8765	0.0023	-3.6976
gamma-Linolenic acid	1.7662	0.8148	0.0103	-1.1410
9,10-Epoxyoctadecenoic acid	1.7268	0.6914	0.0127	-1.0407
Arachidonic acid	1.2792	0.8889	0.0025	-1.3008

4.7 Effects of *Parkia speciosa* Pod Extract (PSPE) on Metabolic Responses in 3T3-L1 Adipocyte Differentiation by Metabolomics Approaches

4.7.1 Metabolites Profiling in 3T3-L1 Adipocyte Cells Treated with PSPE

A comprehensive analysis identified 33 significant metabolites in 3T3-L1 adipocyte cells from the differentiated, PSPE, and simvastatin-treated groups (Table 4.11). These metabolites were from 19 sub-pathways related to the pathways in lipid metabolism, amino acid metabolism, carbohydrate metabolism, nucleotide metabolism, and the metabolism of cofactors and vitamins.

Among these pathways, lipid metabolism exhibited the most substantial alteration, with a high number of compounds significantly affected. This suggests that lipid metabolism is particularly responsive to the treatments applied in this study. The identified metabolites displayed a variety of physiochemical properties and were selected based on the passing criteria: a corrected p-value < 0.05 and a fold change cut-off of ± 2.0.

Table 4.11 provides a detailed list of these 33 metabolites, including their respective pathways, sub-pathways, mass, and KEGG IDs. This comprehensive dataset highlights the extensive metabolic alterations occurring in response to PSPE treatment in 3T3-L1 adipocyte cells.

Table 4.11
Altered Metabolites in PSPE-Treated 3T3-L1 Adipocyte Cells.

Pathway	Sub-pathway	Compound	Mass	KEGG ID
Lipid metabolism	Sphingolipid metabolism	Phytosphingosine	317.2935	C12144
		Sphingosine	299.2815	C00319
		3-Dehydrosphinganine	299.2822	C02934
	Linoleic acid metabolism	9,10-Epoxyoctadecenoic acid	296.2357	C14825
		gamma-Linolenic acid	278.2236	C06426
		Linoleic acid	280.2406	C01595
	Steroid biosynthesis	3beta-Hydroxypregn-5-en-20-one sulfate	396.1956	C18044
		4-Methylpentanal	100.0888	C02373
		7-Dehydrodesmosterol	382.3257	C05107
	Glycerophospholipid metabolism	Phosphorylcholine	481.3185	C04230
		Phosphatidylethanolamine (PE)	765.5304	C00350
	Fatty acid biosynthesis	Dodecanoic acid	200.1776	C02679
		Capric acid	172.1461	C01571
		Myristic acid	228.2085	C06424
		Palmitoleic acid	254.2246	C08362
	Primary bile acid biosynthesis	Chenodeoxycholic acid glycine conjugate	449.3162	C05466
		7a-Hydroxy-cholestene-3-one	400.3340	C05455
	Arachidonic acid metabolism	Arachidonic acid	304.2412	C00219
Amino acid metabolism	Cysteine and methionine metabolism; Metabolic pathways	5'-Methylthioadenosine	297.0905	C00170
	Tyrosine metabolism	Hydroquinone	110.0370	C00530
	Tryptophan metabolism	Indoleacetic acid	175.0633	C00954
	Phenylalanine metabolism	N-Acetyl-L-phenylalanine	207.0894	C03519
		Phenylalanine	165.0789	C00079
Carbohydrate metabolism	Pyruvate metabolism	S-Lactoylglutathione	379.1047	C03451
	Fructose and mannose metabolism	L-Rhamnulose	164.0684	C00861
	Glyoxylate and dicarboxylate metabolism	Phosphoglycolic acid	155.9819	C00988

	Pentose and glucuronate interconversions	Uridine diphosphate glucuronic acid	580.0367	C00167
Nucleotide metabolism	Purine metabolism	Guanosine triphosphate	522.9924	C00044
		Guanosine diphosphate	443.0257	C00035
	Pantothenate and CoA biosynthesis	2-dehydropantoate	146.0580	C00966
Metabolism of cofactors and vitamins	Biotin metabolism	Biocytin	372.1804	C05552
	Riboflavin metabolism	FAD	785.1564	C00016
		Lumichrome	242.0807	C01727

4.7.2 Cluster Analysis

4.7.2.1 Principle Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA)

The classification of samples into three distinct clusters was analysed using PCA and PLS-DA score plots. Principal Component Analysis (PCA) statistically divided all detected metabolites into smaller groups known as principal components (PC) to identify potential biomarkers. However, the PCA score plots for the metabolites detected in 3T3-L1 adipocyte cells did not reveal a clear separation among the studied groups. This lack of distinct clustering suggests that several significant metabolites were shared across the differentiated, PSPE-treated, and simvastatin-treated groups, indicating overlapping metabolic profiles (Figure 4.14a).

In contrast, the supervised component analysis using Partial Least Squares Discriminant Analysis (PLS-DA) provided a more distinct separation of the samples. The PLS-DA score plots identified three clusters corresponding to the differentiated 3T3-L1 adipocyte cells, PSPE-treated groups, and simvastatin-treated groups (Figure 4.14b). This distinction underscores the effectiveness of PLS-DA in differentiating the metabolic profiles among the groups, highlighting the unique metabolic alterations induced by each treatment. To further investigate the contributors to this separation, a biplot was generated (Figure 4.14c and Figure 4.14d).

The biplot analysis revealed that key metabolites from various metabolic pathways played a significant role in the observed separation. Specifically, metabolites involved in lipid metabolism, such as linoleic acid, arachidonic acid, dodecanoic acid, and sphingosine, were prominent contributors. Additionally, metabolites from amino

acid metabolism, including phenylalanine and indoleacetic acid, as well as those involved in the metabolism of cofactors and vitamins, such as biocytin and flavin adenine dinucleotide (FAD), were also significant.

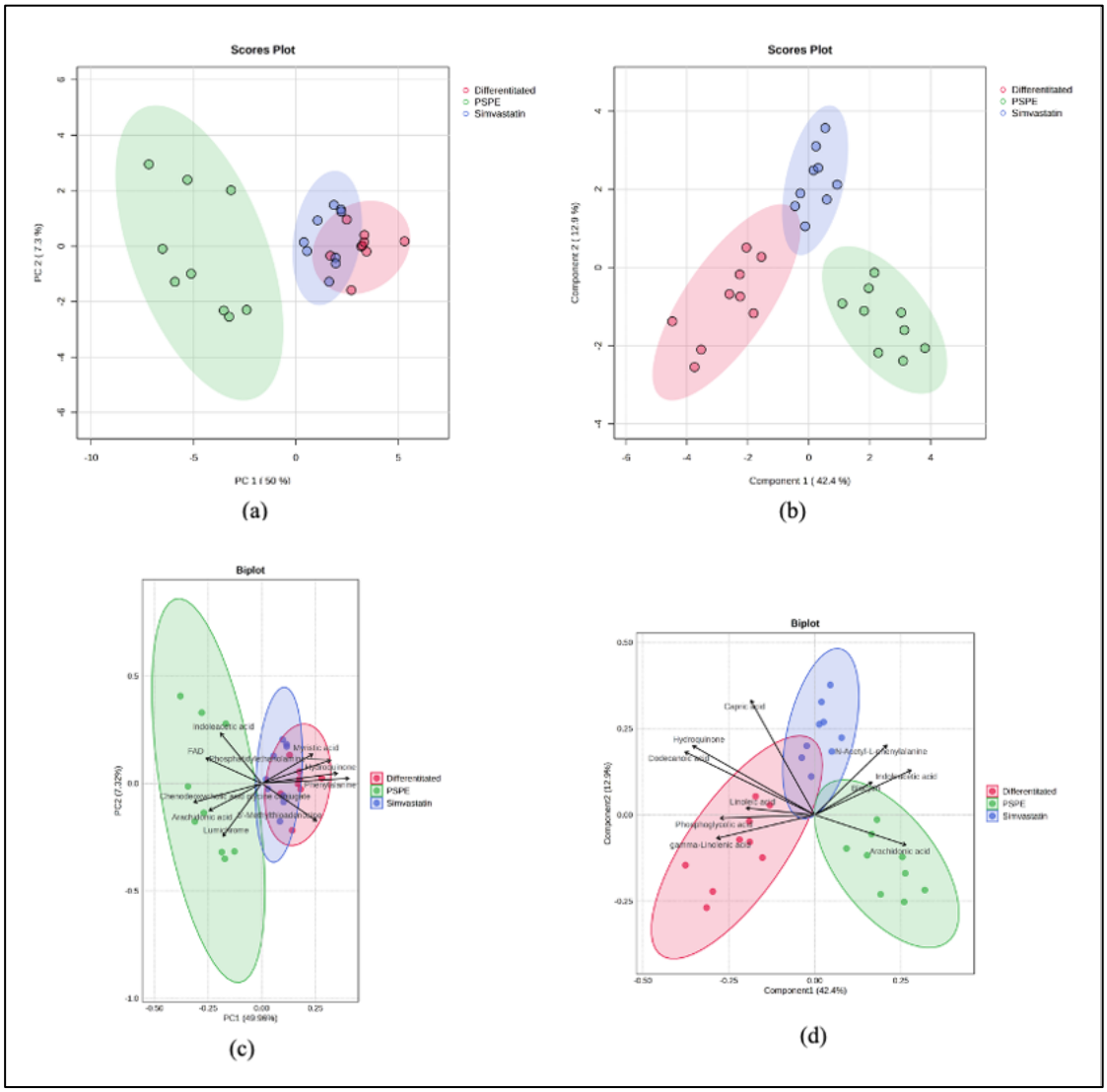

Figure 4.14 Cluster Analysis of Differentiated, PSPE, and Simvastatin-Treated 3T3-L1 Adipocyte Cells Utilising Metaboanalyst's Data Annotation Tools. a) PCA of Detected Metabolites. b) PLS-DA of Detected Metabolites. c) Significant Changes of the Metabolites Illustrated in the PCA Biplot. d) Significant Changes of the metabolites Illustrated in the PLS-DA Biplot.

4.7.2.2 Hierarchical Clustering

The quantitative variation of identified metabolites in differentiated 3T3-L1 adipocyte cells, PSPE-treated groups, and simvastatin-treated groups was thoroughly analysed and visualised using a combination of heat maps and agglomerative

hierarchical clustering (Figure 4.15). The hierarchical tree structure displayed at the top of the heat map illustrates the clustering relationships among the samples. Several samples from the differentiated 3T3-L1 adipocyte cells were found to cluster closely with samples from the simvastatin-treated group. This suggests a degree of similarity in the metabolic profiles between these two groups.

On the other hand, samples from the PSPE-treated group formed distinct clusters separate from both the differentiated and simvastatin-treated groups. This clear separation indicates significant differences in their metabolite profiles, highlighting the unique metabolic alterations induced by PSPE treatment. These clustering patterns are consistent with the observations made in the PCA score plots, where similar grouping trends were noted. Further analysis identified several key metabolites that were highly expressed in the samples from the PSPE-treated group. Notable among these were FAD, 7α-hydroxy-cholestene-3-one, 3-dehydrosphinganine, sphingosine, lumichrome, chenodeoxycholic acid glycine conjugate, and 7-dehydrodesmosterol.

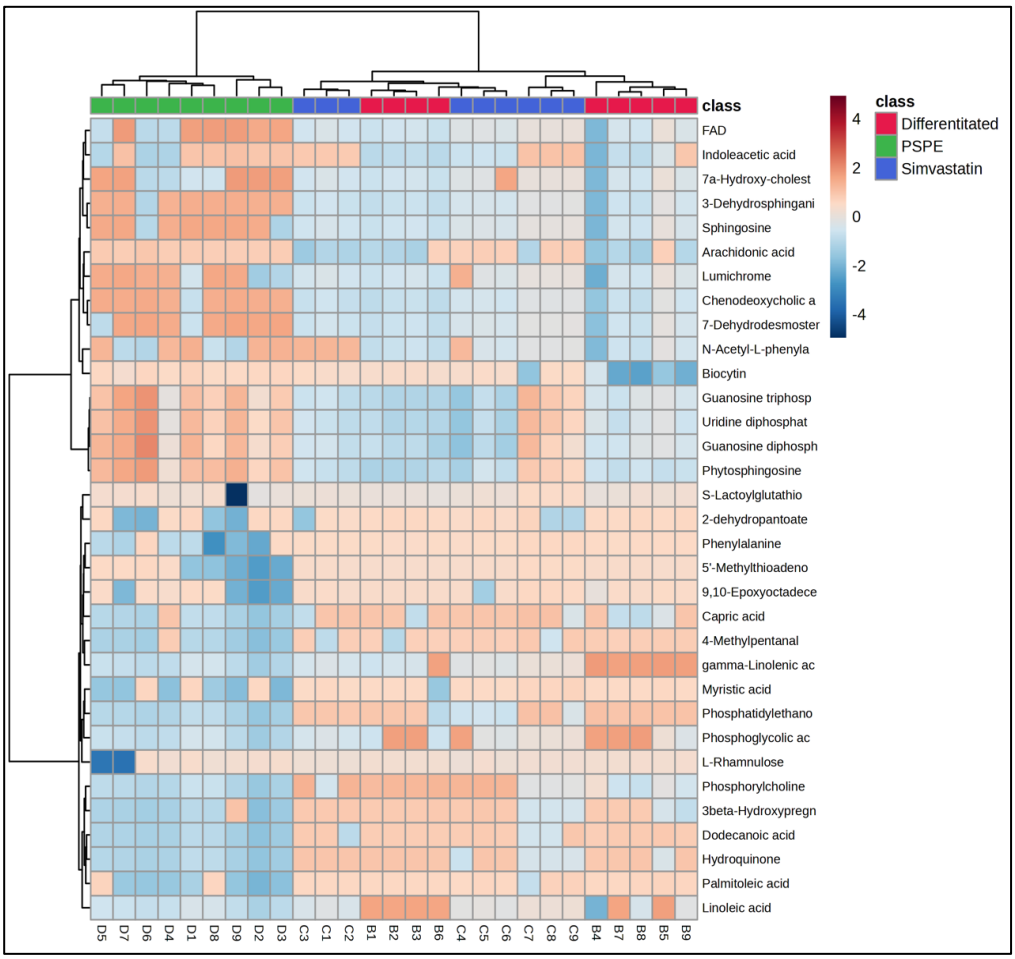

Figure 4.15 Heat Map of the Detected Metabolites in Differentiated, PSPE, and Simvastatin-Treated 3T3-L1 Adipocyte Cells. The Columns Represent Samples, and the Row Represent Detected Metabolites. Color Key Indicates Metabolites Expression Value, Blue: Lowest, Red: Highest.

4.7.3 Metabolomics Pathway Analysis (MetPA)

The pathway analysis conducted using MetPA identified a total of 23 significant metabolic pathways in 3T3-L1 adipocytes (Table 4.12). This comprehensive analysis revealed several metabolic pathways that were notably affected by PSPE in the adipocyte cells. Among the significant pathways detected, linoleic acid metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, phenylalanine metabolism, and arachidonic acid metabolism were highlighted as particularly noteworthy (Figure 4.16). These pathways were found to be significantly altered in response to the PSPE treatment applied in the study.

Table 4.12
Results of MetPA Pathway Analysis for PSPE-Treated 3T3-L1 Adipocyte Cells.

Pathway	Compound	Expected	Hits	Raw *p*	FDR	Impact
Linoleic acid metabolism	5	0.0884	2	0.0029	0.2332	1.00
Phenylalanine, tyrosine and tryptophan biosynthesis	4	0.0707	1	0.0689	0.9192	0.50
Phenylalanine metabolism	10	0.1768	1	0.1638	1	0.36
Arachidonic acid metabolism	43	0.7603	1	0.5407	1	0.30
Ascorbate and aldarate metabolism	10	0.1768	1	0.1638	1	0.16
Biotin metabolism	10	0.1768	1	0.1638	1	0.15
Glycerophospholipid metabolism	36	0.6365	2	0.1314	1	0.12
Sphingolipid metabolism	32	0.5658	3	0.0174	0.6366	0.10
Pentose and glucuronate interconversions	19	0.3360	1	0.2889	1	0.10
Folate biosynthesis	27	0.4774	1	0.3848	1	0.09
Pyruvate metabolism	23	0.4067	1	0.3386	1	0.05
Primary bile acid biosynthesis	46	0.8134	2	0.1942	1	0.04
Purine metabolism	71	1.2554	2	0.3601	1	0.03
Cysteine and methionine metabolism	33	0.5835	1	0.4485	1	0.02
Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	15	0.2652	1	0.2357	1	0.01
Amino sugar and nucleotide sugar metabolism	42	0.7426	1	0.5322	1	0.01
Biosynthesis of unsaturated fatty acids	36	0.6365	3	0.0239	0.6366	0
Fatty acid biosynthesis	47	0.8310	3	0.0477	0.9192	0
Riboflavin metabolism	4	0.0707	1	0.0689	0.9192	0
Steroid hormone biosynthesis	79	1.3969	2	0.4118	1	0
Glyoxylate and dicarboxylate	32	0.5658	1	0.4383	1	0

metabolism						
Steroid biosynthesis	41	0.7250	1	0.5235	1	0
Tryptophan metabolism	41	0.7250	1	0.5235	1	0

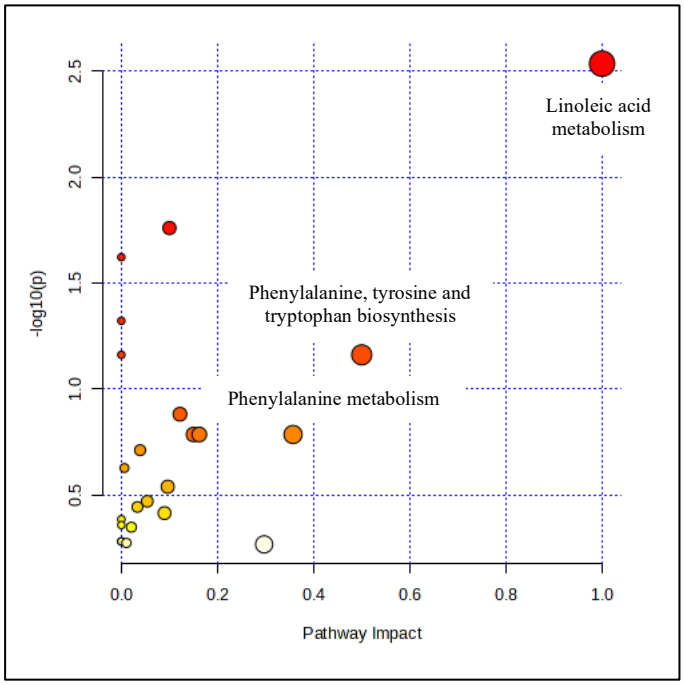

Figure 4.16 Potential Pathways for PSPE-Treated 3T3-L1 Adipocyte Cells Using Metaboanalyst.

4.7.4 Potential Metabolic Biomarkers in 3T3-L1 Adipocyte Cells Treated with PSPE by Variable Importance in Projection (VIP) and Area Under the ROC Curve (AUROC)

The metabolites differentially expressed between the differentiated 3T3-L1 cells, PSPE-treated groups, and simvastatin-treated groups were selected based on their Variable Importance in Projection (VIP) values. Metabolites with VIP values greater than one were identified as potential biomarkers. To further evaluate the classifying power of these selected biomarkers, a Receiver Operating Characteristic (ROC) curve analysis was conducted. The ROC values are often summarised into a single metric known as the Area Under the Curve (AUC), which provides a measure of the biomarker's discriminative ability. According to Xia et al. (2013), the utility of a biomarker based on its AUC value can be categorised as follows: 0.9-1.0 = excellent; 0.8-0.9 = good; 0.7-0.8 = fair; 0.6-0.7 = poor; and 0.5-0.6 = fail.

Figure 4.17 presents the VIP scores for the detected metabolites, highlighting those with a VIP value of more than one. These metabolites include indoleacetic acid,

N-acetyl-L-phenylalanine, gamma-linolenic acid, linolenic acid, biocytin, phosphoglycolic acid, arachidonic acid, and 2-dehydropantoate, lumichrome, dodecanoic acid, and 7a-hydroxy-cholestene-3-one. The selection of these metabolites as potential biomarkers indicates their significant roles in distinguishing between the different treatment groups. The performance of these potential biomarkers was further assessed using the AUC values derived from the ROC curve analysis. The AUC values, along with the corresponding p-values and fold changes, were summarised in Table 4.13.

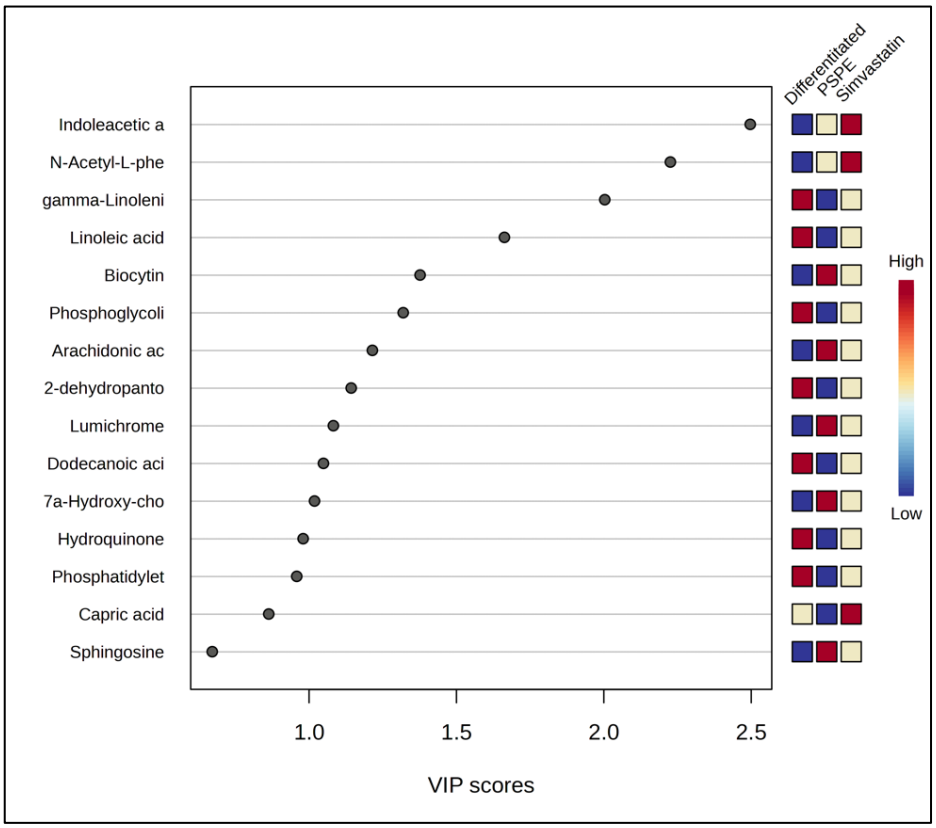

Figure 4.17 VIP Scores for Detected Metabolites in PSPE-treated 3T3-L1 Adipocyte Cells.

Table 4.13
VIP value, AUC value, p-value and Fold Change Analysis of 11 Metabolites Identified Using VIP and AUROC. Green Indicates Increased Fold Change in Differentiated Group; Red Indicates Reduced Fold Change in Differentiated Group.

Metabolites	VIP value	p-value	AUC value (Differentiated vs PSPE)	AUC value (Differentiated vs Simvastatin)	Fold Change (Differentiated vs PSPE)	Fold Change (Differentiated vs Simvastatin)
Indoleacetic acid	2.4970	0.0269	0.7037	0.8889	-8.0740	-8.4123
N-Acetyl-L-phenylalanine	2.2259	0.0294	0.6420	0.9383	-7.6527	-6.4909
gamma-Linolenic acid	2.0032	0.0034	0.9630	0.7037	6.8289	6.5800
Linoleic acid	1.6626	1.14E-04	0.8642	0.7037	9.5903	9.3414
Biocytin	1.3764	0.0173	0.9506	0.8395	-7.3734	-6.1700
Phosphoglycolic acid	1.3190	0.0082	0.9506	0.6914	8.0360	6.2266
Arachidonic acid	1.2144	0.0025	1.0	0.6667	-11.0587	-4.9258
2-dehydropantoate	1.1425	1.61E-04	0.5803	0.7778	-13.5624	-5.0797
Lumichrome	1.0820	0.0029	0.7531	0.8025	-7.6964	-1.5680
Dodecanoic acid	1.0483	2.76E-06	1.0	0.5556	-12.1227	-0.1117
7a-Hydroxy-cholestene-3-one	1.0180	0.0127	0.6914	0.8025	-6.9156	-1.5778

CHAPTER 5
DISCUSSION

5.1 Extraction Yield

Extraction yield refers to the proportion of extractable material obtained from a given amount of plant material after undergoing a specific extraction process, typically expressed as a percentage of the dry weight of the starting material. It serves as a key indicator of the efficiency of the extraction method and is influenced by factors such as solvent type, solvent-to-sample ratio, extraction time, temperature, and the physical and chemical properties of the plant matrix (Jha & Sit, 2022). The extraction yield of *Parkia speciosa* pods using 70% ethanol in this study was determined to be 37.83%. The extraction procedure involved soaking 600 g of dried *P. speciosa* pod powder in 1.2 L of 70% ethanol, followed by continuous shaking at room temperature using an orbital shaker for 3 to 4 days. This process was repeated by replacing the solvent until it turned colourless, ultimately resulting in a final extraction yield of 37.83%. Interestingly, previous studies have reported varying extraction yields for *P. speciosa* pod extract (PSPE), highlighting the influence of methodological and biological factors on extraction efficiency. For instance, Fithri et al. (2019) reported an extraction yield of 19.66%, while Kamisah et al. (2017) recorded a yield of 24.7%.

Despite utilising a similar solvent concentration and extraction method from Fithri et al. (2019), our study achieved a comparatively higher yield. One possible explanation for this difference lies in the geographical origin of the *P. speciosa* samples, as Fithri et al. (2019) sourced their pods from Indonesia, whereas our samples were collected from Malaysia. The phytochemical composition and moisture content of plant material are known to vary with location, climate, soil type, and seasonal factors, all of which can influence extraction yield (Mugao, 2024). Although Kamisah et al. (2017) employed a different solvent and extraction technique, their yield was comparatively closer to the one obtained in this study. This observation further underscores the complex relationship between extraction parameters and phytochemical content.

Siddiqui et al. (2023) emphasised that several factors contribute to variations in extraction yield, including the type and polarity of the solvent, extraction technique, duration, temperature, solvent-to-solid ratio, and, importantly, the origin and maturity

stage of the plant material. For example, Ng et al. (2020) demonstrated that even slight differences in solvent polarity can markedly affect the recovery of bioactive compounds from plant tissues. Moreover, the environmental conditions in which *P. speciosa* is grown, such as rainfall, temperature fluctuations, and soil nutrient availability, are likely to modulate its secondary metabolite profile, thereby affecting extraction outcomes (Ghasemzadeh et al., 2018).

Consistent with these findings, the discrepancies observed between our study and previous reports suggest that a combination of these factors, particularly the geographical source of *P. speciosa* pods, could explain the variation in extraction yield. This highlights the critical importance of considering plant origin and environmental conditions, alongside standardized extraction methods, when comparing phytochemical extraction studies. It also reinforces the necessity for optimised, reproducible extraction protocols when evaluating the biological activity of plant-based extracts, particularly in metabolomics and functional food research, where yield and phytochemical consistency are pivotal.

5.2 Phytochemical Analysis of *P. speciosa* Pod Extract (PSPE)

5.2.1 HPLC Analysis

The phytochemical composition of the ethanolic extract of *Parkia speciosa* pods (PSPE) was elucidated through high-performance liquid chromatography (HPLC) analysis. By comparing the chromatogram of PSPE against known standards, several peaks were identified, with two distinct peaks corresponding to gallic acid and p-coumaric acid. In contrast, kaempferol and catechin, both of which have been previously reported in other parts of *P. speciosa* or related species, were not detected in the present extract. While gallic acid and p-coumaric acid were successfully identified, challenges such as peak complexity and overlapping peaks limit the definitive characterisation of other phytoconstituents present in the extract.

The identification of gallic acid and p-coumaric acid in the PSPE is consistent, to some extent, with earlier work by Ko et al. (2014), who previously reported gallic acid, chlorogenic acid, ellagic acid, kaempferol, vanillic acid, and epicatechin in *P. speciosa* pod extracts. Additionally, catechin and quercetin were detected by Kamisah et al. (2017), Ko et al. (2014), Mustafa et al. (2018), and Siti et al. (2021), while rutin

was later reported by Siti et al. (2021). Notably, p-coumaric acid had not been documented in any of these earlier HPLC-based investigations, suggesting that its detection in this study may be attributed to methodological differences. The use of 70% ethanol as the extraction solvent in the present study likely played a fundamental role in the detection of this previously unreported compound.

Quantitative analysis of these phytochemicals revealed gallic acid concentrations of 53.97 ± 0.76 µg/mL. The concentration was determined using calibration curve constructed from standard solution, ensuring reliable quantification. The ability to successfully quantify the component enhances the phytochemical profiling in PSPE and establishes a clearer link between extraction parameters and phytochemical outcomes. Meanwhile, the concentration of the detected p-coumaric acid was unable to be quantified as it was below the detection value. When compared to previous findings on extraction yields, it becomes evident that the type and concentration of extraction solvent significantly influence both the yield and composition of plant extracts, corroborating reports by Nawaz et al. (2020), who emphasised solvent polarity as a critical factor in phytochemical extraction.

In previous studies, varying solvents such as 95% ethanol (Ko et al., 2014; Siti et al., 2021), 100% ethanol (Mustafa et al., 2018), and 100% methanol (Kamisah et al., 2017) were used for *P. speciosa* pods extraction, each resulting in different phytochemical profiles. The absence of p-coumaric acid in these earlier works further highlights the influence of extraction solvent and concentration on compound detectability. Our findings align with the study conducted by Martin et al. (2014), who demonstrated that 70% ethanol offers superior extraction efficiency for a broader range of phytochemicals compared to absolute solvents, owing to its intermediate polarity which facilitates the dissolution of both hydrophilic and moderately lipophilic compounds. This was further supported by Nawaz et al. (2020), who found that solvent polarity affects extraction yield and antioxidant properties, suggesting a combination of polar and non-polar solvents for optimal results. Thus, this characteristic of 70% ethanol may explain the successful detection of p-coumaric acid in the current study.

Nonetheless, despite the broader extraction potential of 70% ethanol reported by Martin et al. (2014), certain phytochemicals such as catechin and kaempferol were not detected in this study. This absence may reflect natural variation in phytochemical profiles due to factors such as plant part, maturity stage, environmental conditions, and post-harvest handling, as well as the intrinsic selectivity of any chromatographic

method. In HPLC analysis, parameters such as detection wavelength, mobile phase composition and gradient, stationary phase type, flow rate, column temperature, and run time inherently determine which compounds are most effectively resolved and detected (Santhosh et al., 2014; Adel et al., 2021). In this study, chromatographic detection was performed at 280 nm, a wavelength commonly employed for monitoring general phenolic compounds due to the strong absorbance of aromatic rings at this region. However, it is well established that not all phenolics and flavonoids exhibit optimal absorbance at 280 nm. In particular, flavonoids such as kaempferol, a flavonol, typically show maximum absorbance in the 320–370 nm range, while catechin, a flavan-3-ol, may exhibit variable absorbance depending on solvent system and conjugation state (Markham, 1982; Crozier et al., 2009). Consequently, detection at a single wavelength may limit sensitivity toward certain flavonoid subclasses, potentially resulting in non-detection despite their presence at low concentrations. Thus, the non-detection of kaempferol and catechin in the present study does not necessarily indicate their complete absence but may reflect methodological limitations related to wavelength selection and extract complexity. These methodological characteristics are not limitations per se but reflect the optimisation choices made to target specific compounds of interest within the broader phytochemical spectrum.

Furthermore, Mustafa et al. (2018) emphasised the substantial effects of plant collection site, seasonality, extraction conditions, and identification methods on phytochemical composition findings, which mirror those of Fithri et al. (2019), who demonstrated that *P. speciosa* pods collected from different regions exhibited notable differences in bioactive compound content. These observations underscore the importance of carefully considering environmental factors, extraction protocols, and solvent systems when investigating the phytochemical profiles of botanicals.

Collectively, this study not only confirms the presence of gallic acid in *P. speciosa* pods as reported previously but also reports the presence of p-coumaric acid, thus expanding the known phytochemical composition of this plant. This highlights the critical role that solvent polarity and extraction techniques play in influencing the qualitative and quantitative outcomes of phytochemical investigations. As such, it is recommended that future research further examine the impact of solvent selection and extraction protocols on the comprehensive profiling of PSPE, particularly in relation to its bioactivity in metabolomics-based anti-adipogenic studies.

5.2.2 Determination of Total Phenolic Content and Total Flavonoid Content

The determination of the total phenolic content (TPC) and total flavonoid content (TFC) in the ethanolic extract of *Parkia speciosa* pods (PSPE) provides valuable insights into the phytochemical composition and potential bioactivity of the plant. In the present study, PSPE demonstrated a broad TPC range, from 8.567 ± 0.12 to 267.5 ± 33.57 µg gallic acid equivalents (GAE)/mL of extract, alongside a TFC up to 30.53 ± 0.52 µg quercetin equivalents (QE)/mL. These detections of the compounds are in line with existing literature highlighting the phenolic and flavonoid content in *P. speciosa* pods, which contributes to their well-documented antioxidant and potential health-promoting properties (Kamisah et al., 2013; Ko et al., 2014; Ghasemzadeh et al., 2018; Saleh et al., 2021; Azemi et al., 2022).

However, there were variations in TPC and TFC values reported in these previous studies, which are reflective of multiple factors influencing phytochemical yields, including solvent type, extraction conditions, and geographic origin of the plant material. The use of 70% ethanol in this study likely facilitated the extraction of both polar and moderately non-polar bioactive compounds, accounting for the substantial phenolic and flavonoid yields observed. Martin et al. (2014) previously confirmed that aqueous ethanol mixtures significantly enhance the recovery of a broader range of bioactive compounds when compared to absolute ethanol or methanol, a conclusion further supported by Mustafa et al. (2018) and Siddiqui et al. (2023), who reported notable variations in phytochemical content as a function of environmental factors and extraction techniques.

Significantly, the comprehensive HPLC profiling performed in parallel with TPC and TFC assays in this study provides additional specificity and depth to the understanding of the phytochemical profile in PSPE. While TPC and TFC analyses offer cumulative estimations of total phenolic and flavonoid content, HPLC analysis identified individual phenolic constituents such as gallic acid and p-coumaric acid within PSPE. These compounds are well-documented in the literature for their biological activities and are often used as phytochemical markers in quality assessments of plant extracts (Mradu et al., 2012). The detection of these specific compounds supports the TPC and TFC values obtained and highlights the contribution of these phenolic acids to the overall phytochemical richness of PSPE.

Moreover, findings from previous studies reinforce the relevance of TPC and TFC determinations in *P. speciosa*. For instance, Fithri et al. (2016) reported significant levels of phenolic and flavonoid compounds in methanolic extracts of *P. speciosa* pods, while Ko et al. (2014) highlighted the rich flavonoid profile of the plant, supporting the current findings using a different solvent system. The strong presence of these phytochemicals suggests a well-established pattern of bioactive compound accumulation in this species, which may have implications for its biological properties, including potential antioxidant, anti-adipogenic and metabolic regulatory effects.

In summary, the overall TPC and TFC data with HPLC findings in this study offer a comprehensive evaluation of the phytochemical constituents in PSPE. These results not only affirm the phytochemical wealth of *P. speciosa* but also establish a solid foundation for subsequent investigations into the biofunctional properties of these compounds, particularly in relation to adipocyte differentiation and metabolic regulation. Therefore, identifying and measuring the phenolic and flavonoid content of PSPE is an essential step in understanding how its bioactive compounds may influence cellular processes, and it also provides valuable background for the metabolomics investigations that were further carried out in this study.

5.3 Antioxidant Activities of *P. speciosa* Pod Extract (PSPE)

5.3.1 DPPH Radical Scavenging and FRAP Assay

The assessment of antioxidant activity is a crucial step in evaluating the potential health-promoting properties of plant extracts. In this study, the antioxidant capacity of PSPE was determined using two widely accepted methods, which are the DPPH radical scavenging assay and the ferric reducing antioxidant power (FRAP) assay. These complementary assays provide valuable insights into the free radical scavenging ability and reducing power of the extract, respectively.

In the DPPH assay, the IC_{50} values recorded for PSPE and the positive control, ascorbic acid, were 57.05 ± 0.22 µg/mL and 23.13 ± 0.33 µg/mL, respectively. These results indicate that PSPE exhibits an antioxidant potency of approximately 0.4 times in scavenging DPPH radicals when compared to ascorbic acid, a well-known natural antioxidant. While the extract exhibited lower scavenging activity compared to ascorbic acid, this finding still reflects a moderate antioxidant capacity, consistent with its

phytochemical profile. Interestingly, our findings differed slightly from those reported by Ko et al. (2014) and Fithri et al. (2020), who observed marginally higher IC_{50} values for PSPE. These discrepancies are likely attributable to differences in the extraction solvent, plant material origin, and environmental growing conditions, all of which are known to influence phytochemical content and, subsequently, antioxidant activity (Mustafa et al., 2018; Siddiqui et al., 2023). As such, the standardization of extraction procedures and careful documentation of plant source conditions are crucial for reliable comparisons and reproducibility in antioxidant research.

Furthermore, the antioxidant potential observed here aligns closely with our earlier TPC, TFC, and HPLC findings. Phenolic and flavonoid compounds are well-recognised for their radical scavenging and reducing properties due to their hydroxyl groups, which can donate electrons or hydrogen atoms to neutralize reactive species (Zheng et al., 2010; Meo et al., 2013). Our HPLC profiling further identified key phenolic compounds such as gallic acid and p-coumaric acid in PSPE. Both of these compounds have been widely reported for their strong antioxidant, anti-inflammatory, and lipid-lowering activities (Kiokias et al., 2020), suggesting that their presence substantially contributes to the overall antioxidant profile of the extract.

In addition to the DPPH results, the FRAP assay was employed to assess the reducing capacity of PSPE. Our data showed that PSPE achieved a maximum FRAP value of 325.3 ± 4.85 µg $FeSO_4$/mL, a value comparable to that of ascorbic acid across several concentrations. Although slightly lower than the positive control, the reducing activity observed indicates the effectiveness of PSPE in converting ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). These findings support the potential of PSPE as a reducing agent, an important mechanism in combating oxidative stress through the stabilization of reactive oxygen species (ROS). This outcome is in line with previous reports by Gan & Latiff (2011) and Ko et al. (2014), who similarly demonstrated notable reducing capacities in *P. speciosa* pod extracts.

Importantly, while gallic acid and p-coumaric acid identified in our HPLC analysis are likely contributors to the antioxidant properties of PSPE, the moderate IC_{50} value observed in the DPPH assay suggests that other unidentified compounds, or possible synergistic interactions among phytochemicals, may also play significant roles in the overall antioxidant effect of the extract. This hypothesis is supported by prior studies indicating that the antioxidant activity of plant extracts often results from the

combined actions of multiple constituents rather than a single dominant compound (Ghasemzadeh et al., 2018; Skroza et al., 2022).

Overall, these findings not only highlight the antioxidant potential of PSPE but also reinforce the relevance of its phenolic and flavonoid content, as previously quantified through TPC, TFC, and characterised by HPLC. The consistent presence of antioxidant activity across multiple assays validates the phytochemical richness of PSPE and provides a strong rationale for its further investigation, particularly in the context of adipogenesis and oxidative stress-related metabolic disturbances.

5.4 *In Vitro* Anti-adipogenic Activities of *P. speciosa* Pod Extract (PSPE)

5.4.1 *P. speciosa* Pod Extract (PSPE) Dose Optimisation

5.4.1.1 Cell Viability Assay

The MTT assay served as an essential preliminary step in evaluating how PSPE influences the viability of mature 3T3-L1 adipocytes, providing preliminary insights before exploring its functional effects on adipocyte differentiation and metabolism. In this study, the cells were treated with a range of PSPE concentrations (31.25–1000 µg/mL) for 72 hours. The non-cytotoxic concentrations of the extract were determined based on the cell viability percentages obtained relative to differentiated control cells.

The results of the cell viability assay revealed a concentration-dependent manner, with cell viability consistently remaining above 80% at concentrations between 31.25 and 250 µg/mL, indicating that these doses were well-tolerated by mature adipocytes. However, a marked reduction in viability was observed at the highest tested concentration (1000 µg/mL), where cell viability decreased to 59.62 ± 14.48 %. This trend suggests the existence of a threshold beyond which PSPE begins to exert cytotoxic effects on adipocytes, reflecting a classic dose-response relationship commonly seen with plant extracts.

When comparing these results to both the untreated differentiated control and the positive control group which was treated with 4.19 µg/ml simvastatin, it was evident that lower PSPE concentrations, particularly 31.25 µg/mL and simvastatin maintained high cell viability levels, exceeding 99%. This indicates that at lower doses, PSPE exerts minimal adverse effects on mature adipocytes, which is crucial when considering its

potential therapeutic applications. In contrast, the decline in viability at higher concentrations aligns with the concentration-dependent cytotoxic patterns reported in other plant extract studies. This was proven by research done by Chang et al. (2016) on plant extracts and compounds, which shows dose-dependent effects on adipocyte viability and function, where low concentrations of resveratrol (1-10 μM) inhibit adipogenesis and lipolysis, while higher doses (10-100 μM) induce cytotoxicity in 3T3-L1 cells.

Since there are currently no published studies specifically examining the effects of PSPE on 3T3-L1 mature adipocyte viability, this finding highlights the novelty of the present work. However, similar observations have been reported in studies using different plant extracts. For instance, Etesami et al. (2020) demonstrated a significant reduction in 3T3-L1 adipocyte viability at higher concentrations of *Berberis vulgaris* extract, while Li et al. (2020) reported comparable effects with the ethanolic extract of *Litchi chinensis* seeds. Both studies noted that increased extract concentrations led to decreased cell viability, supporting the idea that adipocytes are particularly sensitive to high concentrations of plant-derived bioactive compounds.

The decrease in viability observed at elevated PSPE concentrations could be attributed to several factors, including the potential presence of cytotoxic phytochemicals, the concentration of the extract, and the prolonged exposure period (Tabatabaei et al., 2015). While the MTT assay is a valuable tool for assessing cell viability, it does not elucidate the underlying mechanisms responsible for the observed cytotoxicity (Kim et al., 2009; Hoogstraten et al., 2022). Future investigations should aim to isolate and characterise specific bioactive components within PSPE that may contribute to this effect and explore their possible cytotoxic mechanisms in adipocytes, whether through oxidative stress, apoptosis, or alterations in lipid metabolism pathways.

Relating these findings to the phytochemical composition of PSPE, as identified through HPLC analysis, provides additional context for the observed effects. The extract contains bioactive phenolics, including gallic acid and p-coumaric acid, both of which have been reported to exert dual activities, acting as antioxidants at lower concentrations but showing cytotoxic potential at higher levels (Rajashekar, 2023; Kalinowska et al., 2024). This duality may help explain the concentration-dependent impact of PSPE on adipocyte viability observed in this study. Specifically, the reduction in cell viability at higher doses reflects the dose-responsive patterns often reported in

phytochemical research, where phenolic compounds that are beneficial at moderate concentrations can shift toward pro-oxidant or cytotoxic effects when present in excess (Lapidot et al., 2002).

In conclusion, PSPE shows a dose-dependent effect on the viability of mature 3T3-L1 adipocytes through MTT assay. Lower concentrations exhibit minimal cytotoxicity, while higher doses significantly impair cell viability, likely due to the cumulative effect of bioactive compounds within the extract. These results lay an important foundation for subsequent functional studies, highlighting the need for careful consideration of PSPE dosing in future *in vitro* and *in vivo* study.

5.4.1.2 Oil Red O Staining Assay

Following the MTT assay, the dose optimisation was further performed across a range of PSPE concentrations (31.25–125 µg/mL) to determine the most effective dose for evaluating its anti-adipogenic potential in 3T3-L1 adipocytes, guided by findings from earlier cell viability assays. This step was crucial to ensure that the selected concentration would exhibit anti-adipogenic effects without inducing significant cytotoxicity, thereby maintaining cell viability throughout the differentiation process. Following this optimisation, the oil red O (ORO) staining assay was employed to assess intracellular lipid accumulation, a key indicator of adipogenesis, and to establish the effective concentration for further analysis.

ORO staining was utilised to both visualise and quantify lipid droplet accumulation, providing qualitative and quantitative data to assess the anti-adipogenic efficacy of PSPE over time. In this assay, the cells were treated with 31.25 to 125 µg/mL at varying treatment periods, which were 24, 48, and 72 hours, with differentiated cells serving as the baseline control. The results demonstrated a clear time-dependent and concentration-specific effect on lipid accumulation. No significant morphological differences were observed at 24 hours; however, by 48 and 72 hours, cells treated with 62.5 and 125 µg/mL of PSPE, alongside the simvastatin-positive control, exhibited visibly reduced lipid droplets compared to the untreated differentiated controls. The group of adipocytes that were treated with 62.5 µg/mL PSPE for 48 hours showed a high percentage of cell viability with a significant reduction in the lipid content. Therefore, it was determined as the optimal treatment concentration and exposure period.

To further support the microscopic observation, quantitative analysis of lipid content after elution at 48 and 72 hours confirmed a significant reduction in lipid accumulation in PSPE-treated cells at both concentrations (62.5 and 125 µg/mL). These findings are consistent with previous reports by Li et al. (2020) and Oh et al. (2022), who demonstrated comparable reductions in lipid accumulation following treatment with pomegranate flower extract and red pepper leaf extracts, respectively. Both studies attributed these effects to the presence of bioactive phytochemicals capable of modulating adipogenic pathways, providing valuable context for the present results. To date, no previous studies have directly assessed dose optimisation of *Parkia speciosa* pod extract using oil red O staining in 3T3-L1 adipocytes. This underscores the novelty of the current study, which provides a systematic assessment of PSPE dose optimisation using both viability and oil red O staining assays, further supported by phytochemical analysis.

Integrating these findings with the earlier antioxidant assays and cell viability assays highlights the interconnected nature of biological effects in PSPE. The dose-dependent reduction in lipid accumulation, in parallel with the reduction in cell viability at higher doses, suggests a complex relationship between the antioxidant capacity, cytotoxicity, and anti-adipogenic potential of PSPE. Collectively, these results emphasise the importance of dose optimisation when evaluating the therapeutic potential of plant extracts, ensuring maximal bioactivity while maintaining cellular viability and functionality.

5.4.2 Effect of PSPE on Lipid Accumulation by Oil Red O Staining Assay

Confirmatory testing of the chosen concentration of PSPE was then performed following dose optimisation. To validate the selection of 62.5 µg/mL as the optimal concentration of PSPE for evaluating its anti-adipogenic effect in mature 3T3-L1 adipocytes, the oil red O (ORO) staining assay was again employed. ORO assay is a widely accepted method for quantifying intracellular lipid accumulation during adipocyte differentiation. Similarly, after 48 hours treatment period, microscopic evaluation of ORO-stained cells at 100X magnification did not reveal apparent morphological differences in lipid accumulation between the experimental groups. Nonetheless, a slight reduction in lipid droplet accumulation was observed in both the

PSPE- and simvastatin-treated groups when compared to the differentiated control, providing preliminary visual evidence of anti-adipogenic potential in PSPE.

To substantiate these observations, quantitative analysis of lipid accumulation was conducted by eluting the intracellular ORO dye and measuring absorbance at 490 nm using a microplate reader. The results demonstrated that, although neither the PSPE- nor simvastatin-treated groups reduced lipid content to the level of the undifferentiated control, both treatments significantly attenuated lipid accumulation relative to the differentiated control. Specifically, treatment with PSPE resulted in a 17.1 ± 0.01 % reduction, while simvastatin, used as a pharmacological positive control, achieved a 19.4 ± 0.01% reduction in lipid content. Notably, no significant difference was detected between the PSPE- and simvastatin-treated groups, indicating that PSPE at this concentration exhibits a comparable anti-adipogenic effect to the established anti-lipogenic agent.

These findings are consistent with the earlier dose optimisation data, reinforcing the evidence that 62.5 µg/mL of PSPE is effective in reducing adipogenic differentiation in 3T3-L1 adipocytes. The comparable lipid-reducing effect of PSPE to simvastatin, a widely used lipid-lowering drug, highlights the potential of PSPE as a natural intervention with therapeutic relevance in obesity management. This is particularly relevant given that simvastatin has been reported to exert anti-adipogenic effects via inhibition of HMG-CoA reductase and modulation of adipogenic transcription factors (Jakab et al., 2021), suggesting that PSPE may act through similar or complementary pathways. Although direct studies using *P. speciosa* pod extract and oil red O staining in 3T3-L1 adipocytes are limited, these findings align closely with Oh et al. (2019) and Li et al. (2020), that utilised ORO staining to assess plant extract effects on lipid accumulation in 3T3-L1 adipocytes. Those studies reported similar reductions in lipid content, underscoring the general anti-adipogenic potential of bioactive constituents within *P. speciosa*.

The anti-adipogenic effects, specifically the reduction in lipid accumulation observed in this study, may be attributed to the bioactive phenolic compounds detected in PSPE, particularly gallic acid and p-coumaric acid. These compounds have been widely recognised for their ability to inhibit adipocyte differentiation and lipid accumulation. Aranaz et al. (2019) highlighted the inhibitory role of phenolic compounds, including gallic acid, on adipogenesis, while Kang et al. (2013) reported similar findings for p-coumaric acid and other related phenolic compounds. The

presence of these bioactive compounds in PSPE provides a plausible explanation for the reduction in lipid droplets observed in this study. Previous studies have reported that gallic acid suppresses adipogenesis by downregulating key transcription factors such as PPARγ and C/EBPα (Park et al., 2014), while p-coumaric acid has demonstrated inhibitory effects on adipocyte differentiation by reducing lipid accumulation, decrease triglyceride synthesis, and modulate the expression of key adipogenic regulators such as PPARγ and C/EBPα (Kang et al., 2013; Ilavenil et al., 2016). The presence of these compounds in PSPE provides mechanistic support for the reductions in lipid accumulation observed in this study.

Taken together, these findings offer compelling evidence for the anti-adipogenic potential of PSPE at an optimised dose of 62.5 µg/mL, delivering effects comparable to a pharmacological agent while maintaining cell viability. The integration of these data with previous antioxidant and phytochemical analyses suggests that the anti-adipogenic activity of PSPE is likely driven by its phenolic composition and associated biological properties. These results contribute to the growing body of evidence supporting the potential application of *Parkia speciosa* in managing obesity and metabolic disorders.

5.4.3 Effect of PSPE on Triglyceride Level

The triglyceride assay was performed to quantify the intracellular triglyceride content and evaluate the impact of PSPE on lipid accumulation within 3T3-L1 adipocytes. The results revealed a significant reduction in triglyceride levels in cells treated with both PSPE and simvastatin, showing decreases of 34.8% and 39.6%, respectively, compared to untreated differentiated control cells. These findings highlight the lipid-lowering potential of PSPE, demonstrating effects comparable to simvastatin which is a widely prescribed statin drug used in the clinical management of dyslipidemia and obesity-related metabolic disorders.

Comparable trends have been documented in several plant-based studies. For instance, a study by Les et al. (2020) investigated the anti-adipogenic effects of *Jasonia glutinosa* (Rock tea) extract at a concentration of 50 µg/mL on 3T3-L1 adipocytes, reporting a significant 25% reduction in triglyceride accumulation. This effect was attributed to the rich phenolic acid and flavonoid content from the extract, known contributors to anti-lipogenic activity through modulation of adipogenic gene expression and lipid metabolism. Similarly, Geum et al. (2021) examined the effects of

Heracleum moellendorffii root extract on triglyceride accumulation at a higher concentration of 100 µg/mL, observing a substantial 60% reduction. The authors linked this effect to the presence of bioactive compounds, including phenolic acids and flavonoids, which are thought to suppress adipogenesis by inhibiting key transcription factors such as PPARγ and C/EBPα, and enhancing lipolytic pathways.

As there is currently no published study specifically assessing the triglyceride-lowering effects of *Parkia speciosa* pod extract on 3T3-L1 adipocytes using a triglyceride assay, the findings from the present study fill this gap, offering the first quantitative evidence of its lipid-lowering efficacy. The substantial reduction in triglyceride content observed here can plausibly be attributed to the phenolic acids and flavonoids detected in PSPE, particularly gallic acid and p-coumaric acid, both previously reported to exert anti-adipogenic effects (Kang et al., 2013; Park et al., 2014; Aranaz et al., 2019). These compounds have been shown to interfere with lipid accumulation by downregulating adipogenic transcription factors and reducing oxidative stress, mechanisms likely contributing to the lipid-lowering effects observed in this study.

Taken together, these results reinforce the growing body of evidence that plant-derived phenolic-rich extracts possess significant anti-adipogenic properties. The triglyceride assay findings presented here not only align with earlier results from dose optimisation and oil red O staining assays but also underscore the potential of PSPE as a natural alternative for managing lipid accumulation in adipocytes.

5.5 Metabolic Responses in 3T3-L1 Adipocyte Differentiation

Metabolomics is a field of research that investigates the complex and diverse metabolic processes in biological systems (Wang & Huang, 2021). This approach involves a comprehensive analysis of metabolites present in biological specimens (Clish, 2015). In this study, a metabolomic analysis was initially conducted between the differentiated and undifferentiated 3T3-L1 adipocyte cell groups to validate the success of the cell differentiation process. This preliminary comparison was to establish a reliable reference framework for the model and control groups, which was essential for interpreting the subsequent analysis of the treatment groups. The detected metabolites in both groups were thoroughly analysed to identify the significant metabolites. According to Roberts et al. (2009), when comparing the metabolic changes between

adipocytes in pre- and post-differentiation phases, there were some metabolites alterations, and this reflects the changes of the 3T3-L1 cells during differentiation. The alterations in cellular metabolites may also serve as metabolic triggers that initiate or support the pathways required for the process of adipocyte differentiation.

The study successfully identified significant metabolites and metabolic pathways that distinguish differentiated from undifferentiated 3T3-L1 adipocytes. A total of 14 metabolites were significantly altered between the two cellular states, and these metabolites were from lipid metabolism, amino acid metabolism, carbohydrate metabolism, nucleotide metabolism, and glycan metabolism. Based on the VIP scores, four metabolites, which are gamma-linolenic acid (GLA), arachidonic acid (AA), 9,10-epoxyoctadecenoic acid (9,10-EpOME), and dolichyl b-D-glucosyl phosphate were identified as potential biomarkers. These metabolites were found to be involved primarily in linoleic acid metabolism and N-glycan biosynthesis, which are the key pathways in both differentiated and undifferentiated adipocytes. Thus, the major metabolic pathways identified can be categorised into two main pathways which are lipid metabolism and glycan metabolism.

5.5.1 Lipid Metabolism

Lipid metabolism encompasses a wide range of biochemical pathways that govern the synthesis, modification, and degradation of lipids and is fundamental to adipocyte biology (Morigny et al., 2021). During adipogenesis, preadipocytes undergo complex metabolic alterations to support the transition from a proliferative to a lipid-storing phenotype. This metabolic shift is characterised by increased lipogenesis, altered fatty acid oxidation, and dynamic changes in lipid intermediates, all of which are tightly regulated by transcriptional networks and signalling pathways (Oates & Antoniewicz, 2022). According to Qian et al. (2020), transcriptional regulation, particularly by PPARγ, is essential in adipocyte differentiation and metabolism, with various lipids serving as key regulators. Such transitions are crucial not only for lipid accumulation but also for establishing the functional identity of mature adipocytes, which are specialized for energy storage and endocrine signalling (Dinel et al., 2010).

In the present metabolomics study, a distinct lipidomic profile was identified between adipocytes and preadipocytes, highlighting the functional divergence in their metabolic requirements. Notably, three metabolites which are GLA, AA and 9,10-

EpOME were significantly downregulated in differentiated adipocytes. All of these metabolites are considered important intermediates in linoleic acid metabolism, a pathway that yields bioactive lipid mediators, which are polyunsaturated fatty acids (PUFA)-derived signalling molecules such as prostaglandins, leukotrienes, and epoxy fatty acids (Dyall et al., 2022). These mediators exert profound effects on adipocyte signalling and differentiation.

GLA, an omega-6 polyunsaturated fatty acid, plays a critical role in early adipocyte development. It serves as a precursor to dihomo-γ-linolenic acid (DGLA), which can be further metabolized into prostaglandins with anti-inflammatory and cell regulatory properties (Fan & Chapkin, 1998). The reduced levels of GLA in differentiated adipocytes compared to preadipocytes observed in this study likely reflect the increased demand for membrane synthesis and remodelling in preadipocytes which are essential during rapid cell proliferation. GLA also contributes to maintaining membrane fluidity and may participate in lipid-mediated signalling pathways that prime preadipocytes for differentiation (Azain, 2004). Thus, considering the metabolic state of proliferating preadipocytes, GLA may provide essential substrates for bioactive lipid mediators involved in coordinating cellular growth and early lineage commitment. Notably, GLA has previously been reported to be upregulated during adipocyte differentiation as part of the lipid accumulation process (Roberts et al., 2009). However, to date, no study has specifically quantified GLA levels on a stand-alone basis in adipogenesis. Thus, this highlights the need for targeted metabolomics approaches to more precisely evaluate its changes in adipogenesis.

Arachidonic acid, another omega-6 polyunsaturated fatty acid was also found to be downregulated in differentiated adipocytes in this study. It is known as a precursor to a wide array of eicosanoids, including prostaglandins, leukotrienes, and thromboxanes. These lipid mediators are integral to immune regulation, inflammation, and cell signalling (Calder, 2001; Bennett & Gilroy, 2016). During the early phases of adipogenesis, arachidonic acid has been shown to promote differentiation by stimulating cyclic AMP and prostacyclin signalling pathways, which activate early transcription factors such as C/EBPβ and C/EBPδ (Massiera et al., 2003; Schmitz & Ecker, 2008). Therefore, the downregulated arachidonic acid in the differentiated cells may reflect a shift away from these differentiation-associated signalling pathways, as the cells transition from a regulatory to a storage-focused phenotype. According to Green et al. (2021), as cells differentiate, arachidonic acid levels decrease. This

interpretation is supported by previous findings showing that arachidonic acid-derived prostaglandins, while essential for initiating adipogenic gene expression, are less prominent once terminal differentiation is achieved (Khan et al., 2016). Thus, the reduced abundance of arachidonic acid in mature adipocytes likely mirrors the diminished need for eicosanoid-mediated signalling, as the metabolic priorities of the cell become centered on lipid storage and energy balance. Interestingly, both gamma-linolenic acid and arachidonic acid were found to be downregulated in differentiated adipocytes. Given that GLA serves as an upstream precursor in the biosynthetic pathway leading to arachidonic acid, this coordinated decrease is expected. The reduction in GLA availability may contribute to the diminished pool of arachidonic acid, thereby reflecting a broader metabolic shift away from lipid signalling toward energy storage as adipocytes reach terminal differentiation.

Similarly, the observed downregulation of 9,10-epoxyoctadecenoic acid (9,10-EpOME), a linoleic acid-derived epoxide, further suggests a reduced engagement of lipid-mediated signalling mechanisms in the mature adipogenic state. EpOMEs, including 9,10-EpOME, along with other epoxyoctadecenoic acid isomers have been implicated in modulating the activity of peroxisome proliferator-activated receptors (PPARs), particularly PPARγ, a transcription factor central to adipogenesis (Thomson et al., 2012; Korbecki et al., 2019). The downregulated of this metabolite in adipocytes compared to preadipocytes may indicate the activation of lipid-derived signalling mechanisms in preadipocytes that prepare the cellular environment for PPARγ induction. Additionally, epoxide fatty acids are recognised for their cytoprotective effects, counteracting oxidative stress and inflammation, conditions that often accompany extracellular matrix remodelling during early adipogenic commitment (Zeldin, 2001). Therefore, the decline in 9,10-EpOME levels in mature adipocytes may correspond with a diminished need for cytoprotective and differentiation-promoting lipid signals, aligning with a metabolic transition toward lipid accumulation and maintenance of adipocyte identity.

Collectively, these findings highlight a dynamic alteration of lipid metabolism that governs adipocyte differentiation. The elevated levels of gamma linolenic acid, arachidonic acid, and 9,10-EpOME in preadipocytes suggest an active lipid signalling environment conducive to early adipogenic commitment, while their downregulation in mature adipocytes aligns with a shift toward lipid storage and metabolic homeostasis.

This stage-specific metabolite distribution highlights the critical role of linoleic acid-derived lipid mediators in coordinating the adipocyte lineage progression.

5.5.2 Glycan Metabolism

Glycan metabolism refers to the synthesis, modification, and breakdown of glycans in cells. It involves a complex network of enzymatic reactions that create diverse glycan structures, attach them to proteins and lipids, and regulate their turnover. Glycans or also known as complex carbohydrates have long been recognised to play their significant metabolic, structural and physical roles within biological systems (Varki, 2017). They can be classified into various types based on their structural characteristics such as N-linked glycans (attached to proteins via asparagine), O-linked glycans (linked to serine or threonine residues) (Harvey, 2003), and glycolipids (sugar chains attached to lipids) (Shivatare et al., 2022). These structures, collectively known as glycoconjugates, are formed through the process of glycosylation, a crucial mechanism for numerous cellular functions. One of the most well-studied forms of glycosylation is N-glycan biosynthesis, a complex process that occurs primarily in the endoplasmic reticulum (ER) and golgi apparatus. During this process, N-linked glycans are assembled on a lipid carrier called dolichyl phosphate, which facilitates the transfer of a pre-assembled glycan chain onto the asparagine residues of nascent proteins.

In this study, one of the potential biomarkers, dolichyl b-D-glucosyl phosphate, was observed to be downregulated in differentiated cells compared to undifferentiated cells. This metabolite is involved primarily in N-glycan biosynthesis. According to Aebi (2013), N-glycan biosynthesis is a highly regulated process in which N-glycans are added to asparagine residues on developing proteins in the ER. These glycans are important for the proper folding, stability, and function of glycoproteins. The correct folding of glycoproteins ensures that they achieve their functional conformation, which is crucial for various cell surface receptors, enzymes, and signalling molecules involved in regulating growth and differentiation (Ferris et al., 2014). Lannoo and Van Damme (2015) highlighted the crucial role of dolichyl b-D-glucosyl phosphate in the early stages of N-glycan biosynthesis. It acts as a sugar donor in the transfer of glucose to the growing lipid-linked oligosaccharide precursor during glycoprotein assembly in the ER.

In the context of preadipocytes, these cells are actively growing and proliferating, requiring a high rate of glycosylation for the production of glycoproteins

that are necessary for cell signalling, growth, and differentiation (Araujo, 2015). The observed downregulation in differentiated adipocytes compared to preadipocytes reflects the increased demand for glycosylation during differentiation phase. Enhanced N-glycan biosynthesis in preadipocytes supports the formation of functional glycoproteins essential for cell adhesion, growth factor receptor activation, and extracellular matrix organization, all of which are critical for preparing the cell for differentiation. Once preadipocytes have differentiated into adipocytes, their primary role shifts towards lipid storage and metabolic regulation. This transition results in the decreased demand for N-glycan biosynthesis, which leads to lower levels of dolichyl b-D-glucosyl phosphate. The downregulation of dolichyl b-D-glucosyl phosphate in adipocytes compared to preadipocytes reflects the distinct metabolic needs of these cells. Preadipocytes, which are preparing to differentiate, necessitate high levels of glycosylation to effectively regulate cellular processes, while differentiated adipocytes focus more on lipid metabolism and storage, therefore reducing their glycosylation requirements.

5.6 Effects of *Parkia speciosa* Pod Extract (PSPE) on Metabolic Responses in 3T3-L1 Adipocyte Differentiation

In this part, a comprehensive metabolomics analysis was further conducted to investigate the metabolic alterations induced by PSPE treatment in differentiated 3T3-L1 adipocytes. This comparative analysis aimed to elucidate the underlying metabolic responses associated with natural intervention to inhibit adipogenesis. By examining the differentiated, PSPE-treated, and simvastatin-treated groups, the study sought to identify potential biomarkers and disrupted pathways that may contribute to the anti-adipogenic effects of PSPE.

A total of 33 significantly altered metabolites were identified across the three groups, encompassing 19 sub-pathways related to lipid metabolism, amino acid metabolism, carbohydrate metabolism, nucleotide metabolism, and the metabolism of cofactors and vitamins. Among these, lipid metabolism showed the most pronounced changes, suggesting its central role in the cellular response to PSPE treatment. Based on the VIP scores, specifically those exceeding a value of one (VIP > 1), the metabolites were prioritized as potential biomarkers. These included linoleic acid, gamma-linolenic acid, arachidonic acid, dodecanoic acid, 7α-hydroxy-cholestene-3-one, indoleacetic

acid, N-acetyl-L-phenylalanine, phosphoglycolic acid, 2-dehydropantoate, biocytin, and lumichrome. These compounds were found to play key roles in distinguishing the metabolic profiles of treated and untreated adipocytes.

5.6.1 Lipid Metabolism

Lipid metabolism plays a central role in maintaining adipocyte function and energy balance (Skrzypski & Kołodziejski, 2023). During adipogenesis, processes such as lipid biosynthesis, uptake, and remodelling are tightly coordinated to support the transition from preadipocytes to mature adipocytes. However, modulation or disruption of this regulatory network can significantly influence lipid accumulation and the progression of adipocyte differentiation (Cristancho & Lazar, 2011; Siersbæk et al., 2012; Miehle et al., 2020). In this study, metabolomics profiling revealed notable shifts in several lipid-related pathways in response to PSPE treatment, compared to untreated differentiated adipocytes. Specifically, linoleic acid and gamma-linolenic acid were both significant intermediates in the linoleic acid metabolism pathway that were downregulated, while arachidonic acid, dodecanoic acid, and 7α-hydroxy-cholestene-3-one, representing potential biomarkers from arachidonic acid metabolism, fatty acid biosynthesis, and primary bile acid biosynthesis, respectively, were upregulated in PSPE-treated group.

Linoleic acid, an essential omega-6 polyunsaturated fatty acid, serves dual functions in adipocyte metabolism. It acts as a precursor for long-chain fatty acids such as arachidonic acid and is incorporated into triglycerides during lipogenesis (Choque et al., 2014). Gamma-linolenic acid (GLA), a desaturation product of linoleic acid, is further converted into dihomo-gamma-linolenic acid (DGLA) and ultimately arachidonic acid (Kapoor & Huang, 2006; Sergeant et al., 2016). Typically, both linoleic acid and GLA increase during adipocyte differentiation to support membrane synthesis and lipid droplet formation (Fan & Chapkin, 1998; Hutley et al., 2003). In contrast, both metabolites were significantly downregulated in PSPE-treated cells in this study. This reduction may indicate inhibition of lipid synthesis and desaturation processes, thereby limiting the supply of lipogenic precursors required for full adipocyte maturation. Consistent with this, previous reports have shown that decreased availability of linoleic acid can impair adipogenesis by weakening PPARγ activation, a key transcriptional regulator of fat cell development (Hutley et al., 2003). Thus, the

downregulation of linoleic acid and its derivative GLA in treated cells suggests a suppression of key lipogenic and differentiation pathways essential for adipocyte maturation.

On the other hand, despite the downregulation of linoleic acid and GLA, arachidonic acid, a downstream product of linoleic acid metabolism, was significantly upregulated. While commonly associated with pro-inflammatory signalling (Caldari-Torres & Trinh, 2022), arachidonic acid also plays an important role in adipocyte regulation via prostaglandin synthesis (Pisani et al., 2014). Certain prostaglandins, such as PGF2α, are known to inhibit adipogenesis by interfering with PPARγ signalling (Liu & Clipstone, 2007; Fujimori, 2012). Thus, elevated arachidonic acid may reflect a compensatory increase in substrate availability for anti-adipogenic prostaglandins. According to Laaksonen et al. (2006), simvastatin has been shown to influence eicosanoid production and inflammatory pathways, which may partly explain the upregulated of this metabolite. Polyphenol-rich extracts such as PSPE may exert similar modulatory effects, thereby contributing to the observed metabolic shifts.

Interestingly, dodecanoic acid or also known as lauric acid, is a medium-chain saturated fatty acid with 12 carbon atoms, involved in fatty acid biosynthesis. This metabolite was also found to be upregulated in PSPE-treated group. As mentioned by Jørgensen et al. (2001), medium-chain fatty acids (MCFAs) like dodecanoic acid are metabolized more efficiently than long-chain fatty acids, as they are rapidly oxidized in mitochondria and less readily stored as triglycerides. Elevated levels of MCFAs have been linked to enhanced energy expenditure and reduced fat accumulation (Montgomery et al., 2013). According to Montgomery et al. (2017), in both cell culture and animal models, MCFAs have been shown to improve mitochondrial function, reduce lipid accumulation, and limit oxidative stress compared to long-chain fatty acids. The observed increase in dodecanoic acid in PSPE group may therefore indicate a shift toward fatty acid oxidation and energy dissipation, consistent with an anti-adipogenic effect.

Similarly, 7α-hydroxy-cholestene-3-one, an intermediate in bile acid biosynthesis, was also elevated in PSPE-treated adipocytes. Beyond their classical role in lipid absorption (Trauner et al., 2010; Donkers et al., 2019), bile acids are increasingly recognised as metabolic signalling molecules that act via nuclear receptors like Farnesoid X Receptor (FXR) and membrane-bound receptors such as G-protein coupled bile acid receptor (TGR5) (Wei et al., 2009). These signalling pathways have

been associated with improved insulin sensitivity, reduced lipid storage, and increased energy expenditure (Trauner et al., 2010; Ma & Patti, 2014). The rise in this bile acid intermediate may therefore contribute to a more metabolically active state in adipocyte under PSPE treatment.

Taken together, the changes in these lipid metabolites point to a coordinated alterations of lipid metabolism under PSPE treatment. The downregulation of linoleic acid and GLA suggests a suppression of lipid biosynthesis and storage, while the upregulation of arachidonic acid, dodecanoic acid, and 7α-hydroxy-cholestene-3-one indicates a shift toward prostaglandin-driven inhibition of adipogenesis, fatty acid oxidation, and bile acid-mediated signalling. Although these metabolites arise from distinct sub-pathways, their collective response supports a metabolic phenotype that limits lipid accumulation and favors energy expenditure.

5.6.2 Amino Acid Metabolism

Amino acid metabolism, particularly branched-chain amino acids (BCAAs), plays an essential role in adipocyte differentiation and function. BCAAs are rapidly consumed during adipogenesis and are considered critical for the differentiation process (Salinas-Rubio et al., 2018). As adipocytes mature, they undergo significant metabolic reprogramming, which includes increased glucose uptake, enhanced glycolysis and the citric acid (TCA) cycle activity, as well as altered glutamine metabolism (Oates & Antoniewicz, 2022). Beyond their classical roles as building blocks for proteins, amino acids and their derivatives also regulate gene expression through various mechanisms, including transcriptional modulation, epigenetic mechanisms, and signalling pathways (Van Winkle & Ryznar, 2019; Sah et al., 2021; Chen et al., 2024). Several amino acids function as signalling molecules that influence key regulatory networks such as mTORC1, AMPK, and MAPK (Paudel et al., 2021). In adipocyte biology, shifts in the levels of specific amino acid-derived metabolites often reflect changes in differentiation state, metabolic activity, and cellular stress response (Green et al., 2016; Salinas-Rubio et al., 2018; Oates & Antoniewicz, 2022). Within this metabolic framework, tryptophan and phenylalanine are two essential aromatic amino acids that contributes to pathways known to influence adipogenesis and metabolic balance. In this study, two detected potential biomarkers, indoleacetic acid (IAA) and N-acetyl-L-phenylalanine were significantly upregulated in 3T3-L1 adipocytes treated with PSPE, compared to the

differentiated control group. These metabolites are intermediates of tryptophan and phenylalanine metabolism, respectively, and their upregulation suggests that PSPE may exert regulatory effects through amino acid pathways.

IAA is a catabolite of tryptophan and is best known as a plant hormone produced by various microorganisms through several biosynthetic pathways, including the indole-3-pyruvate, indole-3-acetamide, and indole-3-acetonitrile routes (Patten et al., 2013; Tang et al., 2023). While extensively studied in plant biology, IAA is also produced in mammalian systems, particularly under the influence of gut microbiota or cellular stress. In mammalian cells, IAA and other indole derivatives act as ligands for the aryl hydrocarbon receptor (AhR), a transcription factor involved in detoxification, immune signalling, and adipocyte differentiation (R. Shinde & McGaha, 2018). Prior studies have shown that AhR activation by indole metabolites can inhibit adipogenesis by suppressing key transcriptional regulators such as PPARγ and C/EBPα (Shimba et al., 2001a). The elevated levels of IAA observed in PSPE-treated adipocytes may therefore reflect an upregulation of tryptophan metabolism toward anti-adipogenic signalling, potentially mediated through AhR pathways.

Similarly, N-acetyl-L-phenylalanine is a metabolite formed through the acetylation of phenylalanine and is part of the phenylalanine metabolism pathway. This pathway contributes to the biosynthesis of neurotransmitters and other phenylacetic compounds with metabolic relevance (Pascual et al., 2016). Although the specific function of N-acetyl-L-phenylalanine in adipocytes is not yet well defined, its accumulation may reflect a shift in phenylalanine usage under treatment-induced metabolic stress or redox adaptation. Additionally, phenylalanine-derived metabolites have been linked to improved insulin sensitivity and reduced adiposity in several models of metabolic dysfunction (Adams, 2011; Irving et al., 2015; Yang et al., 2018). The elevated levels observed here may therefore be indicative of treatment-induced metabolic reprogramming.

The consistent upregulation of both IAA and N-acetyl-L-phenylalanine in PSPE-treated adipocytes suggests that this intervention may influence amino acid metabolism as part of their anti-adipogenic mechanism. For IAA, this likely involves AhR activation and downstream suppression of adipogenic gene expression. Meanwhile, in the case of N-acetyl-L-phenylalanine, the accumulation may point to altered phenylalanine metabolism, potentially linked to reduced lipid biosynthesis or increased oxidative stress resistance. Notably, simvastatin has been shown to impact

amino acid metabolism through mechanisms involving AMPK activation and mitochondrial biogenesis (Trupp et al., 2012), while the diverse phytochemical content of PSPE may trigger similar regulatory responses via antioxidant or signalling pathways. Together, these findings suggest that tryptophan and phenylalanine metabolism may contribute to the anti-adipogenic effects observed in treatment group. Although the underlying mechanisms may differ, both pathways appear to support a shared metabolic outcome characterised by reduced lipid accumulation.

5.6.3 Carbohydrate Metabolism

Carbohydrate metabolism represents a highly complex network of biochemical pathways essential for cellular energy production and metabolic homeostasis (Dashty, 2013; Salih et al., 2022). It encompasses processes such as glycolysis, the citric acid (TCA) cycle, and the pentose phosphate pathway, which coordinate the breakdown of glucose and other monosaccharides to generate ATP and key intermediates for biosynthetic processes (Dashty, 2013). In addition to these core processes, carbohydrate metabolism also involves more specialized intermediary pathways such as glyoxylate and dicarboxylate metabolism. Although the glyoxylate cycle is classically associated with plants and certain microorganisms, components of the glyoxylate and dicarboxylate metabolism pathway have been identified in mammalian cells, particularly in the context of mitochondrial and peroxisomal function. This pathway plays a pivotal role in processing small dicarboxylic acids, including glyoxylate and oxalate, and contributes to redox homeostasis, energy regulation, and the detoxification of by-products arising from lipid and amino acid metabolism (Salido et al., 2012).

Among the notable intermediates in this pathway is phosphoglycolic acid, a metabolite involved in the detoxification of glycolate and glyoxylate (Dellero et al., 2016). In mammalian systems, phosphoglycolic acid is believed to be produced through mitochondrial and peroxisomal enzymatic activities, where it may serve as a transient carrier of two-carbon units or act as a regulatory molecule during oxidative stress responses (Nordgren & Fransen, 2014). While its functions have been more thoroughly studied in plant photorespiration (Bauwe et al., 2010), its relevance in mammalian metabolism has been associated with mitochondrial oxidative processes and peroxisomal activity, especially in cells that are metabolically active or undergoing changes such as differentiation. In this context, phosphoglycolic acid may serve as a

marker of oxidative flux and could indirectly support lipid metabolism by aiding the regeneration of redox cofactors.

In the present study, phosphoglycolic acid was found to be significantly downregulated in PSPE-treated adipocytes compared to the differentiated control group. This downregulation suggests that PSPE may reduce oxidative metabolic activity during adipogenesis. The presence of complex mixture of antioxidant-rich phytochemicals in PSPE including flavonoids and phenolic acids, is believed to reduce oxidative stress (Ko et al., 2014; Balaji et al., 2015; Saleh et al., 2021) and alter metabolic fluxes associated with adipocyte differentiation.

The observed downregulation of phosphoglycolic acid may thus reflect a coordinated suppression of oxidative and anabolic processes typically activated during adipogenesis (Llobet et al., 2015). Given the role of glyoxylate and dicarboxylate metabolism in maintaining redox regulation and supporting lipid biosynthesis, the inhibition of this pathway could impair the availability of key cofactors and intermediates necessary for lipid accumulation. This metabolic shift is consistent with the anti-adipogenic effects observed in PSPE intervention, and it highlights the potential of phosphoglycolic acid as a potential metabolic biomarker of reduced oxidative activity during adipocyte differentiation. Collectively, these findings suggest that treatment with PSPE may alter carbohydrate metabolism to lower oxidative flux, thereby contributing to the inhibition of adipocyte maturation.

5.6.4 Nucleotide Metabolism

Nucleotide metabolism is essential for maintaining cellular function, as it supports fundamental biological processes such as nucleic acid synthesis, energy transfer, and intracellular signalling (Stasolla et al., 2003; Lane & Fan, 2015). In recent years, nucleotides have been recognised for roles beyond serving as building blocks for DNA and RNA. They also contribute to oncogenic signalling, stress response, and energy regulation, particularly in rapidly dividing or metabolically active cells (Shi et al., 2023). In adipocytes, nucleotide metabolism is involved not only in nucleic acid synthesis but also in the production of important coenzymes, including nicotinamide adenine dinucleotide (NAD^+), flavin adenine dinucleotide (FAD), and Coenzyme A (CoA), which are important in redox regulation, fatty acid metabolism, and energy homeostasis (Yamaguchi et al., 2019). The regulation of nucleotide metabolism is

especially critical during adipogenesis, where the metabolic demands of proliferating preadipocytes and lipid-storing mature adipocytes differ significantly (Shinde et al., 2023).

Within this broader metabolic context, pantothenate and CoA biosynthesis represents a significant sub-pathway in nucleotide metabolism. This pathway includes five enzymatic steps that convert pantothenate (vitamin B5) into CoA, a vital cofactor in acyl group transfer, lipid metabolism, and the citric acid (TCA) cycle (Leonardi & Jackowski, 2007; Martinez et al., 2014). One of the critical intermediates in this pathway is 2-dehydropantoate, a keto acid formed from the condensation of α-ketoisovalerate. This metabolite is an essential precursor in the synthesis of pantothenic acid, which is ultimately used to produce CoA. The availability of 2-dehydropantoate directly influences CoA production, thereby affecting downstream metabolic processes including fatty acid oxidation, lipogenesis, and mitochondrial energy generation.

In this study, 2-dehydropantoate was found to be significantly upregulated in the PSPE-treated group compared to the differentiated control group. This finding suggests that PSPE enhances the activity of the pantothenate and CoA biosynthesis pathway. As a central metabolic cofactor, CoA plays a vital role in mitochondrial β-oxidation of fatty acids, leading to the production of acetyl-CoA, NADH, and $FADH_2$, which feed into the TCA cycle to generate cellular energy (Mattozzi et al., 2016; Adeva-Andany et al., 2019). The increased levels of 2-dehydropantoate may indicate an adaptive shift towards boosting CoA availability, supporting a metabolic environment that favors catabolic energy-generating processes over lipid storage (Leonardi et al., 2010; Garcia et al., 2012).

Previous studies have shown that increasing CoA biosynthesis can attenuate lipid deposition and enhance mitochondrial oxidative metabolism, which are favorable outcomes in the context of obesity and adipocyte hypertrophy (Adeva-Andany et al., 2019). Simvastatin has also been reported to influence CoA-dependent metabolic pathways, including those involved in lipid metabolism (Fernandes Silva et al., 2022). The similar upregulation of 2-dehydropantoate observed with PSPE treatment suggests that its bioactive compounds may engage similar nucleotide-linked biosynthetic mechanisms to regulate adipocyte metabolism.

In sum, the elevated levels of 2-dehydropantoate in PSPE-treated adipocytes suggest that PSPE may promote CoA biosynthesis. This process may enhance fatty acid oxidation and reduce lipogenesis, shifting the metabolic balance from storage toward

energy expenditure. This highlights the relevance of nucleotide-coenzyme biosynthesis in adipocyte metabolic reprogramming and positions 2-dehydropantoate as a potential biomarker for evaluating the metabolic effects of anti-adipogenic interventions.

5.6.5 Metabolism of Cofactors and Vitamins

Metabolism of cofactors and vitamins play a crucial role in cellular metabolism, acting as enzyme coenzymes or precursors in various biochemical reactions. They are essential for maintaining normal health and supporting plant development, metabolism, and stress responses (da Fonseca-Pereira et al., 2023). Within the broader context of adipogenesis, the metabolism of vitamin-derived cofactors becomes increasingly relevant, as these molecules support essential processes such as fatty acid biosynthesis, oxidative phosphorylation, and cellular redox balance (Landrier et al., 2012). In this study, two metabolites which are biocytin and lumichrome emerged as significantly upregulated in adipocytes treated with PSPE, when compared to untreated differentiated adipocytes. This finding suggests a potential alteration in vitamin and cofactor metabolism in response to natural intervention.

Biocytin is an intermediate in the biotin metabolism pathway and represents the biotinylated form of lysine, typically generated during the degradation of biotin-dependent carboxylases. Its accumulation often reflects enhanced biotin turnover or recycling activity. Biotin itself is an essential water-soluble B-vitamin that acts as a coenzyme for carboxylases involved in key metabolic processes, including acetyl-CoA carboxylase in fatty acid synthesis and pyruvate carboxylase in gluconeogenesis (Rodríguez Meléndez, 2000). Increased levels of biocytin observed in the PSPE and simvastatin groups may indicate elevated recycling or utilisation of biotin in these treated adipocytes, likely due to higher demand for biotin-dependent enzymatic functions that regulate lipid metabolism. Supporting this, previous studies demonstrated that biotin supplementation has been shown to inhibit adipocyte differentiation and lipid accumulation through various mechanisms. Studies have demonstrated that biotin reduces serum triglycerides and downregulates lipogenic gene expression, including SREBP1c and PPARγ, in liver and adipose tissues (Larrieta et al., 2010). In 3T3-L1 adipocytes, biotin supplementation decreased fatty acid synthesis while increasing fatty acid oxidation and uptake (Moreno-Méndez et al., 2019). A biotin analog inhibited acetyl-CoA carboxylase activity and adipogenesis in 3T3-L1 cells, blocking the

induction of PPARγ expression (Levert et al., 2002). This aligns with the observed anti-adipogenic effects seen in PSPE-treated group.

In parallel, lumichrome, a degradation product of riboflavin (vitamin B_2), was also found to be elevated in the same treatment group. Riboflavin serves as a precursor to two key flavocoenzymes including flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) which are central to redox reactions in mitochondrial energy metabolism, including β-oxidation and the electron transport chain (Balasubramaniam & Yaplito-Lee, 2020; Henriques & Gomes, 2020). While lumichrome itself is not a functional coenzyme, its increased levels can signify heightened riboflavin turnover, possibly due to increased mitochondrial activity or oxidative stress adaptation. Interestingly, recent research suggests that lumichrome may also possess bioactive properties, including anti-inflammatory and antioxidant effects, which could further support its relevance in adipocyte metabolic regulation (Chantarawong et al., 2019). Notably, simvastatin has been shown to stimulate AMP-activated protein kinase (AMPK) signalling in various tissues, including vascular endothelial cells and arteries (Choi et al., 2008; Kou et al., 2009), which leads to numerous beneficial effects such as enhanced mitochondrial biogenesis and lipid catabolism (Wu & Zou, 2020). This also could help explain the upregulation of riboflavin metabolism and its associated by-products in PSPE-treated adipocytes.

While biocytin and lumichrome originate from different vitamin pathways, which are biotin and riboflavin, respectively, they may be functionally linked through their broader role in regulating lipid and energy metabolism. Both metabolites are associated with enzymatic systems that either synthesize or oxidize fatty acids, and their simultaneous elevation suggests a metabolic adaptation aimed at rebalancing lipid fluxes under treatment conditions. In essence, their upregulation may reflect a coordinated enhancement of cofactor availability to support enzymatic demands. From a biological standpoint, these findings highlight the capacity of PSPE to modulate metabolism of cofactor and vitamin as part of their anti-adipogenic mechanism in 3T3-L1 cells. The increased levels of biocytin and lumichrome imply an upsurge in vitamin-dependent metabolic processes, potentially contributing to reduced lipid storage and enhanced energy expenditure.

5.7 Effects of PSPE on 3T3-L1 Adipocytes in Inhibiting Adipogenesis

Based on the findings, this study proposes that *Parkia speciosa* pod extract (PSPE) inhibits adipogenesis in 3T3-L1 adipocytes through a multi-pathway metabolic alterations mechanism, involving the alteration of lipid, amino acid, carbohydrate, nucleotide, as well as metabolism of cofactors and vitamin. These effects are driven by the presence of phytochemicals, particularly phenolics and flavonoids, which are present in PSPE and were confirmed through HPLC profiling. This is supported by the amount of total phenolic content (TPC) and total flavonoid content (TFC) exhibited. The strong antioxidant potential of PSPE, evidenced by its DPPH and FRAP activities, may contribute to reduction of oxidative stress during early adipogenic commitment, thereby disrupting redox-sensitive transcriptional regulators such as PPARγ and C/EBPα, which are critical for adipocyte differentiation (Rahman et al., 2016).

Furthermore, PSPE reduced several key metabolites in lipid metabolism, specifically in the linoleic acid metabolism pathway, including linoleic acid and gamma-linolenic acid (GLA), both of which are known to facilitate adipocyte differentiation by promoting membrane biosynthesis and activating PPARγ (Goto et al., 2015). The downregulation of these lipids suggests that PSPE interferes with early lipid signalling and structural remodelling necessary for adipocyte maturation. At the same time, PSPE upregulated arachidonic acid, a polyunsaturated fatty acid that produces prostaglandins known to suppress adipogenesis (Fujimori, 2012; Pisani et al., 2014). The increase in dodecanoic acid, a medium-chain fatty acid, implies enhanced fatty acid oxidation, favoring lipid catabolism over storage (Shibata et al., 2012). The rise in 7α-hydroxy-cholestene-3-one, a bile acid precursor, also suggests activation of bile acid–dependent signalling via FXR and TGR5, which are linked to reduced lipid accumulation and improved mitochondrial function (Jadhav et al., 2018).

Additionally, PSPE altered amino acid metabolism by increasing indoleacetic acid and N-acetyl-L-phenylalanine, intermediates in the metabolism of tryptophan and phenylalanine, respectively. These metabolites are linked to activation of the aryl hydrocarbon receptor (AhR), which negatively regulates adipogenic transcriptional programs (Shimba et al., 2001). The increase in these metabolites suggests that PSPE may suppress the expression of PPARγ and C/EBPα at the transcriptional level through AhR-mediated mechanisms. PSPE also downregulated phosphoglycolic acid, an intermediate in glyoxylate and dicarboxylate metabolism, indicating a reduction in

oxidative metabolic activity and biosynthetic flux, a shift that aligns with decreased adipocyte proliferation and differentiation. Conversely, the upregulation of 2-dehydropantoate, biocytin, and lumichrome, metabolites related to pantothenate, biotin, and riboflavin metabolism, respectively, suggests enhanced coenzyme biosynthesis and mitochondrial cofactor availability, contributing to energy efficiency and fatty acid oxidation during adipocyte inhibition.

Collectively, PSPE inhibits adipogenesis through several integrated mechanisms: (i) suppression of lipid-derived signalling metabolites; (ii) antioxidant action that lowers ROS and disrupts redox-sensitive transcription; (iii) activation of fatty acid oxidation and bile acid pathways; (iv) transcriptional inhibition via amino acid-regulated AhR signalling; and (v) increased coenzyme biosynthesis supporting mitochondrial adaptation and metabolic resilience. These metabolomics alterations ultimately result in reduced lipid accumulation in adipocytes, as confirmed by a significant reduction in lipid droplet formation and intracellular triglyceride levels following PSPE treatment, as demonstrated by oil red O staining and triglyceride quantification assays. These results provide functional evidence that the metabolic shifts induced by PSPE effectively suppress adipocyte differentiation and lipid storage, highlighting its potential as a natural anti-adipogenic agent in the context of obesity-associated disorders. Figure 5.1 illustrates a comprehensive visual summary of the major findings obtained in the present study, integrating the observed effects of *Parkia speciosa* pod extract on 3T3-L1 adipocyte differentiation and its underlying metabolomic alterations. However, despite these insights, further in-depth investigations are necessary to elucidate the specific molecular mechanisms and signalling pathways through which the identified phytochemicals exert their modulatory effects on adipogenesis.

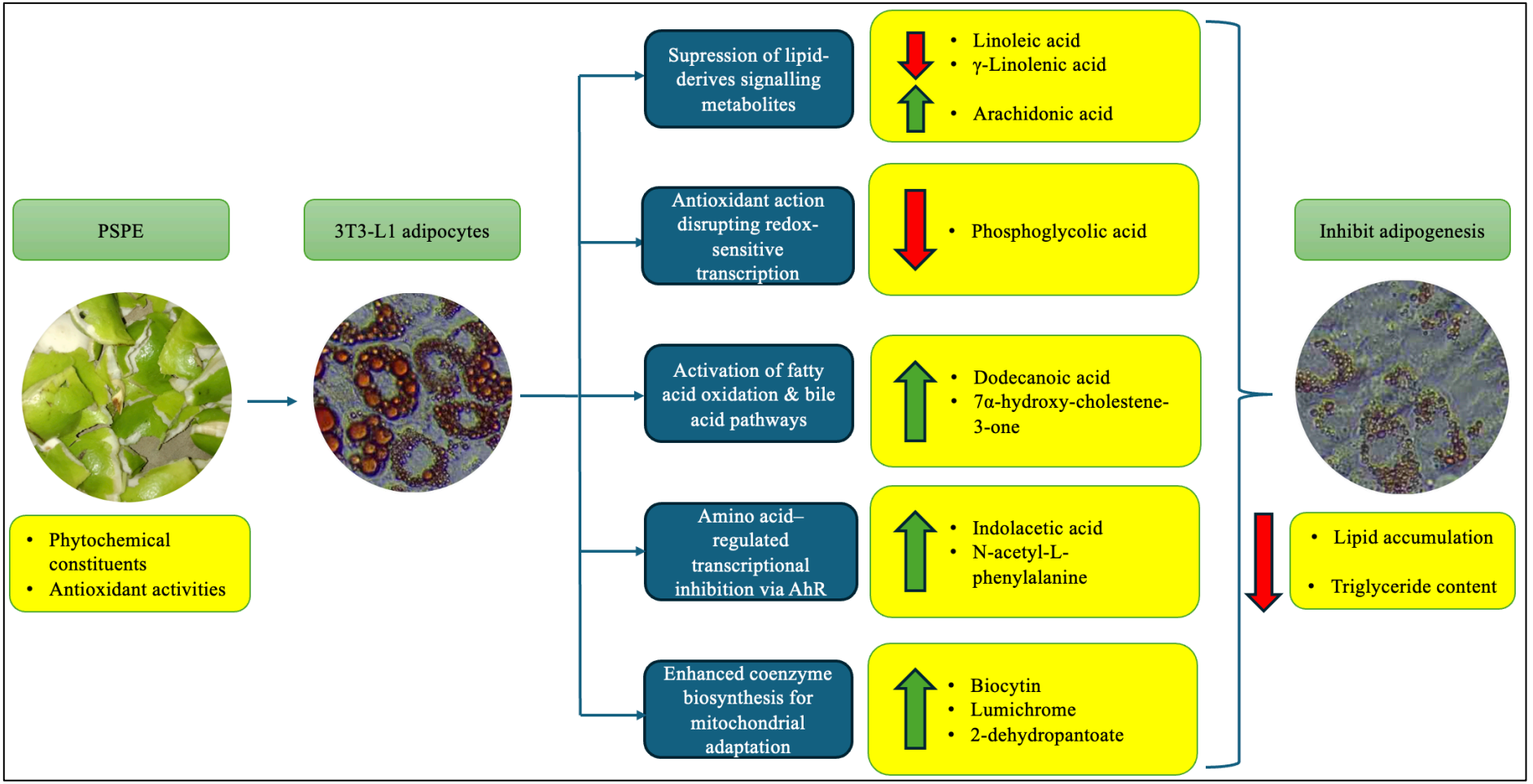

Figure 5.1 The Schematic Diagram of Adipogenesis Inhibition by *P. speciosa* Pod Extract (PSPE).

CHAPTER 6
CONCLUSION

In this study, the extraction of *Parkia speciosa* pod extract (PSPE) was successfully achieved through an exhaustive maceration method using 70% ethanol, resulting in a final yield of 37.83%. Phytochemical profiling via high-performance liquid chromatography (HPLC) enabled the identification and quantification of several bioactive compounds, notably gallic acid (53.97 ± 0.76 µg/mL) and p-coumaric acid (1.74 ± 0.11 µg/mL). Both of these phenolic compounds have been previously associated with anti-obesity activities, providing a strong basis for further investigation into the therapeutic potential of PSPE. Moreover, the extract exhibited a high total phenolic content (242.8 ± 11.62 µg GAE/mL) and total flavonoid content (30.53 ± 0.52 µg QE/mL), further supporting the richness of bioactive constituents within PSPE that could contribute to its biological activities.

In addition to its phytochemical characterisation, the antioxidant properties of PSPE were evaluated using DPPH and FRAP assays. The extract demonstrated notable free radical scavenging activity and ferric reducing antioxidant power, although its potency was slightly lower compared to the ascorbic acid standard. Nevertheless, the antioxidant activity observed suggests that PSPE may have a role in reducing oxidative stress, a condition often linked with the onset and progression of hyperlipidemia, obesity, and related metabolic disorders.

The anti-adipogenic effects of PSPE were further explored using an *in vitro* model of adipogenesis involving 3T3-L1 preadipocyte cells. Cell viability assays confirmed that PSPE was non-cytotoxic at concentrations up to 125 µg/mL, establishing a safe window for further investigation. Subsequent lipid accumulation assays using oil red O staining and triglyceride quantification revealed that treatment with PSPE at 62.5 µg/mL significantly inhibited lipid accumulation in differentiated 3T3-L1 adipocytes. Remarkably, the reduction in triglyceride content induced by PSPE was comparable to that achieved by simvastatin, a clinically used anti-hyperlipidemic agent. These results strongly suggest that PSPE exerts anti-adipogenic effects, likely through the inhibition of key stages in adipocyte differentiation and lipid storage processes.

To provide a more comprehensive understanding of the underlying molecular changes by PSPE, metabolomic profiling was performed using LC/MS-QTOF. The

analysis identified 33 significant metabolites with notable alterations in pathways related to lipid metabolism, amino acid metabolism, carbohydrate metabolism, nucleotide metabolism, and cofactor and vitamin metabolism. Among these, lipid metabolism emerged as the most prominently affected pathway, corroborating the results observed in the lipid accumulation assays. Several potential biomarkers, including gamma-linolenic acid, linolenic acid, arachidonic acid, dodecanoic acid, and others, were significantly altered following PSPE treatment. These alterations suggest that PSPE may exert its anti-adipogenic effects by regulating important lipid biosynthesis and degradation pathways, as well as modulating broader metabolic networks that influence adipocyte function.

Bringing all results together, the findings from this study demonstrate that *Parkia speciosa* pod extract possesses promising anti-adipogenic potential, characterised by its antioxidant properties, the ability to inhibit lipid accumulation in adipocytes, and its significant alteration on metabolic pathways involved in adipogenesis. The combined phytochemical, antioxidant, anti-adipogenic, and metabolomic data provide compelling evidence that PSPE could serve as a valuable natural candidate for the treatment of obesity and associated metabolic disorders.

Limitation and Recommendation

While this study has successfully demonstrated the anti-adipogenic effects of *Parkia speciosa* pod extract (PSPE) in 3T3-L1 adipocyte models, several limitations should be acknowledged. Firstly, the current findings are primarily based on *in vitro* experiments, which, although highly informative and frequently preferred as it is considered a well-documented model system for *in vitro* adipogenesis study, may not fully capture the complexity of adipogenesis *in vivo*. The cellular microenvironment, hormonal influences, and systemic metabolic interactions present in living organisms are not fully represented in the 3T3-L1 cell culture system. Therefore, the translatability of these results to human physiology remains to be validated.

Although PSPE demonstrated anti-adipogenic effects that were comparable in trend to the commercial drug, simvastatin, the extent of these effects was not identical. While both interventions reduced lipid accumulation and modulated relevant metabolic pathways, the degree of change in specific parameters varied, suggesting potential differences in potency, mechanism of action, or target specificity. Therefore, it cannot

be concluded that PSPE possesses equivalent efficacy to simvastatin in all aspects of adipogenesis inhibition. To clarify these differences, additional comparative studies are warranted, particularly those examining the mechanistic overlap and divergence between the two interventions. Such investigations should explore whether PSPE engages similar upstream regulators, downstream effectors, or entirely distinct pathways, thereby providing a more precise understanding of its therapeutic potential relative to existing pharmacological agents.

Secondly, although PSPE was shown to reduce lipid accumulation and significantly modulate key metabolic pathways, the specific molecular targets and signalling pathways underlying these effects were not deeply characterised in this study. In particular, the direct interactions of PSPE or its active constituents with key regulators such as PPARγ, AMPK, or adipogenic transcription factors were not investigated at the mechanistic level. Additionally, while untargeted metabolomics analysis provided valuable insights into altered metabolic pathways, the study did not include targeted validation of specific metabolites using methods such as mass spectrophotometry-mass spectrophotometry (MS-MS) to confirm metabolites altered.

Another limitation concerns the complexity of the PSPE composition. Although major phenolic compounds were identified, the potential synergistic or antagonistic effects among the multiple bioactive constituents remain unclear. The exact contribution of individual compounds such as gallic acid and p-coumaric acid to the overall anti-adipogenic and antioxidant effects needs further clarification.

In light of these limitations, several recommendations for future research are proposed. Firstly, *in vivo* studies using appropriate animal models of diet-induced obesity should be conducted to evaluate the efficacy, bioavailability, and systemic effects of PSPE in a physiologically relevant context. Such studies should include assessments of body weight, adipose tissue mass, plasma lipid profiles, glucose tolerance, and inflammatory markers to fully elucidate the metabolic benefits of PSPE.

Secondly, mechanistic studies should be undertaken to investigate the molecular pathways through which PSPE exerts its anti-adipogenic effects. Techniques such as Western blotting, qPCR, and reporter assays could be employed to examine the modulation of adipogenic transcription factors such as PPARγ, C/EBPα, SREBP1c and key metabolic regulators. Furthermore, targeted metabolomics approaches should be applied to validate and expand upon the untargeted findings reported in this study. The use of isotopic labeling experiments could help trace the metabolic fate of key

metabolites and provide deeper insights into how PSPE modulates critical pathways involved in lipid metabolism, amino acid metabolism, carbohydrate metabolism, and other interconnected metabolic networks. Finally, efforts should be directed towards the isolation, characterisation, and evaluation of individual bioactive compounds from PSPE. This would help in identifying the most potent anti-adipogenic constituents and in understanding possible synergistic effects.

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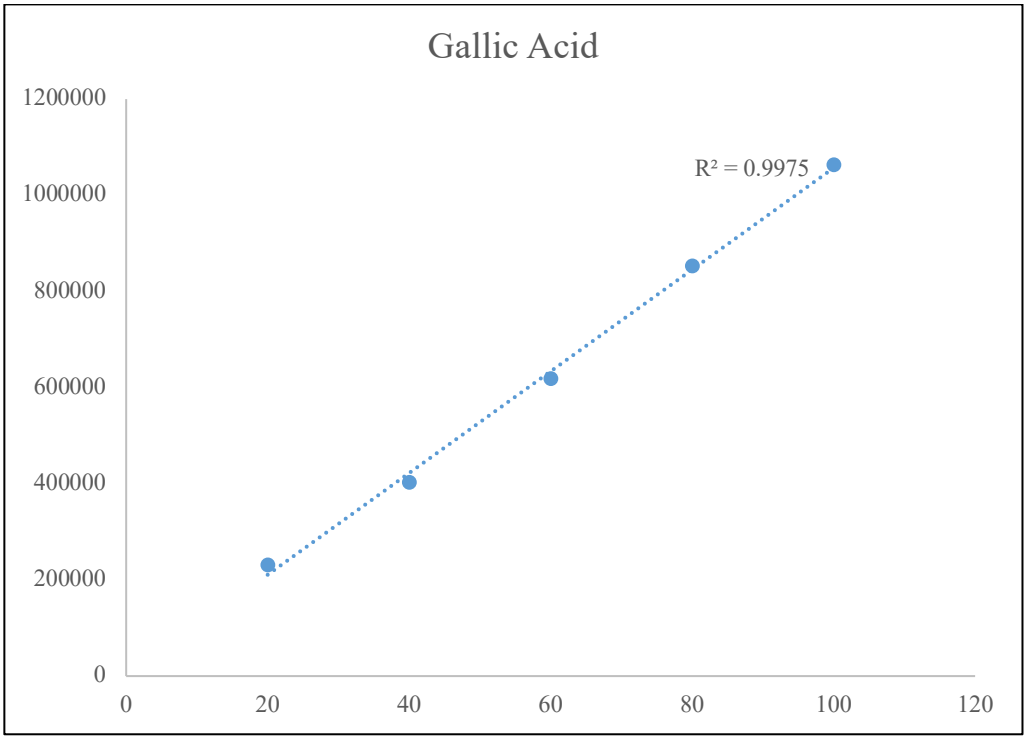
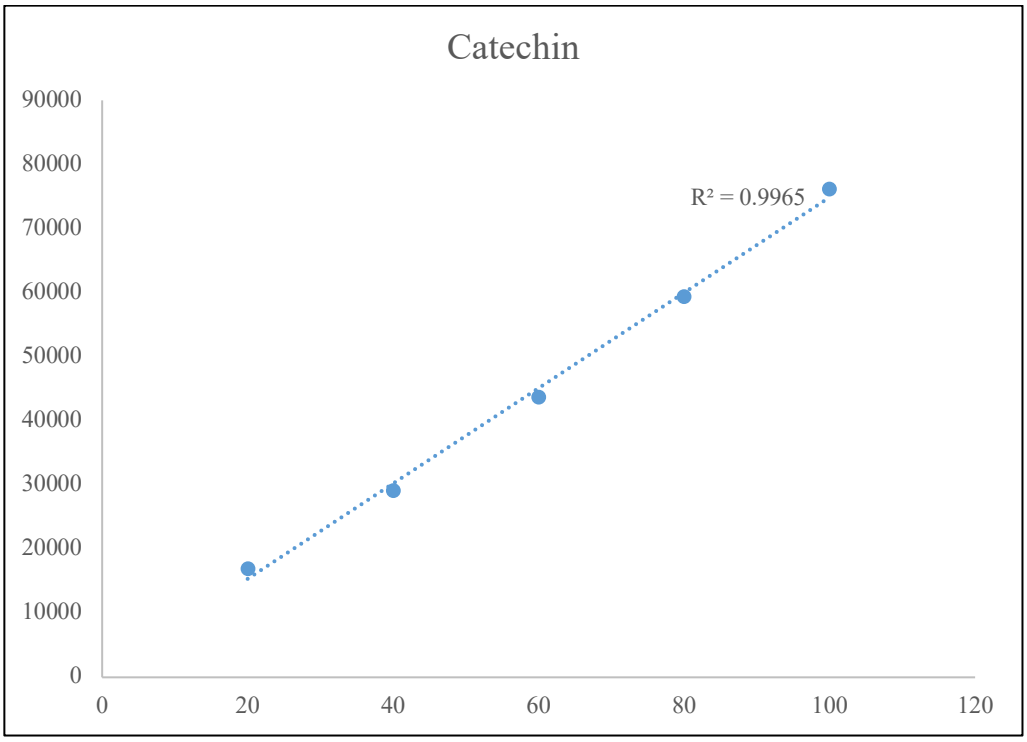
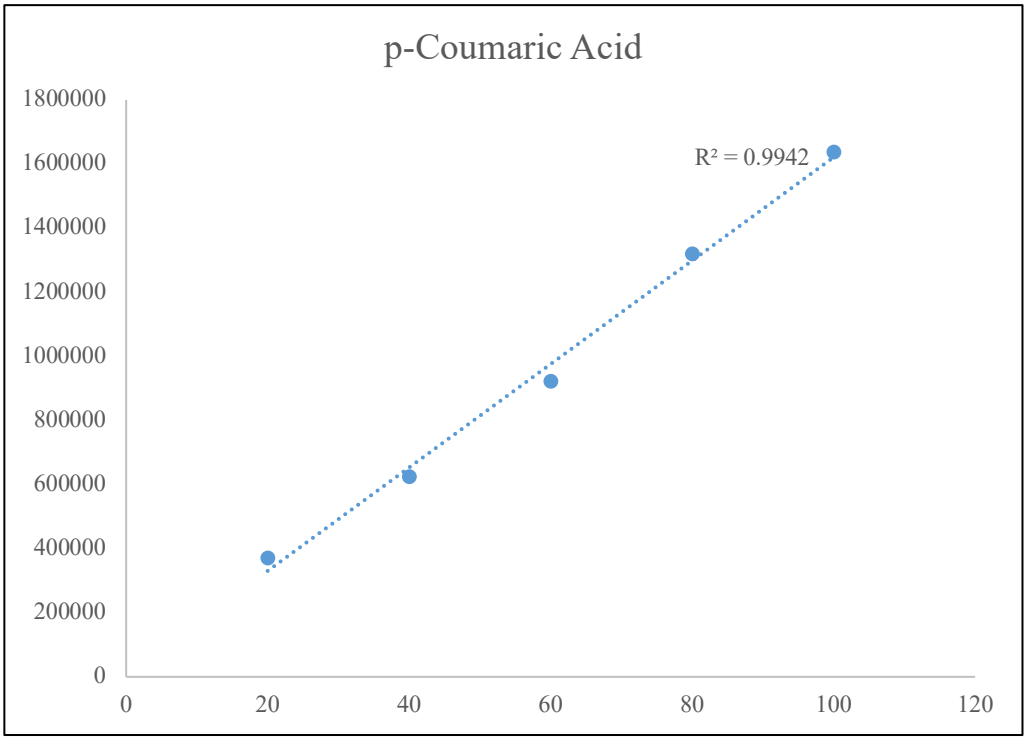
APPENDICES

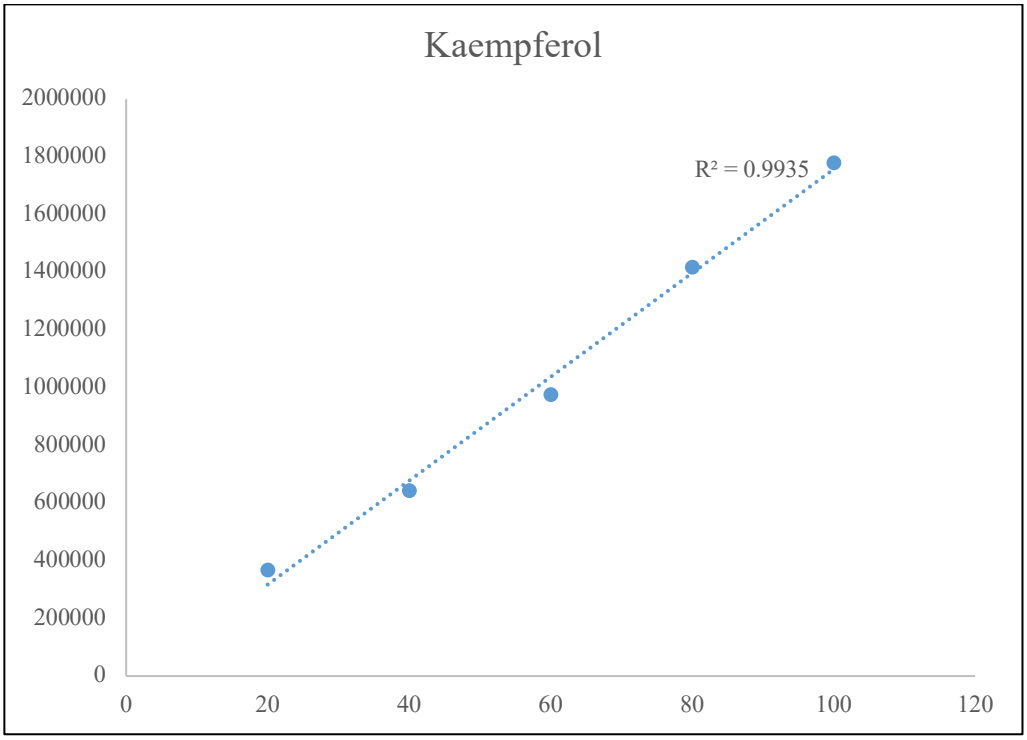
APPENDIX 1

Standard Curves of Compounds in HPLC



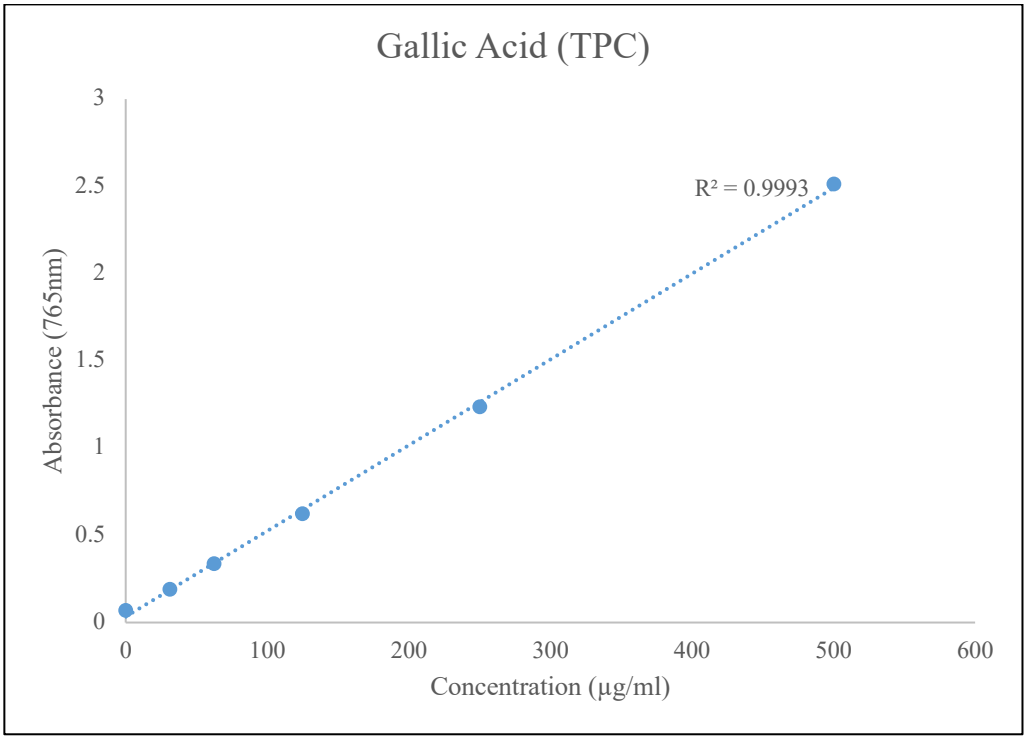


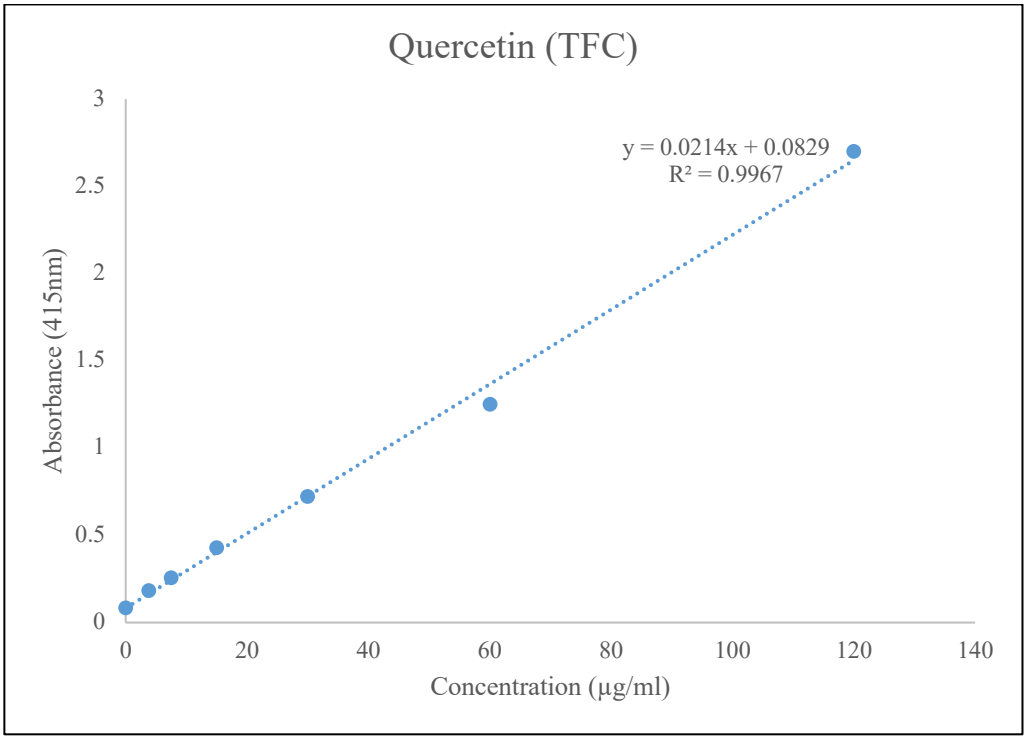

p-Coumaric Acid
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
0
20
40
60
80
100
120
R² = 0.9942

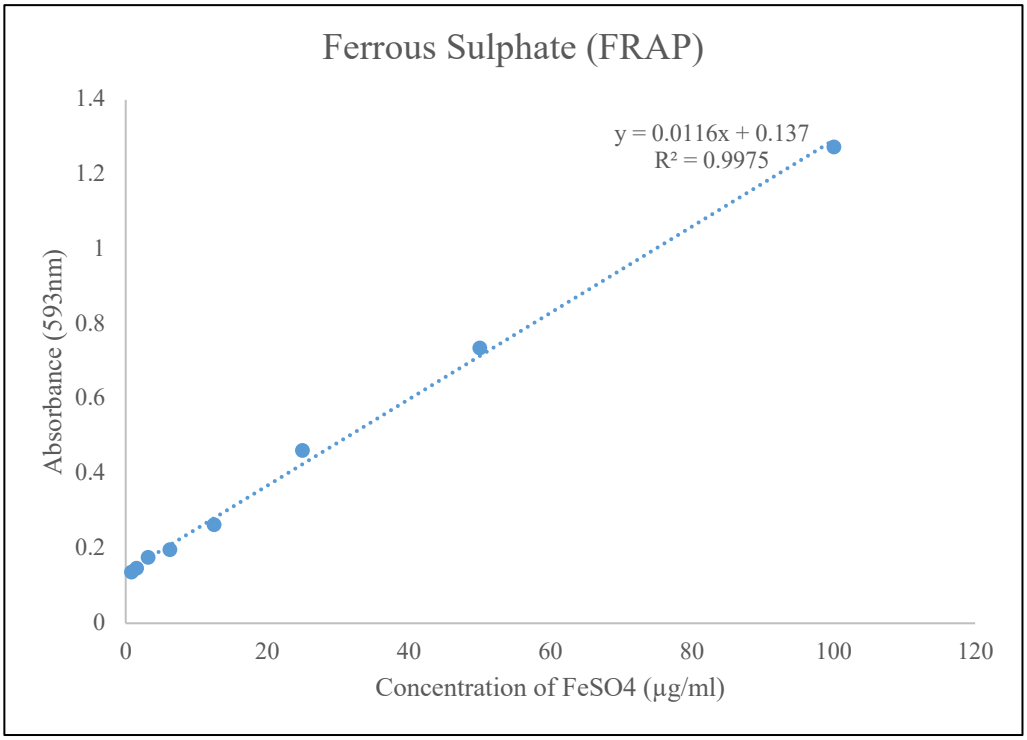

Kaempferol
2000000
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
0
20
40
60
80
100
120
R² = 0.9935

APPENDIX 2

Standard Curve of TPC, TFC and FRAP Assay






Ferrous Sulphate (FRAP)
y = 0.0116x + 0.137
R² = 0.9975
Absorbance (593nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
Concentration of FeSO4 (µg/ml)

AUTHOR'S PROFILE

Anas bin Abdullah obtained his Bachelor of Medical Laboratory Technology (Hons.) in 2021 from Universiti Teknologi MARA, Selangor. He is a dedicated graduate with experience as a Medical Laboratory Technologist and Sales Executive specializing in Histology instruments and reagents, with strong expertise in cell culture techniques, laboratory analysis, and scientific instrumentation. Driven by a passion for the field, he is eager to expand his knowledge and gain a deeper understanding of the subject he is exploring.

LIST OF PUBLICATION

Abdullah, A., Haron, N., Mohamed, E., Mohamad Yusof, M. I., Hassan Cheong, N. D., Camalxaman, S. N., Ishak, A. R., Rahim, S. A., Eshak, Z., & Samat, S. (2025). Anti-adipogenic effects of *Parkia speciosa* Hassk. pods extract containing gallic acid and p-coumaric acid on 3T3-L1 adipocytes. *Plant Science Today*, *12*(2). https://doi.org/10.14719/pst.4421

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Dayana Hassan Cheong, N., Mohamed, E., Haron, N., Nazrina Camalxaman, S., **Abdullah, A.**, Izwan Mohamad Yusof, M., Razali Ishak, A., Ab Rahim, S., Eshak, Z., & Rohim Tualeka, A. (2024). Phytochemical quantification and HPLC analysis of *Parkia speciosa* pod extract. *The Medical Journal of Malaysia, 79,* 34-39.

MAKLUMAT BAHAN (*Information of Materials*):

Bil. *No*	Judul Bahan *Title*
1.	UNTARGETED METABOLOMICS ANALYSIS OF THE INTERVENTION EFFECTS OF *Parkia speciosa* POD EXTRACT (PSPE) ON 3T3-L1 ADIPOCYTES DIFFERENTIATION