THE EFFECTS OF *PLUCHEA INDICA* (L.) TEA ON ANTI-ADIPOGENESIS IN 3T3-L1 CELLS, ANTI-HYPERGLYCEMIC, AND ANTI-HYPERLIPIDEMIC ACTIVITIES IN MICE

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ฤทธิ์ของชากระทาเกลือในการยับยั้งกระบวนการสร้างไขมันในเซลล์ 3T3-L1 และการยับยั้งภาวะน้ำตาลและไขมันสูงในเลือดในหนูไมซ์

นายกิตติพจน์ สิริชัยเวชกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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กิตติพจน์ สิริชัยเวชกุล : ฤทธิ์ของชากระทาเกลือในการยับยั้งกระบวนการสร้างไขมันในเซลล์ 3T3-L1 และการยับยั้งภาวะน้ำตาลและไขมันสูงในเลือดในหนูไมซ์ (THE EFFECTS OF *PLUCHEA INDICA* (L.) TEA ON ANTI-ADIPOGENESIS IN 3T3-L1 CELLS, ANTI-HYPERGLYCEMIC, AND ANTI-HYPERLIPIDEMIC ACTIVITIES IN MICE) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ เภสัชกร ดร.เกรียงศักดิ์ เอื้อมเก็บ, 144 หน้า.

การศึกษาในครั้งนี้นั้นเพื่อวิจัยผลของชากระทาเกลือ (*Pluchea indica* (L.) Less.) ในการยับยั้งอนุมูลอิสระ ยับยั้งการสร้างไขมัน (anti-adipogenesis) ในเซลล์ 3T3-L1 ผลต่อเอนไซม์ไลเปสในตับอ่อน และการยับยั้งความผิดปกติของไขมันในเลือดของหนูไมซ์ที่ถูกเหนี่ยวนำให้เกิดภาวะความผิดปกติของไขมันในเลือดด้วยอาหารไขมันสูง ฤทธิ์ในการต้านอนุมูลอิสระ 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), Hypochlorous acid (HOCl), Nitric oxide, Peroxynitrite และฤทธิ์ในการต้านออกซิเดชันต่อแอลดีแอล-คอเลสเทอรอลซึ่งได้มาจากการสกัดจากเลือดของอาสาสมัครสุขภาพดี พบว่าชากระทาเกลือนั้นสามารถต่อต้านสารอนุมูลอิสระในทุกการทดลองได้เป็นอย่างดี ความสามารถมีชีวิตของเซลล์ 3T3-L1 ไม่ลดลงอย่างมีนัยสำคัญหลังจากได้รับชากระทาเกลือขนาด 200 ถึง 1,000 ไมโครกรัมต่อมิลลิลิตร เมื่อเปรียบเทียบกับกลุ่มควบคุม ($p > 0.05$) ชากระทาเกลือที่ขนาด 750 ถึง 1,000 ไมโครกรัมต่อมิลลิลิตรส่งผลให้การสะสมของไขมันในเซลล์ลดลงอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มควบคุม ($p < 0.05$) ชากระทาเกลือที่ขนาด 250 ถึง 1,000 มิลลิกรัมต่อมิลลิลิตรมีผลยับยั้งเอนไซม์ไลเปสเพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มควบคุม ($p < 0.05$) ผลจากการวิเคราะห์ด้วยเอฟทีไออาร์แสดงให้เห็นว่าพื้นที่ใต้กราฟรวมของไขมัน โปรตีน กรดนิวคลีอิก ไกลโคเจน และคาร์โบไฮเดรตของเซลล์ 3T3-L1ที่ได้รับชากระทาเกลือนั้นน้อยกว่ากลุ่มที่ไม่ได้รับชากระทาเกลืออย่างมีนัยสำคัญ ($p < 0.05$) นอกจากนี้จากการทดสอบความทนทานต่อน้ำตาลกลูโคสในหนูไมซ์พบว่าหนูที่ได้รับชากระทาเกลือที่ขนาด 400 และ 600 มิลลิกรัมต่อกิโลกรัมนั้น แสดงให้เห็นถึงผลในการป้องกันภาวะน้ำตาลในเลือดสูงอย่างมีนัยสำคัญโดยการป้องกันดีขึ้นเมื่อขนาดของชามากขึ้นเมื่อเปรียบเทียบกับกลุ่มที่ไม่ได้รับชากระทาเกลือ นอกจากนั้นหนูที่ได้รับชากระทาเกลือที่ขนาด 400 และ 600 มิลลิกรัมต่อกิโลกรัม วันละ 1 ครั้งเป็นเวลา 4 สัปดาห์สามารถป้องกันภาวะไขมันในเลือดสูงและภาวะอ้วนที่เกิดจากการเหนี่ยวนำจากอาหารที่มีไขมันสูงเมื่อเทียบกับกลุ่มที่ไม่ได้รับชากระทาเกลือ นอกจากนี้การวิเคราะห์ทางเนื้อเยื่อโดยการย้อมเซลล์ไขมันด้วยสีฮีมาทอกไซลินและสีอีโอซินแสดงให้เห็นว่าหนูที่ได้รับชากระทาเกลือนั้นจะมีขนาดของเซลล์ไขมันลดลงและจำนวนของเซลล์ไขมันต่อฟิลด์เพิ่มขึ้นอย่างมีนัยสำคัญ การทดสอบความเป็นพิษของชาต่อหนูไมซ์พบว่าค่า

เอนไซม์อะลานิน อะมิโนทรานส์เฟอเรส เอนไซม์อัลคาไลน์ฟอสฟาเตส ครีเอทินีน จำนวนเม็ดเลือดแดง จำนวนเม็ดเลือดขาว เกล็ดเลือด และน้ำหนักอวัยวะสัมพัทธ์หลังจากได้รับชากระทาเกลือที่ขนาด 400 และ 600 มิลลิกรัมต่อกิโลกรัม วันละ 1 ครั้งเป็น เวลา 4 สัปดาห์ นั้นไม่มีความแตกต่างระหว่างค่าพารามิเตอร์เหล่านี้เมื่อเปรียบเทียบกับกลุ่มควบคุม การหาปริมาณของสารออกฤทธิ์สำคัญในชานั้นได้มีการใช้เครื่องลิควิดโครมาโทรกราฟีแมสสเปกโทรมิเตอร์ (LC-MS/MS) วิเคราะห์และแยกสารสำคัญของชากระทาเกลือ ซึ่งพบว่ามีสาร 4-O-caffeoylquinic acid (4-CQ), 5-O-caffeoylquinic acid (5-CQ), 3,4-O-dicaffeoylquinic acid (3,4-CQ), 3,5-O-dicaffeoylquinic acid (3,5-CQ) และ 4,5-O-dicaffeoylquinic acid (4,5-CQ) เป็นองค์ประกอบหลัก โดยสรุปแล้ว การค้นพบของเราเป็นหลักฐานว่าชากระทาเกลือนั้นมีความปลอดภัยและมีศักยภาพในการพัฒนาเป็นเครื่องดื่มสำหรับต้านอนุมูลอิสระหรือเป็นยารักษาโรค ซึ่งสามารถใช้ในการป้องกันภาวะน้ำตาลในเลือดสูง ไขมันในเลือดสูง และโรคอ้วน

สาขาวิชาปรีคลินิก ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2561 ลายมือชื่ออาจารย์ที่ปรึกษา _____

KITTIPOT SIRICHAIWETCHAKOON : THE EFFECTS OF *PLUCHEA INDICA* (L.) TEA ON ANTI-ADIPOGENESIS IN 3T3-L1 CELLS, ANTI-HYPERGLYCEMIC, AND ANTI-HYPERLIPIDEMIC ACTIVITIES IN MICE. THESIS ADVISOR : ASSOC. PROF. GRIANGSAK EUMKEB, Ph.D. 144 PP.

PLUCHEA INDICA / ADIPOGENESIS / 3T3-L1 CELLS / FTIR MICROSPECTROSCOPY / DYSLIPIDEMIA / MICE / ANTI-OXIDATION / ANTI-LDL OXIDATION

The present study focused on the effect of *Pluchea indica* (L.) Less. tea on free radical scavenging activity, anti-adipogenesis in 3T3-L1 cells, activity on pancreatic lipase and anti-dyslipidemia in high-fat-diet-induced mice. The 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), Hypochlorous acid (HOCl), Nitric oxide, Peroxynitrite scavenging activities and the scavenging activities of Copper (Cu^{2+}), Azobis hydrochloride (AAPH), 3-Morpholinosydnonimine hydrochloride (SIN-1) induced LDL oxidation which isolated from a healthy volunteer were performed. *P. indica* (L.) Less. tea exhibited good antioxidant activity in all test systems. The viability of 3T3-L1 cells was not significantly decreased after exposure to 200 to 1000 μg/ml *P. indica* (L.) Less. tea compared to controls ($p > 0.05$). The *P. indica* (L.) Less. tea at 750 to 1000 μg/ml exhibited a significantly reduced lipid accumulation compared to the control ($p < 0.05$). The inhibitory effects of the *P. indica* (L.) Less. tea at 250 to 1000 μg/ml on lipase activity were significantly increased compared to control ($p < 0.05$). The FTIR results showed that the integrated areas of lipids, proteins, nucleic acids, glycogen and

carbohydrates of the *P. indica* (L.) Less. tea-treated 3T3-L1 adipocytes were significantly lower than the untreated 3T3-L1 adipocytes ($p < 0.05$). In addition, Oral glucose tolerance test (OGTT) in mice was performed and *P. indica* (L) Less. tea at 400 and 600 mg/kg/d orally showed prevention of hyperglycemia with dose-dependent manner compared with the untreated group. Moreover, oral administration of *P. indica* (L) Less. tea at 400 and 600 mg/kg once daily for 4 weeks could protect dyslipidemia and obesity from high fat diet induction with a dose-dependent manner when compared to the untreated group. Furthermore, histological analysis expressed that *P. indica* (L) Less. tea treated group significantly reduced adipocyte size and increased number of adipocytes per field of Hematoxylin & Eosin stained perigonadal fat tissue. Toxicity of the tea was investigated, and the results revealed that ALT, ALP, Creatinine, RBC, WBC, Platelet and relative organ weight after feeding *P. indica* (L) Less. tea at 400 and 600 mg/kg/d orally once daily for 4 weeks did not affect these parameters compared with the control group. *P. indica* (L.) Less. tea was identified and quantified the main active ingredients by LC-MS/MS technique. *P. indica* (L.) Less. tea was found 4-O-caffeoylquinic acid (4-CQ), 5-O-caffeoylquinic acid (5-CQ), 3,4-O-dicaffeoylquinic acid (3,4-CQ), 3,5-O-dicaffeoylquinic acid (3,5-CQ) and 4,5-O-dicaffeoylquinic acid (4,5-CQ). In conclusion, our findings provide evidence that *P. indica* (L.) Less. tea is safe, has potentials to develop to be antioxidant beverage or medicine, and use to prevent hyperglycemia, hyperlipidemia, and obesity.

School of Preclinic Student's Signature _____

Academic Year 2018 Advisor's Signature G. Fumkab.

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Kittipot Sirichaiwetchakoon

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LIST OF ABBREVIATIONS

AAPH	=	2,2’-azobis(2-amidinopropane) dihydrochloride
ABTS	=	2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid
ALP	=	Alkaline Phosphatase
ALT	=	Alanine Transaminase
ATCC	=	American Type Culture Collection
BMI	=	Body Mass Index
BCS	=	Bovine Calf Serum
cAMP	=	Cyclic Adenosine Monophosphate
CBC	=	Complete Blood Count
CHD	=	Coronary Heart Disease
CQ	=	Caffeoylquinic acid
DAG	=	Diacylglycerols
DEX	=	Dexamethasone
DMEM	=	Dulbecco's Modified Eagle's medium
DMSO	=	Dimethyl Sulfoxide
DPPH	=	2,2-diphenyl-1-picrylhydrazyl
DTPA	=	Diethylene-triamine-pentaacetic acid
FBS	=	Fetal Bovine Serum
FFA	=	Free Fatty Acid
FTIR	=	Fourier Transforms Infrared Spectroscopy
g	=	Gram

LIST OF ABBREVIATIONS (Continued)

GLUT4	=	Glucose Transporter 4
HDL	=	High-Density Lipoprotein
H&E	=	Hematoxylin & Eosin
HFD	=	High Fat Diet
HEPES	=	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
IBMX	=	Isobutyl-Methylxanthine
i.p.	=	intraperitoneal
LABG	=	Laparoscopic Adjustable Gastric Banding
LC-MS/MS	=	Liquid chromatography-Mass spectrometry/Mass spectrometry
LDL	=	Low-Density Lipoprotein
MAG	=	Monoacylglycerols
MDA	=	Malondialdehyde
mg	=	Milligram
MRM	=	Multiple Reaction Monitoring
MTT	=	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NaOCl	=	Sodium hypochlorite
NED	=	Naphthylethylenediamine dihydrochloride
OGTT	=	Oral Glucose Tolerance Test
PBS	=	Phosphate Buffer Saline
PCA	=	Principal Component Analysis
PIT	=	*P. indica* (L.) Less. tea
PPAR	=	Peroxisome Proliferator-Activated Receptor

LIST OF ABBREVIATIONS (Continued)

RNS	=	Reactive Nitrogen Species
ROS	=	Reactive Oxygen Species
SIM	=	Simvastatin
SIN-1	=	3-Morpholinosydnonimine hydrochloride
TAG	=	Triacylglycerols
TBARS	=	Thiobarbituric acid reacting substance
TCA	=	Trichloroacetic acid
TFC	=	Total Flavonoid Content
TPC	=	Total Phenolic Content
WHO	=	World Health Organization

CHAPTER I

INTRODUCTION

1.1 Introduction

Obesity is a global public health problem, and the prevalence of obesity is increasing worldwide, rapidly (World Health Organization, 2000). Obesity is defined as abnormal or excessive fat accumulation, caused by an imbalance of energy intake and expenditure (Guo et al., 2015). The World Health Organization (WHO) estimates that over 1.5 billion adults are overweight based on Body Mass Index (BMI) ≥ 25 and over 400 million of them are obese based on BMI ≥ 30 (World Health Organization, 2000). Obesity significantly increases the risk of morbidity and mortality (Pi-Sunyer, 2009) and metabolic syndrome that relevant with dyslipidemia, diabetes mellitus and cardiovascular (CV) disease (Haffner et al., 1992; Isomaa et al., 2001; Trevisan et al., 1998). Obesity can be treated by the decreasing of lipid accumulation by reducing calorific intake, inhibiting adipocyte differentiation, stimulating energy expenditure by increased physical activity but also regulating lipid metabolism, the surgical option such as laparoscopic adjustable gastric banding (LABG) in morbid obesity (Chapman et al., 2004; Guo et al., 2015; Iordache et al., 2002) and inhibiting pancreatic lipase. Pancreatic lipase is an important enzyme that can hydrolyse dietary triacylglycerol to glycerol and fatty acids in the intestine (Birari and Bhutani, 2007). Glycerol and fatty acids are regarded as the end products of lipid digestion in the gut, the inhibition of pancreatic lipase may be considered as a fat

reducing absorption therapy (Birari and Bhutani, 2007; Moreno et al., 2003) is one mechanism of obesity treatment (Mohamed et al., 2014).

Oxidative stress is defined as a disturbance in the balance between the production of free radical and antioxidant defense mechanism which is a natural physiological process in the biological systems (Dhawan, 2014). The major causes of oxidative stress are the excesses of intracellular reactive oxygen species (ROS) and reactive nitrogen species (RNS) that can progress the development of chronic and degenerative diseases such as cancer, arthritis, aging, autoimmune disorders, cardiovascular and neurodegenerative diseases (Pham-Huy et al., 2008). Furthermore, oxidative stress may also injure biomolecules, including proteins, lipids, and DNA (Toyokuni, 1999). The high level of free radical can damage to human low-density lipoproteins which the process is called LDL oxidation. LDL oxidation is a process that free radical oxidizes low-density lipoproteins transform to oxidized low-density lipoproteins. The high level of oxidized low-density lipoproteins may affect the progression of atherosclerosis, and it can progress further to cardiovascular dysfunction (Bahorun et al., 2006).

Diabetes mellitus is a group of metabolic disorder characterized by high levels of blood sugar that may occur by the insufficiency of secretion or action of endogenous insulin (Kataoka et al., 1983; Like et al., 1979; Paik et al., 1982). The symptoms of a diabetes patient are increased thirst and hunger and frequent urination. The long term of uncontrolled diabetes may damage, dysfunction and failure of different organs such as eyes, kidneys, nerves, heart, and blood vessels (Maritim et al., 2003). Diabetes patient can become coma or death by acute complication when blood sugar is higher that called diabetes ketoacidosis (Kitabchi et al., 2009). In 2015, an estimated 415

million people had diabetes worldwide, separate to type 2 Diabetes is over 90% of all that equal rate when to compare with women and men. Moreover, trends of Diabetes patient increase continuously (Zimmet, 2017).

Dyslipidemia is one of metabolic disorder characterized by an abnormal amount of lipids such as Triglyceride, LDL-cholesterol, Cholesterol and HDL in the blood (Ascaso et al., 2007). Dyslipidemia is one of the major cardiovascular risk factors that precipitate coronary heart disease (CHD) and atherosclerosis (Mensink et al., 2003; Miller, 2009). Dyslipidemia can be treated by lifestyle intervention by reduction in total calorie intake and increased physical activity with associated weight loss (Grundy, 2001; Lichtenstein et al., 2006) and using of lipid-lowering drugs such as statin, niacin, fibrate, and Omega-3 are used for cardiovascular disease prevention from dyslipidemia (Miller, 2009).

Nowadays, we currently have conventional medications for dyslipidemia, diabetes mellitus and obesity treatment such as bile-acid sequestrants, fibrates, nicotinic acid, cholesterol absorption inhibitors, HMG-CoA reductase inhibitors for dyslipidemia and metformin, sulfonylurea and synthetic insulin for diabetes mellitus and Orlistat for obesity that are widely used. However, some medications have limited efficacies, critical adverse effects such as Orlistat, often associated with rebound weight gain after the cessation of the drug and many patients cannot tolerate its gastrointestinal side effects (Cheung et al., 2013). Simvastatin, an anti-hyperlipidemic drug, can cause severe adverse events such as rhabdomyolysis (Omar and Wilson, 2002) and hypoglycemia from insulin and sulfonylurea (Bodmer et al., 2008; Shorr et al., 1997). At present, the using of plants in therapy instead of synthetic drugs has become increasingly popular during the last decade (Kelly et al., 2005) because it may

have minor adverse effects and traditional medicinal plants are often cheaper and easily consumable (Amin and Nagy, 2009).

Herbal supplements from vegetables and fruits are widely used for healthy enhancement purpose, and some antioxidant agents could be found from a natural source (Slavin and Lloyd, 2012). One of the most popular herbal supplements is herbal beverage such as herbal tea infusion. Tea is the most widely consumed beverage in the world, second only to water (Koo and Noh, 2007). Some herbal tea infusions have an evidence prove that may have a strong antioxidant capacity (Li et al., 2014) and it has a potential to prevent diseases which occur with oxidative stress, and it could prevent atherosclerosis and enhance cardiovascular health (Babu et al., 2008; Miura et al., 2001).

The plant *Pluchea indica* (L.) Less. (*P. indica*) (Family: Asteraceae) is a large evergreen shrub found abundantly in salt marshes. It is widely distributed in India, Southern China, and Southeast Asia. In Thailand, *P. indica* (L.) Less. herbal tea has been commercially available as a health-promoting drink (Kongkiatpaiboon et al., 2018). *P. indica* leaves have been reported some biological activities including the diuretic effect of its methanolic extract (Pramanik et al., 2007), anti-inflammatory activity of the ethyl acetate fraction of ethanol extract of *P. indica* in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages (Buapool et al., 2013), antioxidant activities of volatile oil of *P. indica* (Widyawati et al., 2013) and hypoglycemic and antihyperglycemic activities of *P. indica* methanolic extract in mice (Pramanik et al., 2006).

Pluchea indica (L.) Less. tea is one of potential herbal for anti-hyperglycemic, anti-dyslipidemic and anti-oxidation effects, and it lacks the investigation to prove its

effects. The purposes of this study were to investigate the inhibitory effect of the *Pluchea indica* (L.) Less. tea on pancreatic lipase activity and adipogenesis in 3T3-L1 cells, dyslipidemia and hyperglycemia prevention effects, anti-obesity and toxicity of *P. indica* (L.) Less. tea in mice and to examine the free radical scavenging and LDL oxidation prevention. The biochemical profile in 3T3-L1 adipocytes was also investigated.

1.2 Research objectives

1.2.1 To investigate the effects of *Pluchea indica* (L.) Less. tea on anti-oxidation, anti-human LDL oxidation, anti-adipogenesis in 3T3-L1 cells, anti-hyperglycemic and anti-dyslipidemic activity in mice.

1.2.2 To investigate the active ingredient of *Pluchea indica* (L.) Less. tea.

1.2.3 To investigate the sub-chronic toxicity of *Pluchea indica* (L.) Less. tea in mice.

1.3 Research hypothesis

Pluchea indica (L.) Less. tea had the effects on anti-oxidation, anti-human LDL oxidation, anti-adipogenesis in 3T3-L1 cells, anti-hyperglycemic and anti-dyslipidemic activity in mice, which did not show any toxicity in 3T3-L1 cells, and mice.

1.4 Scope and limitation of the study

This research focused on anti-oxidation, anti-LDL oxidation in human LDL, anti-adipogenesis in the 3T3-L1 adipocytes, anti-hyperglycemic, anti-dyslipidemic activities and sub-chronic toxicity of *Pluchea indica* (L.) Less. in mice.

1.5 Significant of the study

The results showed that *P. indica* (L.) Less. tea had the effects on free radical scavenging effect, anti-LDL oxidation, anti-adipogenesis in 3T3-L1 adipocytes, reduced hyperglycemia and hyperlipidemia in mice. These findings suggest that this tea may be further developed to use as herbal medicine. These data could be used for further investigation about the pharmacological activity and safety in human.

CHAPTER II

LITERATURE REVIEW

Oxidative stress occurs by the damaging effects of free radicals which are by-products of cell metabolism in natural physiological processes in the biological systems (Ames et al., 1993; Shenoy and Shirwaikar, 2002). There is increasing evidence that the major causes of oxidative stress are the excesses of intracellular reactive oxygen species (ROS) and reactive nitrogen species (RNS) that are responsible for causing the development of chronic and degenerative diseases including Alzheimer's disease (Smith et al., 2000), mild cognitive impairment (Guidi et al., 2006), Parkinson's disease (Bolton et al., 2000), cancer (Kinnula and Crapo, 2004), neural disorders (Sas et al., 2007), cardiovascular disease (Singh and Jialal, 2006) and atherosclerosis (Upston et al., 2003). Furthermore, oxidative stress may also injure biomolecules, including proteins, lipids, and DNA (Toyokuni, 1999).

2.1 Biological antioxidant capacity

Free radicals may be either oxygen-derived (ROS, reactive oxygen species) or nitrogen derived (RNS, reactive nitrogen species). The oxygen-derived molecules are O^{-}_{2} (superoxide), ROO (peroxyl), RO (alkoxyl), HO (hydroxyl), HO_2 (hydroperoxyl) and H_2O_2. Nitrogen derived oxidant species are NO (nitric oxide), NO_2 (nitrogen dioxide), ONOO (peroxy nitrate) and N_2O_3 (dinitrogen trioxide). Free radicals can be protected by natural enzymatic and non-enzymatic antioxidant defense mechanisms in

the human body which counteracts the free radicals and other oxidants which is appropriate oxidant: antioxidant balance. However, the defense mechanisms may be an imbalance when radical species are increased or lack of antioxidants. The protection against free radicals can be enhanced by dietary or natural antioxidants from plants. Nowadays, more evidence indicates that foods and plants containing antioxidants may have an important role in disease prevention.

2.1.1 The evaluation of antioxidant capacity

Various methods are used to investigate the antioxidant property of samples (diets, plant extracts, commercial antioxidants, etc.). The example of these methods is as follows:

2.1.1.1 DPPH Scavenging Assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a free radical stable at room temperature, which produces a violet solution in methanol. The DPPH scavenging assay is a decolorization assay that measures the capacity of antioxidants, which directly react with DPPH radicals by measuring the decolorization at 517 nm with a spectrophotometer. The scavenging reaction between DPPH and an antioxidant (HA) can be written as,

$$(DPPH) + (H\text{-}A) \longrightarrow DPPH\text{-}H + (A)$$

Antioxidants react with DPPH and reduce it to DPPH-H and its color changes to light yellow.

2.1.1.2 ABTS Scavenging Assay

The ABTS (2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) radical is a stable radical not found in the human body. The ABTS scavenging activity

can be measured based on colorimetry by the loss of color when an antioxidant is added to the blue-green chromophore ABTS radical.

2.1.1.3 Hypochlorous acid Scavenging Assay

Hypochlorous acid is a weak acid that could generate harmful ROS. Hypochlorous acid could inactivate the antioxidant enzyme catalase by which breaks down the heme prosthetic group. The hypochlorous acid scavenging can be evaluated by measuring the decrease in absorbance of catalase at 404 nm.

2.1.1.4 Nitric oxide Scavenging Assay

Nitric oxide radical is a free radical and weak oxidant that can be generated in biological tissues by specific nitric oxide synthases. The nitric oxide scavenging can be investigated the reducing of nitric oxide radical after challenge with an antioxidant by using Griess reagent (0.33% sulphanilamide in 20% glacial acetic acid at room temperature for 5 min with 1 mL of naphthyl-ethylene-diamine dichloride (0.1% w/v)). The decolorization of the reaction between Griess reagent and nitric oxide radical can be measured at 546 nm, and the amount of nitric oxide radical inhibition is calculated following this equation:

$$\% \text{ inhibition of NO radical} = [A_0 - A_1]/A_0 \times 100$$

Where A_0 is the absorbance before the reaction and A_1 is the absorbance after the reaction has taken place with Griess reagent.

2.1.1.5 Peroxynitrite Scavenging Assay

Peroxynitrite (ONOO) is cytotoxic with strong oxidizing properties which is one of the nitrogen-containing species. Generation of excess peroxynitrite can attack toward various cellular constituents, including sulphydryls, lipids, amino acids and nucleotides and can cause crucial pathogenic mechanical in conditions such

as stroke, myocardial infarction, chronic heart failure, diabetes, circulatory shock, chronic inflammatory diseases, cancer, and neurodegenerative disorders (Pacher et al., 2007). Peroxynitrite Scavenging can be evaluated by Evans blue bleaching assay. This assay measures the decolorization of the reaction at 611 nm.

2.1.2 LDL oxidation

LDL oxidation is the process that low-density lipoproteins transform to oxidized low-density lipoproteins by the high level of free radical. The high level of oxidized low-density lipoproteins may affect the progression of atherosclerosis, and it can progress further to cardiovascular dysfunction (Bahorun et al., 2006). LDL oxidation may be occurred within the artery wall and also in the peripheral sites of inflammation. LDL oxidation is always catalyzed by transition metal ions and several free radicals including RNS and ROS. The anti-LDL oxidation can be measured the lipid peroxidation products in cells, tissues, and body fluids by using thiobarbituric acid reactive substances (TBARS) technique. The assay is performed in the malondialdehyde (MDA) standards as reference. MDA is reacted with the enzyme degradation of polyunsaturated fatty acids in cells. The secondary end product of the oxidation of polyunsaturated fatty acids reacts with two molecules of thiobarbituric acid (TBA) and produce a pinkish-red color which detects by spectrophotometry at 532 nm.

2.2 Obesity

Obesity can cause health problems, due in part to an overabundance of fat cells. Obesity is considered a major risk factor for noninsulin-dependent diabetes mellitus

(NIDDM) (Moller and Flier, 1991) and hypertension (Spiegelman et al., 1993). Besides, obesity has also been linked to some types of cancers, immune dysfunction (Spiegelman et al., 1993). Obesity is identified into two main types; the first is increased adipose tissue mass that results from both increased fat-cell number (hyperplasia) and the second is increased fat-cell size (hypertrophy) (Couillard et al., 2000). Hypertrophy, to a certain degree, is characteristic of all overweight and obese individuals. Hyperplasia is correlated more strongly with obesity severity and is most marked in severely obese individuals. The knowledge and understanding of the differentiation process would allow for manipulation of adipocyte cell numbers and control of certain diseases. It can lead to a find the substances that can use to treat obesity and hyperlipidemia. The molecular mechanism study of adipocyte development may propose important patterns for comparison to other developmental systems and may provide an understanding of the physiology (Couillard et al., 2000).

However, research in human has many limitations such as ethics, the cost of doing research and risk of human harmful or toxicity that may happen from the new novel herbal. So, using of cell line experiment is a good alternative to reduce risks of toxicity and to evaluate the efficacy of herbal before further studies *in vivo*. Various models are used for studying the preadipocyte differentiation process that one of the best models is a 3T3-L1 cell line.

2.3 Preadipocyte differentiation process

Egg transition to the determination and conversion of adipocyte precursor cells into mature adipocytes produce in a series of stages as shown in Figure 2.1.

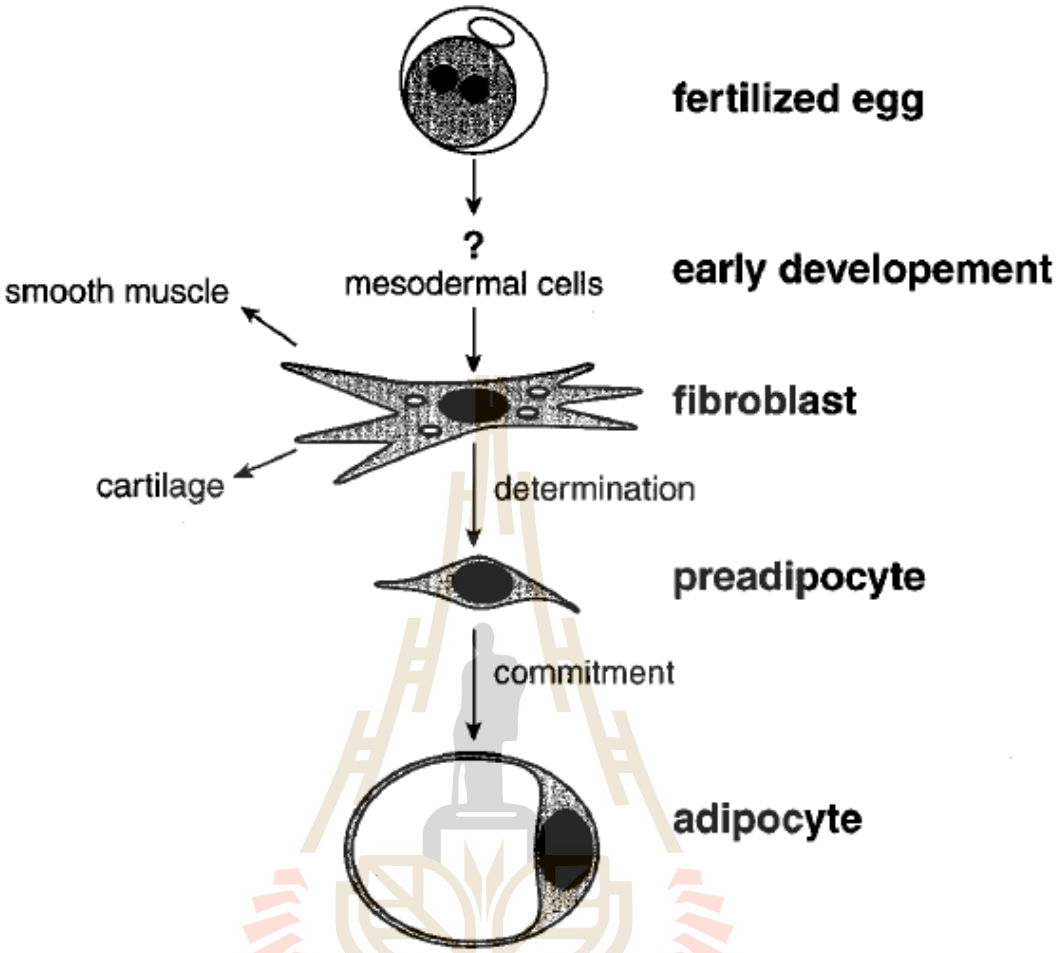


Figure 2.1 From egg to mature adipocyte. A model for the development of adipocytes from a fertilized mammalian egg. Darkened shapes represent nuclei or pronuclei. The developmental stages of determination and commitment were depicted (Oliveira et al., 2013).

The full developmental program of preadipose tissue from a fertilized egg is unknown, but the multiple developmental lineages and multicellular organism start from a single fertilized egg give rise to nearly 200 different cell types. The fibroblasts are known to have mesodermal origins (Cornelius et al., 1994) and can differentiate into committing preadipocytes, cartilage, bone or muscle tissue. Preadipocytes of humanity start to differentiate into adipose tissue during late embryonic development,

with a majority of the differentiation occurring shortly after birth (Burdi et al., 1984) that difference from rat and mouse preadipocytes do not initiate conversion into the adipose tissue until after birth (Ailhaud et al., 1992). All species have the ability to differentiate preadipocytes up to the body's fat storage demands.

2.4 Animal Cell Models

Pre-adipose cells and mature adipocytes have been used over the last many years, especially pre-adipose cells from different animals and the most widely used always collected from rodents, however, feline or porcine cells still have been used but to a lesser extent. Studies in animal models of obesity and related metabolic diseases are well modeled. Nevertheless, their application to humans is limited by the existing differences in their metabolism and physiology (Lee and Fried, 2014).

Primary preadipocytes are fibroblast-shaped cells that can differentiate into mature adipocytes under the proper conditions. Adipogenesis can be divided into two main phases, first is the commitment phase and the second is the terminal differentiation phase. Adipogenesis is a process that preadipocytes have induced the differentiation to an adipocyte phenotype that expressing some markers that include the PPAR and C/EBP family of regulators. Adipogenesis process has a transcriptional cascade which is activated that induces the expression of metabolic genes and adipokines associated with the adipocyte phenotypes, such as fatty acid-binding protein 4 (FABP4, also known as AP2), glucose transporter 4 (GLUT4, also known as SLC2A4), leptin, and adiponectin (Cristancho and Lazar, 2011). Murine preadipocytes have several major advantages of primary cultures because they can be derived from animals of different ages to examine depot- or age-dependent

adipogenic or secretory mechanisms are widely used for the study of adipocyte biology and its mechanism (Hausman et al., 2014). However, these models have some limitations such as they are more difficult to transfect with DNA, they do not propagate in culture, they have some of the triacylglycerol stores that interfere with biochemical and microscopy analyses (Wolins et al., 2006).

2.5 3T3-L1 Cell

The 3T3-L1 cell line is a pre-adipose cell line which is developed from murine Swiss 3T3 cells (Green and Meuth, 1974). The 3T3-L1 cells are derived from disaggregated 17 to 19-day-old Swiss 3T3 mouse embryos and contain only a single cell type (Green and Kehinde, 1974) that has a fibroblast-like morphology, but it can differentiate into an adipocyte-like phenotype under an appropriate condition.

Some models and techniques are being used to evaluate and understand adipocyte biology (Poulos et al., 2010). Because of the 3T3-L1 cell have the potential to differentiate from fibroblast to complete adipocytes. Therefore, the 3T3-L1 cell line has been used widely in more than 5000 published articles on adipogenesis and the biochemistry of adipocytes for the last 30 years (Zebisch et al., 2012).

The 3T3-L1 cell line has some advantages; it is especially easier to culture and less costly to use when compared with freshly isolated cells, such as mature adipocytes, although freshly isolated cells allow for various comparisons, such as the *in vitro* evaluation of differences *in vivo* conditions. Furthermore, they can tolerate an increased number of passages, and the cell populations are still homogeneous that present a homogeneous response following treatments and changes in experimental conditions (Poulos et al., 2010).

3T3-L1 cells can be used to investigate the effects of compounds or nutrients on adipogenesis, to establish the underlying molecular mechanisms of adipogenesis and to evaluate the potential application of various compounds and nutrients in the treatment of obesity (Kang et al., 2016; Lai et al., 2016; Tutino et al., 2016). Moreover, 3T3-L1 cells can study a lot of key molecular markers including transcription factors and various pathways during preadipocyte differentiation (Poulos et al., 2010). The 3T3-L1 cell can be induced differentiation of pre-adipocytes to adipocytes by many protocols. The most common to convert 3T3-L1 cells from their fibroblast phenotype into adipocytes is treat them with adipogenic agents, such as being insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) (Russell and Ho, 1976), which elevates the intracellular cAMP levels in the presence of fetal bovine serum (FBS). 3T3-L1 Pre-adipocytes cell contains a less amount of lipid, but after 10 days induction cell initiates to accumulate lipid and can increase the size and number over the differentiation time as Figure 2.2.

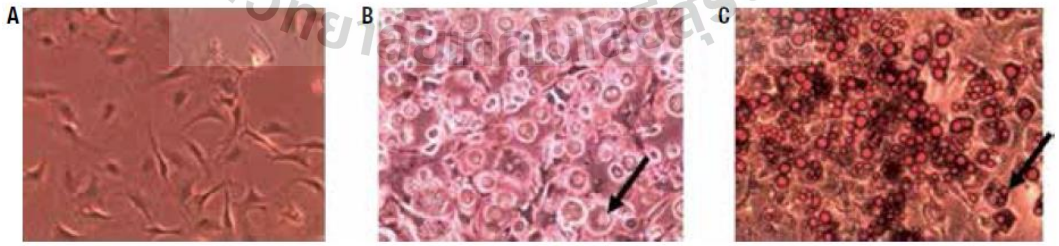


Figure 2.2 3T3-L1 cells. (A) Undifferentiated cells. (B) Cells after 10 days of differentiation. (C) Cells stained with oil red after 10 days of differentiation. The arrows indicate an adipocyte with lipid droplets in the cytoplasm (Oliveira et al., 2013).

Some experiment reported that 3T3-L1 cells could differentiate within 10 to 12 days by additional adipogenic agent rosiglitazone and can persist for at least up to cell culture passage 10 (Zebisch et al., 2012). Furthermore, a new method by using a combination of dexamethasone and troglitazone over fewer days can reduce the span of differentiating promoted time of 3T3-L1 preadipocytes when compared to the combination of IBMX and Dexamethasone with the standard protocol. Also, this method can increase the lipid droplet accumulation to 112% and glucose transporter 4 (GLUT4) mediated a 137% higher glucose uptake compared to by using the traditional method (Vishwanath et al., 2013).

The example of 3T3-L1 cell applications as follow; 3T3-L1 cells have been used to investigate the effect of antioxidants (Calzadilla et al., 2013), melatonin (Kato et al., 2015), reactive oxygen species (ROS) on adipogenic differentiation (Matsuo et al., 2015); The inhibitory effects on adipogenesis in 3T3-L1 adipocytes of compounds such as quercetin (Eseberri et al., 2015; Okabe et al., 2014) and resveratrol (Chang et al., 2015; Patel et al., 2013); Moreover, 3T3-L1 cells have been useful to study the mechanisms of action during the differentiation process to inhibit obesity *in vivo* (Mammi et al., 2016); Also, some endocrine disruptors and obesogenic compounds have also been investigated during the differentiation of 3T3-L1 cells (Regnier et al., 2015). Additionally, the cell can be used to evaluate inflammatory pathways, adipokine synthesis, and secretion of an enzyme's function has been investigated through gene silencing in adipocytes (Abdesselem et al., 2016; An et al., 2012; Choi et al., 2016; Lien et al., 2016; Ma et al., 2012).

However, the 3T3-L1 model has several limitations, for example, the time of initial subculture which requires at least two weeks (Student et al., 1980). Also, if

3T3-L1 cells become confluent or they have been spread widely, they will no longer differentiate into adipocytes. Besides, they are difficult to transfect, and it fails to recapitulate the characteristics of primary cell culture models because these cell lines grow from a single clone (Wolins et al., 2006).

2.6 Lipase Activity

Lipases (triacylglycerol hydrolases) are a group of ubiquitous enzymes that are found in animals, plants, fungi and bacteria which successively hydrolyze triacylglycerols to diacylglycerols and monoacylglycerols, with free fatty acids as coproducts (Beisson et al., 2000). Lipases are normally water-soluble, after the absorption of enzymes to do catalytic reactions that can change into an oil-water interface (Beisson et al., 2000; Dellamora-Ortiz et al., 1997).

In the industrially developed countries. Modern biotechnology has promoted several lipases for some applications such as detergents, foods, and pharmaceuticals (Gupta et al., 2004). Lipase can be produced from several sources such as recombinant human gastric lipase has been produced by insects (Canaan et al., 1998; Wicker-Planquart et al., 1996), yeast (Crabbe et al., 1996), transgenic corn and tobacco have been used as a host to express and produce recombinant dog gastric lipase in seeds (Roussel et al., 2002). Moreover, the discovery of natural lipases and the development of industrial processes for producing commercial lipases.

Various methods have been used to investigate lipase activity by measuring the hydrolytic activity lipase as can be seen from the literature (Jensen, 1983; Tietz and Shuey, 1993). These methods can be classified as follows:

1. Titrimetry
2. Spectroscopy (photometry, fluorimetry, infrared)
3. Chromatography
4. Radioactivity
5. Interfacial Tensiometry
6. Turbidimetry
7. Conductimetry
8. Immunochemistry
9. Microscopy

The general Triacylglycerol hydrolysis reaction catalyzed by lipases can be written as follows:

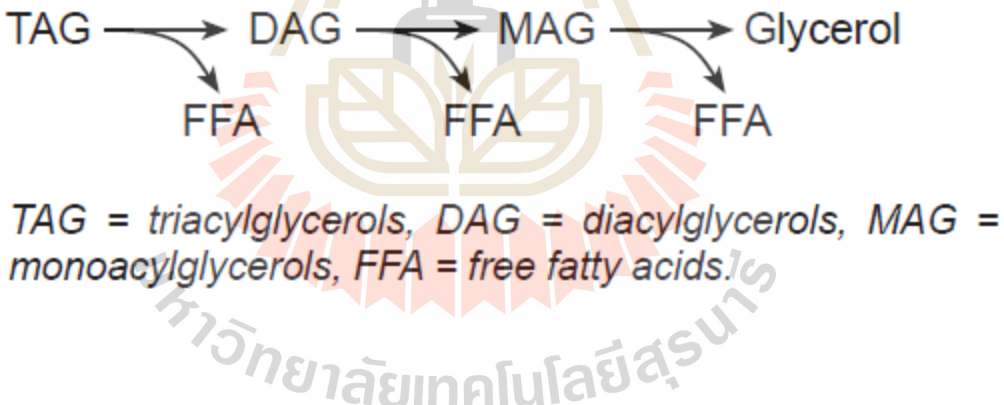


Figure 2.3 The general Triacylglycerol hydrolysis reaction (Beisson et al., 2000).

In summary, the reactions TAG are catalyzed by enzymes; we can investigate lipase activity by measurements using various physicochemical methods of detecting the rest of the substrate or the amount of product compared with a positive control. This method can investigate lipase inhibitory effects which confirm that herbal can treat obesity because lipase inhibitor can inhibit degradation of lipid and reduce lipid

absorption. The example of conventional medicine that uses for treating obesity is Orlistat that acts as a lipase inhibitor.

2.7 *Pluchea indica* (L.) Less.

The plant *Pluchea indica* (L.) Less. (Khlu or Kratagruea) (Family: Asteraceae) is a large evergreen shrub up to 2 meters tall found abundantly in salt marshes. *Pluchea indica* (L.) Less. can be found in Asia and Australia, and it is widespread in the Pacific Islands as an introduced, and often invasive species (Pramanik et al., 2006), but in South-East Asia, including Thailand *Pluchea indica* (L.) has been used in folk medicine. Additionally, *Pluchea indica* (L.) Less. leaves are used in the preparation of herbal tea.

2.7.1 Pharmacological properties of *Pluchea indica* (L.) Less.

Pluchea indica (L.) Less. leaves extract of various solvents (methanol, water, ethyl acetate) showed the anti-diabetic effect that water extract was the most potential as an anti-diabetic agent. *Pluchea indica* (L.) Less. leaves water extract, could reduce blood sugar of *Rattus Norvegicus* Rats Wistra strain 56.37% that were higher than glibenclamide (49.59%) and the other extracts (ethyl acetate extract = 19.11% and methanol extract = 24.27%). Moreover, the water extract of *Pluchea indica* (L.) Less. leaves were also safe to the body healthy of *Mus Musculus* Mice (20-30 g), Balb/c strain. The male mice oral administration of water extract at 2.6 mg/20 g BW increased motoric activity. However, female mice at 1.3 and 2.6 mg/20 g BW, respectively oral administration experienced motoric activity increasing and establishment capacity decreasing. Widyawati et al. concluded that the potency of

Pluchea indica (L.) Less. water extract as an anti-diabetic agent was predicted that there was a cardiac glycoside compounds contribution to reducing blood glucose (Widyawati et al., 2015).

Pluchea indica (L.) Less. extracts had an inhibitory effect against intestinal maltase that can adapt to use for treating diabetes mellitus (Food and Administration) which decrease postprandial hyperglycemia by suppressing carbohydrate digestion using alpha-glucosidase inhibitors. The experiment used the enzyme assay-guided fractionation by chromatography yielded five active caffeoylquinic acid derivatives. About the active constituent, their structures were investigated by mass spectrometry and NMR analysis and completed by comparison with reference data. 3,5-di-O-caffeoylquinic acid (1), 4,5-di-O-caffeoylquinic acid methyl ester (2), 3,4,5-tri-O-caffeoylquinic acid methyl ester (3), 3,4,5-tri-O-caffeoylquinic acid (4), and 1,3,4,5-tetra-O-caffeoylquinic acid (5) were isolated. Arsiningtya et al. suggested that both methyl esterification of quinic acid and the number of caffeate groups in the molecule were important to the inhibitory activity (Arsiningtyas et al., 2014).

The investigation of the hypoglycemic and antihyperglycemic activities of methanolic extract of *Pluchea indica* (L.) Less. (MEPI) leaves showed that it had hypoglycemic and antihyperglycemic activity. The investigation was studied in normal rats and streptozotocin-induced diabetic rats. The blood glucose levels were measured at 1, 4, 8, 16 and 24 h intervals after the treatment. The results showed a % reduction of blood glucose level in normal rat 35.12% and 36.01% for 200 and 400 mg/kg, p.o., respectively, and in streptozotocin-induced diabetic rats 36.10% and 41.87% for 200 and 400 mg/kg, respectively. Furthermore, a toxicity study has been

investigated for the extract, which expressed that the extract was safe to although orally fed (MEPI) at the doses of 3.2 gm/kg of body weight (Pramanik et al., 2006).

The methanol fraction of *Pluchea indica* (L.) Less. showed prevent inflammation and lowest incidences of gastric damage effect when use pretreatment with either *Pluchea indica* (L.) Less. and certain standard drugs that were evaluated on platelet activation factor (PAF)-induced inflammation, gastric necrosis, ulceration and haematological picture that induced by administration of PAF (i.v.) for 20 min (Sen et al., 1996).

About another inflammatory study, Sen et al. reported that they evaluated the influence of the methanol fraction of *Pluchea indica* (L.) Less. root extract for free radical-scavenging activities, they induced lipid peroxidation by CCl_4 and the metabolism of arachidonic acid by lipoxygenase. *Pluchea indica* (L.) Less. root extract expressed significant anti-inflammatory activity against paw edema (*in vivo*) that induced by glucose oxidase, and also inhibited hydroxyl radical and superoxide generation, lysis of erythrocytes induced by hydrogen peroxide, CCl_4-induced lipid peroxidation and also dioxygenase activity of lipoxygenase (both in the presence and absence of hydrogen peroxide) (Sen et al., 2002).

Pluchea indica (L.) Less. showed the antioxidant and acetylcholinesterase inhibition properties of stems and leaves of hexane and methanolic extracts of its. Especially methanolic extract of leaves expressed the highest antioxidant activity (IC_{50} = 24.45 ± 0.34 μg/ml) and total phenolic contents (573.52 ± 6.2 mg GAE/100 g crude extract), in DPPH radical scavenging and Folin-Ciocalteu assay respectively, Nevertheless, in the TLC bioautographic detection experiment it couldn't inhibit acetylcholinesterase. However, other parts of plant extracts, including hexane extract

of leaves and stems, methanolic extract of stems, had the effect to inhibit the acetylcholinesterase properties (Hii et al., 2010).

The study of antioxidant activity that was generated by radical scavenging assay of the hot water extract of *Pluchea indica* (L.) Less. tea leaves (HWEP) against 1,1-diphenyl-2-picrylhydrazyl, superoxide and hydroxyl radical, ferric ion reducing power, as well as ferrous ion chelating. The results showed that HWEP showed good concentration-dependent manner antioxidant activity in all test systems. Moreover, HWEP has potent inhibitory effects on lipopolysaccharide-induced nitric oxide (NO) and prostaglandin E2 (PGE2) production in RAW 264.7 macrophages. In summary, the hot water extract of *Pluchea indica* (L.) Less. leaves contain a source of antioxidants and inhibitors of NO and PGE2 production that can be used as dietary supplements containing good health-promoting effect (Srisook et al., 2012).

The various concentrations of crude aqueous extracts of *Pluchea indica* (L.) Less. leave, and root were investigated cancer cell proliferation and viability of GBM8401 human glioma cells and HeLa cervical carcinoma cells by measuring cell growth curves, trypan blue exclusions, and the tetrazolium reduction assay. Furthermore, the effects of the crude aqueous extracts on focus formation, migration, and apoptosis of cancer cells were studied as well, and the molecular mechanism that investigated the anti-cancer activities of crude aqueous extracts of *Pluchea indica* (L.) Less. root was also investigated by using western blotting analysis. The results expressed that crude aqueous extracts of *Pluchea indica* (L.) Less. leave, and root could suppress proliferation, viability, migration of GBM8401 and HeLa cells. Crude aqueous extracts of *Pluchea indica* (L.) Less. leaves and root could inhibit a significant 75% and 70% on proliferation and viability of GBM8401 and HeLa cancer

cells, respectively after treatment for 48 hours. Furthermore, the crude aqueous extracts of *Pluchea indica* (L.) Less. root showed that it could inhibit focus formation and promoted apoptosis. Pramanik et al. concluded that the *in vitro* anticancer effects of crude aqueous extracts of *Pluchea indica* (L.) Less. leaves and root had sufficient potential to warrant further examination and development as a new anti-cancer agent (Pramanik et al., 2008).

Widyawati reported about the antioxidant activity of *Pluchea indica* (L.) Less. extract by the decrease in absorbance as the result of DPPH which measured the color changed from purple to yellow at 517 nm by using a stopped-flow spectrometer UV-Vis. The results showed that *Pluchea indica* (L.) Less. extract and its fraction had free radical-scavenging activity compared with control antioxidant based on EC (the time needed to reach the steady state) (Widyawati, 2016).

Ethanolic extracts of *Pluchea indica* (L.) Less. root had anti-cancer activities that could strongly inhibit the viability of the human nasopharyngeal carcinoma cells (NPC-TW 01 and NPC-TW 04) in a time- and dose-dependent manner. Ethanolic extracts of *Pluchea indica* (L.) Less. root expressed suppressed effect on migration of cancer cells. Nevertheless, ethanolic extracts of *Pluchea indica* (L.) Less. root significantly increased the occurrence of the cells in the sub-G1 phase and the extent of DNA fragmentation in a dose-dependent manner that indicates ethanolic extracts of *Pluchea indica* (L.) Less. root could increase apoptosis in NPC cells. Ethanolic extracts of *Pluchea indica* (L.) Less. root showed up-regulation of pro-apoptotic Bax protein and down-regulation of anti-apoptotic Bcl-2 protein, thus increasing the ratios of Bax/Bcl-2 protein levels. That implicated that the apoptotic process triggered by ethanolic extracts of *Pluchea indica* (L.) Less. root involved Bax/Bcl-2 protein.

Nevertheless, ethanolic extracts of *Pluchea indica* (L.) Less. root could up-regulate the p53 protein in a concentration-dependent manner. In conclusion, ethanolic extracts of *Pluchea indica* (L.) Less. root could induce the apoptosis-signaling pathway in NPC cells by activation of p53 and by regulation of apoptosis-related proteins (Kao et al., 2015).

Widyawati et al. reported about the antioxidant capacity to scavenge a DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical and inhibit of lipid peroxidation of *Pluchea indica* (L.) Less. leave essential oil that *Pluchea indica* (L.) Less. leave essential oil was prepared by hydrodistillation method, and then their volatile compounds were identified by GC-MS. The volatile compounds in the essential oil of *Pluchea indica* (L.) Less. leave contains 66 components with (10S,11S)-Himachala-3-(12)-4-diene (17.13%) made up the highest proportion of volatile compounds. Total phenol levels in *Pluchea indica* (L.) Less. leave essential oils were 275 ppm. The essential oil of *Pluchea indica* (L.) Less. leaves expressed inhibit DPPH scavenging activity, and it showed inhibition activity of lipid peroxidation in *Pluchea indica* (L.) Less. essential oil (Widyawati et al., 2013).

The ethanolic extract of *Pluchea indica* (L.) Less. leaves at a dose of 300 mg/kg orally exhibited significant and dose-dependent anti-inflammatory activity that elucidated by using the carrageenan-induced edema model and acetic acid induced writhing test. It is also demonstrated that the i.p. administration of *Pluchea indica* (L.) Less. leaves ethanolic extract at a dose of 10, 30, 100 and 300 mg/kg exhibited significant inhibition of abdominal constriction that induced by 0.6% (v/v) acetic acid in a dose-dependent manner. These results implied that *Pluchea indica* (L.) Less.

leaves had significant anti-inflammatory and antinociceptive effects (AK and Ahmad, 2008).

The methanolic extract of the leaves of the *Pluchea indica* (L.) Less. had diuretic activity in Wistar albino rats. Urinary excretion parameters were studied for evaluation of diuretic activity using Furosemide (20 mg/kg, p.o.) as standard. The *Pluchea indica* (L.) Less. extract expressed significant diuretic activity at the doses of 100, 200 and 300 mg/kg. p.o. Moreover, an oral acute toxicity study was elucidated, and the result showed that the LD_{50} value was found to be 2,825 mg/kg body weight (Pramanik et al., 2007).

Pluchea indica (L.) Less. extract had antimalarial properties. The air-dried, powdered plant part (roots) was extracted, sequentially with solvents of increased polarity (methanol, butanol, petroleum ether, and ethyl acetate). The ethyl acetate insoluble fraction and isolated compound extract showed an antimalarial effect in albino mice infected with malaria parasites by administrated with a dose of 100 and 300 mg/kg/d orally when compared with the standard (Chloroquine and Sodium artesunate) (Kundu et al.).

Gomes et al. reported that β-sitosterol and stigmasterol which isolated from the root extract of *Pluchea indica* (L.) Less. could neutralize of viper and cobra venom. The active fraction was isolated and purified by silica gel column chromatography, and the structure was identified by using spectroscopic analysis (EIMS, 1H NMR, 13C NMR) (Gomes et al., 2007).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Materials

Fresh herb of *Pluchea indica* (L.) Less. was collected from Nakhon Ratchasima and North-East region of Thailand. The plant specimen was authenticated by Dr. Paul J Grote. Identification was made in comparison with the voucher specimen (BKF 194428) and deposited at Forest Herbarium, National Park, Wildlife, and Plant Conservation Department, Ministry of Natural Resources and Environment, Thailand. This herb was washed thoroughly; then it was ground and dried in an oven. The production process was performed by the Crystal Biotechnology Co., Ltd and Suranaree University of Technology. *Pluchea indica* (L.) Less. tea dry sample was added to boiling distilled water or PBS, then further heated for 10 min, and then filtered through Whatman No.1 paper. The concentration of *Pluchea indica* (L.) Less. tea (PIT) sample was calculated from the concentration of *Pluchea indica* (L.) Less. tea dry weight in a solvent (µg /ml).

3.1.2 Chemicals and Reagents

3T3-L1 mouse embryonic fibroblasts and bovine calf serum were purchased from the American Type Culture Collection (ATCC, USA). Dulbecco's Modified Eagle's Medium (DMEM) with high glucose, Penicillin, Streptomycin, N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), 3-(4,5-Dimethylthiazol-2-

yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Gibco Invitrogen (Grand Island, NY). Bovine calf serum (BCS), Fetal bovine serum (FBS), Oil Red O was obtained from Hyclone (Logan, Utah). Insulin solution from bovine methyl isobutyl xanthine (IBMX), lipase from porcine pancreas, 4-Nitrophenyl dodecanoate (pNP), Orlistat, Gallic acid, Folin–Ciocalteau reagent, Catechin, A total of 10% neutral buffered formalin solution, sucrose, D-(+)-Glucose, xylenes, Mayer's hematoxylin solution, eosin Y solution, Simvastatin, Glibenclamide, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), potassium persulfate, sodium hypochlorite (NaOCl), catalase, sulfanilamide, naphthylethylenediamine dihydrochloride (NED), 3-Morpholinosydnonimine hydrochloride (SIN-1), Diethylenetriamine-pentaacetic acid (DTPA), Evans Blue, iodixanol (Optiprep^{TM}), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), copper sulfate (Cu_2SO_4), trichloroacetic acid (TCA) and Thiobarbituric acid were obtained from Sigma-Aldrich (St. Louis, USA). 60% High fat mouse diet was purchased from Bio-Serv (Frenchtown, NJ, USA). Dexamethasone (DEX) was obtained from G Bioscience (St. Louis, USA). Dimethyl sulfoxide (DMSO) was obtained from Carlo Erba Reagents S.r.l. (Chaussée du Vexin, Val de Reuil, USA). 4-O-caffeoylquinic acid (4-CQ), 5-O-caffeoylquinic acid (5-CQ), 3,4-O-dicaffeoylquinic acid (3,4-CQ), 3,5-O-dicaffeoylquinic acid (3,5-CQ) and 4,5-O-dicaffeoylquinic acid (4,5-CQ) were purchased from Chengdu Biopurify Phytochemicals Ltd., China. Other reagents used were all analytical grade.

3.2 Methods

3.2.1 Quantitative estimation of chemical constituency

3.2.1.1 Determination of Total Phenolic Content (TPC)

The Folin–Ciocalteu assay has been used for investigating the total phenolic content as previously described by Singleton and Rupasinghe et al. (Rupasinghe et al., 2008; Singleton et al., 1999). In brief, 100 µl of 0.2 N of Folin-Cioculteu was pipetted into a 96 well microtitre plate. This was followed by the addition of either 20 µl of the PIT or various concentrations of gallic acid prepared in methanol (0-0.0625 mg/ml). Finally, 80 µl of 7.5% (W/V) sodium carbonate was added, and the mixture was incubated at room temperature for 2 h. The absorbance of the blue color solution was measured at 765 nm by spectrophotometry, and the total phenolic content was determined using a gallic acid standard curve. The results were expressed as mg gallic acid equivalents (mg GAE/g) per gram of dry weight.

3.2.1.2 Determination of Total Flavonoid Content (TFC)

The aluminium chloride colorimetric assay has been used for investigating the total flavonoid content (Chen and Li, 2007; Settharaksa et al., 2014). Briefly, 125 µl deionized water was pipetted into 96 well microplates. This was followed by either the addition of 25 µl standard catechin at various concentrations (0-0.4 mg/ml) or PIT. Upon completion of the addition of standards or PIT, 10 µl of 5% $NaNO_2$ was also added. The mixture was incubated at room temperature for 6 min. To initiate a color change, 15 µl of 10% $AlCl_3$ solution was added. The solution was allowed to stand for 5 min at room temperature. To prevent a further reaction, 50 µl of 1 M NaOH was added and shaken in microplate reader spectrophotometry for 5 min before measuring absorbance at 595 nm. The total flavonoid content was

determined using a catechin standard curve. The results were presented as mg catechin equivalents (mg CE/g) per gram of dry weight.

3.2.1.3 LC-MS/MS instrument and conditions

The chemical characteristic of PIT was investigated by using LC-MS/MS instrument. The LC-MS/MS system was made up of a combination of chromatographic separation Agilent HPLC 1290 Infinity and the mass analyzer 6490 Triple Quad LC/MS Agilent Technologies equipped with electrospray ionization (ESI) source system, consisting of an auto-sampler, a binary pump, and vacuum degasser. The chromatographic separation was set on Agilent ZORBAX Rapid Resolution High Definition (RRHD) SB-C18, 2.1 mm id x 150 mm (1.8 µm). The mobile phase system used solvent A and solvent B which consisted of 1% formic acid in water and 1% formic acid in acetonitrile, respectively. A combination of both solvents in LC system was set at a ratio of solvent A: solvent B, 100:0 with gradient elution: from 30% solvent B at 10 min and 100% solvent B at 30 min at a flow rate of 0.2 ml/min. The column temperature was maintained at 25 ºC, and the sample injection volume was set at 5 µl. The stock solutions of standards (4-CQ, 5-CQ, 3,4-CQ, 3,5-CQ, and 4,5-CQ) were prepared to a final concentration of 1000 µg/ml by dissolving in methanol, and working solutions were diluted by methanol to obtained the desired concentration and PIT sample working solution was prepared to a final concentration of 150 µg/ml.

3.2.2 3T3-L1 preadipocytes

3.2.2.1 Cell Culture

The 3T3-L1 preadipocytes at a density of 5×10^5 cells/well were seeded in a 6-well plate and cultured in DMEM with high glucose, added with 100 µg/ml

streptomycin, 100 U/ml penicillin and 10% bovine calf serum until confluent. The cells were kept in an incubator at 37 °C in 5% CO_2 and 95% humidity.

3.2.2.2 Differentiation Procedures

After post confluence, two days (day 0), Differentiation process of the 3T3-L1 preadipocyte cells was initiated. Cells were induced into adipocytes by adding differentiation medium containing 1.0 µg/ml insulin, 1.0 µM dexamethasone, 10% FBS, and 0.5 mM of IBMX in DMEM for 48 h (day 2) after that the differentiation medium was changed to maintain medium which consisted of 1.0 µg/ml insulin and 10% FBS in DMEM for 48 h (day 4). The medium was replaced every 48 h until day 10. The 3T3-L1 pre-adipocytes were treated with various concentrations of the PIT at final concentrations (250 - 1000 µg/ml) for 48 h during periods of the differentiation phase (at day 0, 2, 4, 6, and 8). At day 10, the differentiation of 3T3-L1 preadipocytes was observed.

3.2.2.3 *In vitro* Cytotoxic Test (MTT Assay)

The cytotoxic effect of the PIT on cell proliferation was determined using the MTT assay (Denizot and Lang, 1986). Briefly, the cells were seeded in a 96-well plate at a density of 5×10^3 cells/well. The cells were allowed to adhere for 48 h and then were induced to differentiate into adipocytes by adding differentiation medium and treated with PIT between 250 - 1000 µg/ml. The differentiation medium was changed to maintain medium and treated with PIT in various concentrations on day 2, and the maintain media with PIT in various concentrations was replaced every 48 h until day 10. After that, the cytotoxic effect of the PIT on cell proliferation was investigated. The culture medium was removed, and 0.5 mg/ml MTT reagent was added, and the cells were incubated at 37 °C for 4 h. The viable cells formed

formazan crystal and were dissolved in DMSO. The absorbance was measured at 540 nm with a microplate spectrophotometer (Benchmark Plus, Bio-Rad, Japan).

3.2.2.4 Oil Red O and Hematoxylin Staining

The increased amount of lipid accumulation of adipocyte differentiation was measured. Oil Red O Staining can assess the increased amount of lipid accumulation normally associated with adipocyte differentiation (Naowaboot et al., 2012). Briefly, after induced 3T3-L1 preadipocytes to start adipogenesis by standard adipogenic medium and treated with a PIT at various concentrations (250, 500, 750, and 1000 µg /ml) for 48 h, differentiation medium was changed to maintaining medium with different concentrations of the PIT. The medium with various concentrations of the PIT was replaced every 48 h with maintaining medium until day 10. At day 10, the cells were washed with PBS twice and fixed with 10% formaldehyde in PBS for 1h. After that, cells were washed with distilled water twice and stained with 0.5% Oil Red O solution in 60:40 (v/v) isopropanol: distilled water for 30 min at room temperature. The Oil Red O stained cells were washed twice with distilled water and treated with hematoxylin solution for 10 min at room temperature. The lipid droplets were washed twice with 60% isopropanol, eluted with 100% isopropanol and transferred to new 96 well plates. The lipid accumulation was quantified by measuring the absorbance at 490 nm with a microplate spectrophotometer.

3.2.2.5 FPA-FTIR Micro-Spectroscopy

FTIR micro-spectroscopy technique was performed to investigate the effect of the PIT on 3T3-L1 adipocyte cells following the method of Eumkeb et al. and Dunkhuntod et al. (Dunkhunthod et al., 2017; Eumkeb et al., 2012) with minor

modifications. In brief, 3T3-L1 cells were seeded at the density 5×10^5 cells/well in a 24-well plate. The samples were divided into 4 groups, including differentiated group (DIF), PIT at 750 µg/ml (PIT 750), Simvastatin at 1.67 µg/ml (SIM 1.67) and non-differentiate group (ND, preadipocytes). The 3T3-L1 cells were collected after treatment for 10 days and centrifuged at $400\times g$ for 5 min. Cells were washed with 0.85% NaCl and recentrifuged at $400\times g$ for 5 min. Cell pellets were dropped onto Barium Fluoride (BaF_2) optical window 13 mm Ø×2 mm (Crystran, Crystran Ltd) and air vacuum dried for 30 min in a desiccator to eliminate the excess water. The dropped cell slides were kept in a desiccator until analysis with FTIR.

FTIR spectra were performed by using a spectroscopy facility, at the Synchrotron Light Research Institute (Public Organization), Thailand. FTIR spectra were obtained on a Bruker Vertex 27 spectrometer coupled with a Bruker Hyperion 3000 microscope (Bruker Optics Inc., Ettlin-Gen, Germany). The microscope was equipped with nitrogen-cooled 64×64 element MCT, FPA detector, which allowed simultaneous acquisition of spectral data with a 15 x objective.

The spectra were obtained in the transmission mode with the wavenumber range of 4000-700 cm^{-1}. Each of the images used to construct 4×4 binning FTIR image mosaic, 4 cm^{-1} spectral resolution, 64 scans. The area of the sample, from which single spectra were acquired, was approximately 20 µm×20 µm. OPUS 7.2 software (Bruker Optics Ltd, Ettlingen, Germany) was used to acquire FTIR spectral data and control instrument system.

The spectra of DIF, PIT (750), SIM (1.67) and ND groups were identified by Principal Component Analysis (PCA) using variability of the Unscrambler 10.1 software (CAMO Software AS, Oslo, Norway). The spectral range of 3000-2800 cm^{-1}

and 1800-850 cm^{-1} was used for treated cells. The preprocessing of the spectra was performed by second derivative transformations using Savitzky-Golay algorithm (nine smoothing points) and normalized with extended multiplicative signal correction (EMSC) using the spectral regions from 3000-2800 cm^{-1} and 1800-950 cm^{-1}. This method is used for identifying the overlapping of absorption peaks, reducing variation between replicates spectra, and correcting for baseline shift. Score plots (2D) and loading plots were used to represent the different classes of data and relations among variables of the data set, respectively. The integrated peak areas of all groups were analyzed using OPUS 7.2 software (Bruker).

3.2.3 Pancreatic Lipase Assay

Lipase activity assay was based on the lipase cleaving pNP-laureate to produce a colored product. The method was based on the method of Guo et al. (Guo et al., 2015). In brief, 5 mg/ml porcine pancreas lipase type 2 was dissolved in distilled water. The solution was centrifuged at 10,000 × *g* for 5 min, and the supernatant was collected. pNP laurate in reaction buffer (100 mM Tris buffer pH 8.2) was used as a reaction substrate. 0.1% (w/v) pNP laurate was mixed with 5 mM sodium acetate (pH 5.0) containing 1% Triton X-100 and was heated in boiling water for 2 min until all solid matters were dissolved. The solution was then mixed well and cooled to room temperature. All test samples were dissolved in 50% DMSO in reaction buffer. Then mixed 20 µl of the sample and 30 µl of lipase and added to 40 µl reaction buffer, and the reaction was started by adding 30 µl substrate solution. 50% DMSO instead of the sample was performed as a negative control, and the solution of Orlistat was used as a positive control. Sample blank for each test sample was prepared by the reaction solutions without enzyme. The mixtures were incubated at 37 °C for 6 h and

measured at 409 nm using a microplate spectrophotometer. The inhibition rate (%) was described as,

$$\text{Inhibition rate (\%)} = (1 - \frac{OD_{sample} - OD_{sample\ blank}}{OD_{negative\ control}}) \times 100$$

3.2.4 Animals experiment

3.2.4.1 Sample size calculation

The formula for sample size calculation: (Charan and Kantharia, 2013)

$$\text{Sample size} = 2\ SD^2\ (Z^{\alpha/2} + Z^{\beta})^{\ 2}/d^2$$

Where, Standard deviation = from previous studies or pilot study $Z^{\alpha/2} = Z_{\ 0.05/2} = Z_{\ 0.025}$=1.96 (From Z table) at type 1 error of 5% $Z^{\beta} = Z_{\ 0.20}$ = 0.842 (From Z table) at 80% power d = effect size = difference between mean values

Hence the sample size will be = $2 \times 24.06^2 \times (1.96 + 0.842)^2/30^2$ = 10.09.

3.2.4.2 Animals and experimental design

Forty adult male ICR mice were used in each experiment, weighing 30-40 g, aged about 8 weeks, were obtained from the Animal Care Building, Suranaree University of Technology, Nakhon Ratchasima, Thailand. The experimental protocol was approved in accordance with a guideline for the care and use of laboratory animal by animal care and use committee (ACUC), Suranaree University of Technology. Mice were housed in a light, humidity and temperature-controlled room (light on 12h/day, temperature 25 ± 0.5 °C and the humidity 40 ± 2%) at the animal care building at Suranaree University of Technology, Nakhon Ratchasima, Thailand. The mice had free access to food pellets and water except when fasted before blood collection and necropsy.

3.2.4.2.1 The effect of *P. indica* (L.) Less. tea on oral glucose tolerance test (OGTT)

An oral glucose tolerance test was performed to measure how well the body can process a larger amount of sugar. After acclimation for 2 weeks, mice were randomly divided into 4 groups (n=10). After fasting for 16-h, all mice were collected blood for measuring blood glucose before receiving single oral administration of 2 g/kg body weight D-glucose with; The control group (CON) was fed with water. The positive control group (GLI) was fed, a single oral administration, with Glibenclamide at 10 mg/kg. The *P. indica* (L.) Less. tea at a low dose (PIL) and high dose (PIH) treated groups were fed with PIT at 4 0 0 and 6 0 0 mg/kg/day, respectively. Blood glucose was measured by an Accu-Chek glucometer (Roche, Basel, Switzerland) using Accu-Chek test strips at 0, 15, 30, 60 and 120 min that initiated from the oral glucose administration.

3.2.4.2.2 The anti-dyslipidemia and toxicity testing

The anti-dyslipidemia and toxicity testing, The effect of *P. indica* (L.) Less. tea on serum lipid profile had been investigated. Mice were randomly divided into 4 groups (n=10). The control group (CON) was fed with 60% High fat diet (HFD) and water. The positive control group (SIM) was fed with 60% HFD and simvastatin at 2 0 mg/kg /day. The *P. indica* (L.) Less. tea at a low dose (PIL) and high dose (PIH) treated groups were fed with HFD and PIT at 400 and 600 mg/kg/day, respectively. The experimentation was performed for 4 weeks. At the end of the treatment period, all mice were sacrificed under thiopental sodium anesthesia and subjected to necropsy. The blood was collected to analyze total cholesterol, LDL-cholesterol, Triglyceride, and HDL-cholesterol.

3.2.4.2.3 The measurement of body weight, food intake, relative organ and perigonadal fat weight

The body weight of all mice was measured every week, and food intake was assessed daily, and average daily food intakes were calculated. At day 28, mice were sacrificed, and liver, heart, kidney, lung, spleen, and perigonadal fat were collected and weighted. The relative organ and perigonadal fat weight per 100 g of total body weight of each mouse were calculated as follows:

Relative organ weight = Weight of mice organ (g) × 100/Mice body weight

3.2.4.2.4 Biochemical analysis

Before treatment, mice were fasted for 16-h, and blood samples were collected for chemistry analysis. Serum lipid profile (Serum Cholesterol, Triglyceride, HDL-C, and LDL-C) was measured for anti-dyslipidemia analysis. Creatinine, Alanine transaminase (ALT), Alkaline Phosphatase (ALP) and Complete Blood Count (CBC) were measured for toxicity testing. At day 28, mice were sacrificed, and blood was collected. Serum lipid profile, Creatinine, ALT, ALP, and CBC were measured for biochemical analysis compared with pre-treatment.

3.2.4.2.5 Histological analysis

After 4 weeks of treatment, all mice were sacrificed, and histological analysis was performed. The perigonadal fat pad was collected and preserved in 10% (w/v) neutral phosphate buffer formaldehyde. The tissues were fixed in 10% neutral buffered formalin and were embedded in paraffin and microtome sectioned at 5 µm. The sectioned tissues were placed in xylenes and rehydrated through serial alcohol gradients (100%, 95%, 90%, 80%, 70%, and 50%, 2 min each). Hematoxylin and eosin were used for staining. The histopathology of the tissue slide

was examined under a light microscope. The number of adipocytes per each field and the mean areas of each adipocyte was counted and analyzed.

3.2.5 Biological antioxidant capacity experiment

3.2.5.1 DPPH Scavenging Assay

The DPPH scavenging activity of PIT compared with *C. sinensis* tea was evaluated by the method of Brand-Williams et al. (Brand-Williams et al., 1995) with minor modification. Briefly, tea samples were mixed with 0.002% DPPH w/v in methanol at various concentrations (0–300 μg/mL final concentration). The reaction was done in 96-well plate and was shaken suitably. This plate was kept in the dark room at room temperature for 30 min. PBS instead of the extract was mixed with the same volume of DPPH% solution in methanol was used as a control sample. The absorbance of all samples was measured at 515 nm. Radical scavenging activity was repeated for six times and expressed as scavenging percentage.

3.2.5.2 ABTS Scavenging Assay

The ABTS scavenging activity of PIT was measured and compared with *C. sinensis* tea. The regenerated $ABTS^{+}$ radical cation was prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration) in methanol and kept them in the dark at room temperature for 16 h until the reaction was completed. The solution was adjusted absorbance by diluting with ethanol at room temperature to 0.70 (± 0.02) before initiating the reaction. The reaction was done in 96-well plate by mixed 0.9 ml $ABTS^{+}$ radical cation solution with 0.1 ml of the test sample in various concentrations (final concentration 0-40 μg/ml final concentration). The 96-well plate was shaken for 45 seconds, and the absorbance was

measured by spectrophotometry at 734 nm after 15 min of the reaction. The assay was repeated for six times, and the percentage inhibition of absorbance was presented.

3.2.5.3 Hypochlorous acid Scavenging Assay

The hypochlorous acid scavenging assay was performed following Aruoma and Halliwell (Aruoma and Halliwell, 1987) with little modification. Briefly, hypochlorous acid (HOCl) was generated by adjusting the pH of a 10% (v/v) solution of NaOCl to 6.2 with 0.6 M H_2SO_4. Generated HOCl was mixed with 50 mM phosphate buffer (pH 6.8), catalase (7.2 μM) and tea samples various concentration (0–300 μg/ml final concentration) in 96 well-plate and the mixture was incubated at 25 °C for 20 min. The scavenging activity was measured by the decrease in absorbance of catalase at 404 nm. The assay was performed in triplicate.

3.2.5.4 Nitric oxide Scavenging Assay

The nitric oxide in this experiment was generated by RNS generator 3-Morpholinosydnonimine hydrochloride (SIN-1) and was quantified by Griess reaction assay. The test solution was prepared in 96-well plate by mixed 0.25 mM SIN-1 with PBS (pH7.4) and tea samples various doses (0–100 μg/ml final concentration) in a final volume of 40 μl. The test solutions were incubated at 25 °C for 30 min. Then 80 μl of Griess reagent (0.33% sulfanilamide in 20% glacial acetic acid) was added to the incubated solution and was suitably shaken for 5 min before adding 80 μl of 0.1% naphthyl ethylenediamine (NED), and the final solution was incubated at 25 °C for 15 min. The nitric oxide scavenging was measured spectrophotometrically at 540 nm against a blank sample. All tests were repeated for six times.

3.2.5.5 Peroxynitrite Scavenging Assay

Peroxynitrite has been generated by peroxynitrite donor SIN-1, and Evans blue bleaching assay has been used for measuring peroxynitrite scavenging activity. In brief, the assay was performed in a 96-well plate. The reaction mixture contained 50 mM phosphate buffer (pH7.4), 0.1 mM DTPA, 90 mM NaCl, 5 mM KCl, 12.5 µM Evans Blue, 1 mM SIN-1, 37.5 and 75 µg/ml of PIT and *C. sinensis* tea in a final volume of 200 µl. The mixture was incubated at 37 °C for 300 min, and the absorbance was measured at 611 nm every 30 min. The percentage scavenging of peroxynitrite at various times was calculated by comparing the absorbance of tea samples and blank. The assay was performed for six times.

3.2.6 LDL oxidation

3.2.6.1 LDL isolation by ultracentrifugation technique

The experiment was investigated at Liverpool John Moores University (LJMU) Liverpool, the United Kingdom and the protocol has been approved by the ethical committee of LJMU. Whole blood was obtained from the vein of healthy volunteers aged between 24 and 70 years who were normolipidemic, non-smoking, had not taken any medications and supplements within the last two weeks. The 9 parts of whole blood were added into 1 part of 3.8% (w/v) sodium citrate solution as an anticoagulant. Plasma was obtained by centrifugation at 1500 g for 20 min and was transferred to a separate plastic tube. LDL was isolated by density gradient ultracentrifugation using a method developed by Graham et al. (1996) with minor modification (Graham et al., 1996). Briefly, 0.5 ml of 60% (v/v) iodixanol (Optiprep ^{TM}) was mixed with 4.5 ml of plasma and was transferred to an 11.2 ml capacity Optiseal tube. This was over-layered with 5 ml of 12% v/v iodixanol prepared with

human plasma, and PBS was used to fill the rest of the tube. The tubes were ultracentrifuged in a Beckman L8-80 ultracentrifuge using a vertical rotor V65.1 at 350000 g for 3 h with slow acceleration and deceleration. Auto Densi-Flow gradient fractionator (Labconco, UK) has been used to separate the gradient by unloading the fraction from top to bottom of the tube. Each tube was fractionated into 0.5 ml of 20 tubes. All fractions were measured triglycerides, cholesterol, LDL and apoB100 components by using reagents and standards from Randox (Eire). Pooled LDL fraction was measured protein concentration by Bradford assay and was kept in -20 °C until used.

3.2.6.2 AAPH induce LDL Oxidation Assay

AAPH was used for reactive oxygen species (ROS) generating in this experiment. AAPH 20 mM was mixed with 50 µg/ml of LDL protein was used as a sample solution. The 50 µg/ml and 75 µg/ml of PIT and *C. sinensis* tea were added in the test samples. A control sample was 50 µg/ml of LDL protein added with PBS and AAPH-treated sample was 50 µg/ml of LDL cholesterol added with AAPH 20 mM. All samples were incubated at 37 °C and were collected and kept in -20 °C to stop the reaction before measuring plasma oxidation products using the thiobarbituric acid reacting substance (TBARS) formation in various times (0, 30, 60, 90, 120, 150, 180 min). All collected samples at various times determined TBARS concentration by added 20% TCA and spun down by centrifugation at 10,000 RPM for 10 min. The supernatant was collected and added with 1% thiobarbituric acid. The samples were heated at 95 °C for 20 min. TBARS concentration was determined by UV absorption at 532 nm, compared with an MDA standard curve, and results were expressed as MDA equivalence. The assay was measured in triplicate.

3.2.6.3 Copper induces LDL Oxidation Assay

Copper sulfate (Cu_2SO_4) has been used to investigate the inhibition of Copper (Cu^{2+})-mediated LDL oxidation. LDL cholesterol was challenged with Cu^{2+} by adding Cu_2SO_4 at a final concentration of 40 µM to 50 µg/ml of LDL protein in a volume of 1 ml. A control sample was 50 µg/ml of LDL protein added with PBS, and Cu^{2+}-treated sample was 50 µg/ml of LDL protein added with Cu_2SO_4 40 µM. The 15 µg/ml of PIT and *C. sinensis* tea was added in the test samples that contain Cu_2SO_4 40 µM mixed with LDL protein 50 µg/ml. All samples were incubated at 37 °C and were collected and kept in -20 °C to stop the reaction for measuring plasma oxidation products using the TBARS form at various times (0, 30, 60, 90, 120, 150, 180 min). Collected test samples at various times were determined TBARS concentration by TBARS assay as expressed as MDA equivalence. The assay was done in triplicate.

3.2.6.4 SIN-1 induce LDL Oxidation Assay

SIN-1 which is peroxynitrite donor has been used for investigating the SIN-1-mediated LDL oxidation. 1 mM of SIN-1 challenged with 50 µg/ml of LDL protein was used as SIN-1-treated sample and control sample was 50 µg/ml of LDL protein added with PBS. In test samples, 15 µg/ml of PIT and *C. sinensis* tea were mixed 50 µg/ml of LDL protein and 1mM of SIN-1. The samples were incubated at 37 °C for 18 h and were kept in -20 °C to stop the reaction before measuring plasma oxidation products by TBARS assay and the results were presented by MDA equivalence. The assay was repeated for three times.

3.2.7 Statistical Analysis

All the data were expressed as a mean ± standard deviation (SD). The statistical significances difference between treatment and control groups of cell

viability, the amount of lipid accumulation, biomolecular changes, lipase activity, blood toxicity testing, epididymal fat pads, relative organ weight, Peroxynitrite, Cu^{2+}, AAPH and SIN-1 scavenging assay were analysed by One-way analysis of variance (ANOVA) with a Turkey's HSD post-hoc test. The differences of serum lipid profile, food intake, and mouse weight analysis of pre- and post-treatment between groups were calculated by ANCOVA using the Tukey's HSD post-hoc test methods. The statistically significant differences between groups of DPPH, ABTS, HOCl and Nitric oxide scavenging assay were analyzed by independent t-test. Values were considered statistically significant when $p < 0.05$.

CHAPTER IV

RESULTS

4.1 Quantitative estimation of chemical constituency

4.1.1 Total Phenolic Content (TPC) of *P. indica* (L.) Less. tea

The total phenolic content was investigated by using the Folin–Ciocalteu colorimetric assay. Gallic acid was used as a standard of the phenolic compound. A standard calibration curve of gallic acid with an R^2 value of 0.9991 and standard equation y = 51.696x + 0.0624. The total phenolic content was calculated and showed as gallic acid equivalents (GAE)/g of dry weight. The results indicated that total phenolic of the PIT was 107.95 ± 4.87 mg GAE/g of dry weight.

4.1.2 Total Flavonoid Content (TFC) of *P. indica* (L.) Less. tea

Total flavonoid content was investigated by using the aluminium chloride colorimetric assay and expressed regarding catechin equivalent (CE)/g of dry weight. The total flavonoid content was calculated by a standard calibration curve of catechin with an R^2 value of 0.994 and the standard equation of y = 3.3315x + 0.0825. The results showed that the total flavonoid content of PIT was 95.33 ± 0.48 mg CE/g of dry weight.

4.1.3 Chemical Identification of *P. indica* (L.) Less. tea by LC-MS/MS

The negative ion mode was selected for ESI-MS analysis in this study, and the Multiple Reaction Monitoring (MRM) mode has been used for identification. Two pairs of MRM transition were selected at *m/z* 353.1→191.0 and 515→353. MRM chromatograms of PIT and standards (4-CQ, 5-CQ, 3,4-CQ, 3,5-CQ, and 4,5-CQ) of MRM transition at *m/z* 353→191.0 are shown in Figure 4.1a. The result showed that the PIT had been detected 4-CQ and 5-CQ in the extract. Figure 4.1b revealed the chromatograms of PIT and standards of MRM transition at *m/z* 515→353 which explained that 3,4-CQ, 3,5-CQ, and 4,5-CQ were the main ingredient of PIT.

The quantification of the analyses was performed. We focused on the highest peak of chromatogram that was 3,5-CQ, and the resulted from MRM data acquisitions showed that PIT concentration 1500 µg/ml composed of 3,5-CQ 169.93 µg/ml.

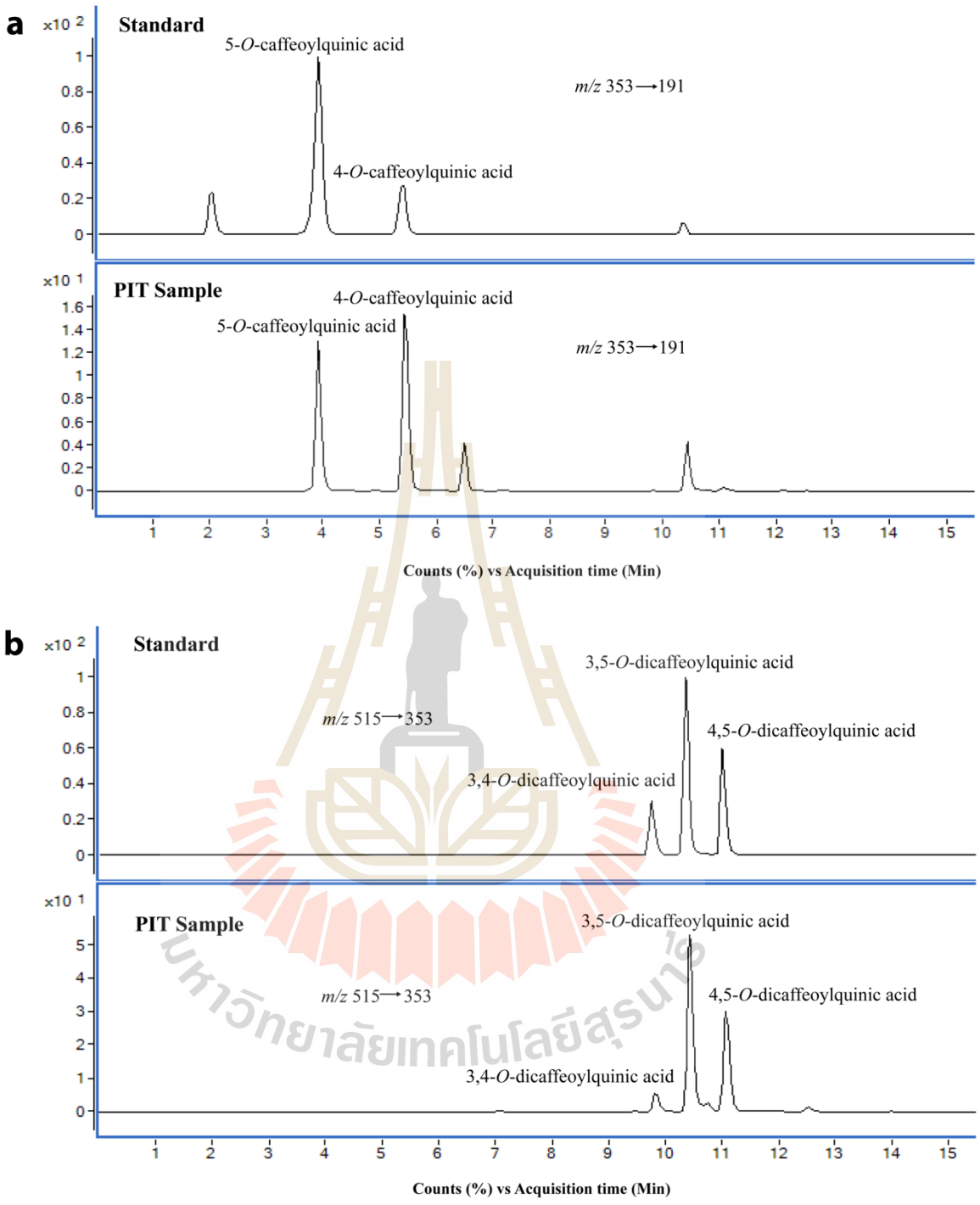


Figure 4.1 MRM chromatograms of PIT and standards (4-CQ, 5-CQ, 3,4-CQ, 3,5-CQ and 4,5-CQ) of MRM transition at m/z 353→191.0 (A) and at m/z 515→353 (B).

4.2 The effects *P. indica* (L.) Less. tea of oxidative stress assays

4.2.1 DPPH Scavenging Assay

The activities of *P. indica* (L.) Less. tea compared with *C. sinensis* tea were investigated. DPPH radical scavenging was calculated from the decolorization of DPPH which was measured spectrophotometrically at 515 nm. The results showed that *P. indica* (L.) Less. tea initiated significantly stronger antioxidant capacity of 75 µg/ml compared to *C. sinensis* tea ($p < 0.05$) (Figure 4.2) and still continuously stronger when the dose was increased up to 300 µg/ml. At concentration 300 µg/ml which was the highest concentration of both teas, *P. indica* (L.) Less. tea displayed 51.19 ± 4.02% DPPH scavenging while *C. sinensis* tea exhibited 41.46 ± 3.83% DPPH scavenging. The IC_{50} value of *P. indica* (L.) Less. tea was less than *C. sinensis* tea, which was 245.85 ± 15.83 µg/ml and 315.41 ± 24.18 µg/ml, respectively.

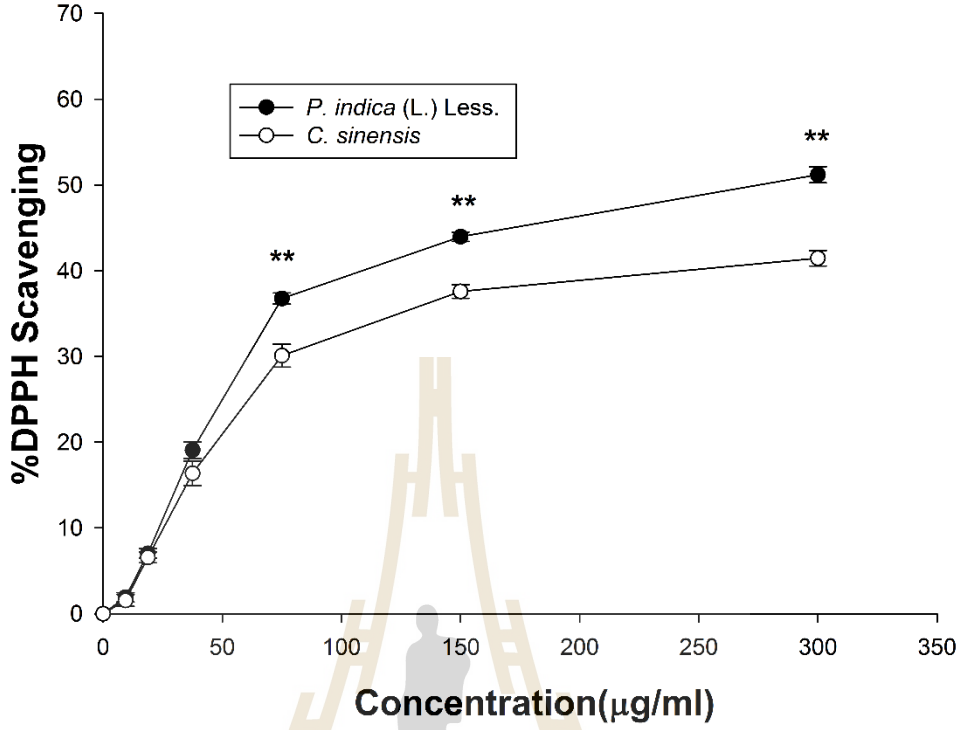


Figure 4.2 The DPPH radical scavenging activity of *P. indica* (L.) Less. tea and *C. sinensis* tea in various concentrations. The data represent the percentage of DPPH inhibition. Each value represents mean ± S.D. (n = 6). ** indicates a statistical difference between the group at $p < 0.01$.

4.2.2 ABTS Scavenging Assay

The ABTS scavenging assay was used to investigate the abilities of the extract to inhibit $ABTS^+$ radical. The extracts interacted with $ABTS^+$ which decrease the absorbance of the $ABTS^+$ radical. The absorbance was measured spectrophotometrically at 734 nm. Figure 4.3 shows the %ABTS radical scavenging of *P. indica* (L.) Less. tea and *C. sinensis* tea. The results displayed that *C. sinensis* tea. significantly inhibited $ABTS^+$ radical stronger than *P. indica* (L.) Less. tea in all tested concentrations ($p < 0.05$). The IC_{50} of *P. indica* (L.) Less. tea and *C. sinensis* tea were 30.47 ± 2.20 and 21.59 ± 0.67 µg/ml, respectively.

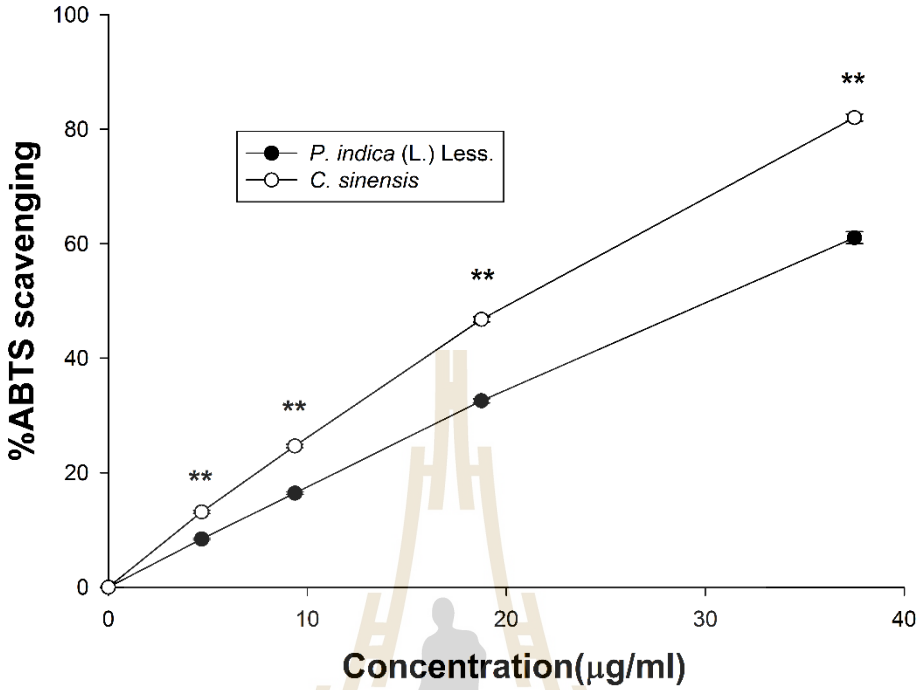


Figure 4.3 The ABTS radical scavenging activity of *P. indica* (L.) Less. tea and *C. sinensis* tea in various concentrations. The data represent the percentage of ABTS inhibition. Each value represents mean ± S.D. (n = 6). ** indicates a statistical difference between the group at $p < 0.01$.

4.2.3 Hypochlorous acid Scavenging Assay

The hypochlorous acid scavenging activity of *P. indica* (L.) Less. tea and *C. sinensis* tea are displayed in Figure 4.4. The results showed that *P. indica* (L.) Less. tea at 18.75 µg/ml exhibited a significantly higher scavenging effect than *C. sinensis* tea ($p < 0.05$). Nevertheless, the other test concentrations of *P. indica* (L.) Less. tea demonstrated some higher scavenging effect than *C. sinensis* tea, but not a significant difference.

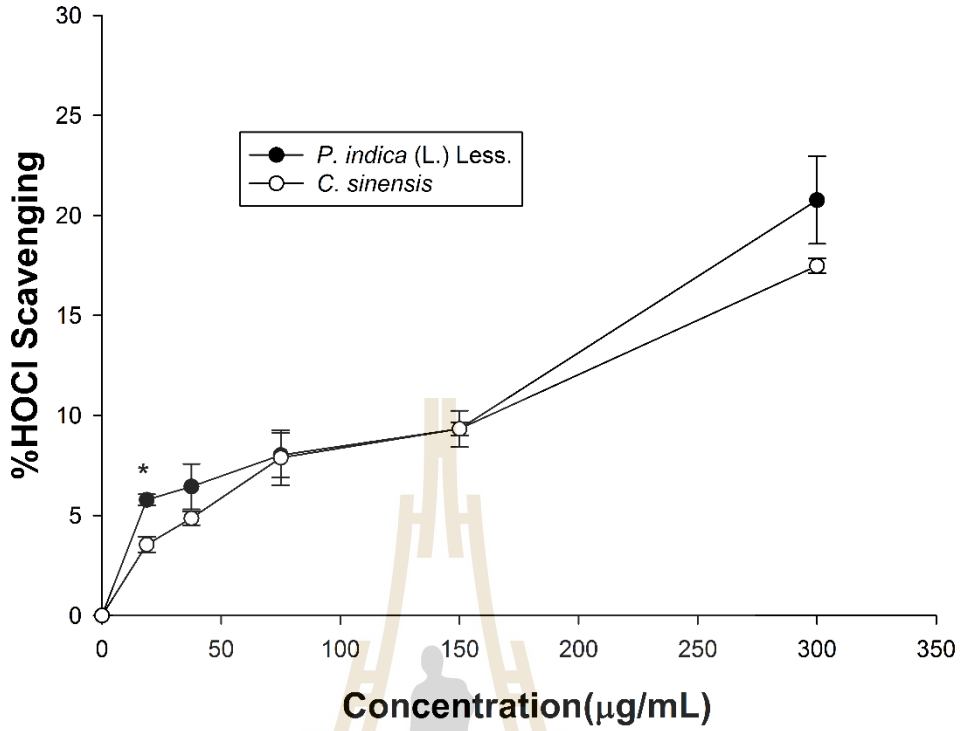


Figure 4.4 The hypochlorous acid radical scavenging activity of *P. indica* (L.) Less. tea and *C. sinensis* tea in various concentrations. The data represent the percentage of HOCl inhibition. Each value represents the mean ± S.D. (n = 3). * indicates a statistical difference between the group at $p < 0.05$.

4.2.4 Nitric oxide Scavenging Assay

Griess reaction method was used to detect nitric oxide radical in this experiment, and the scavenging effect was also measured by the decolorization of the sample. Figure 4.5 expresses the % inhibition of nitric oxide of *P. indica* (L.) Less. tea and *C. sinensis* tea. The results showed that *P. indica* (L.) Less. tea indicated significantly higher % nitric oxide scavenging activities than *C. sinensis* tea start from 20 µg/ml of testing agents ($p < 0.01$). The IC_{50} of *P. indica* (L.) Less. tea and *C. sinensis* tea were 116.48 ± 5.08 µg/ml and 178.42 ± 15.52 µg/ml, respectively.

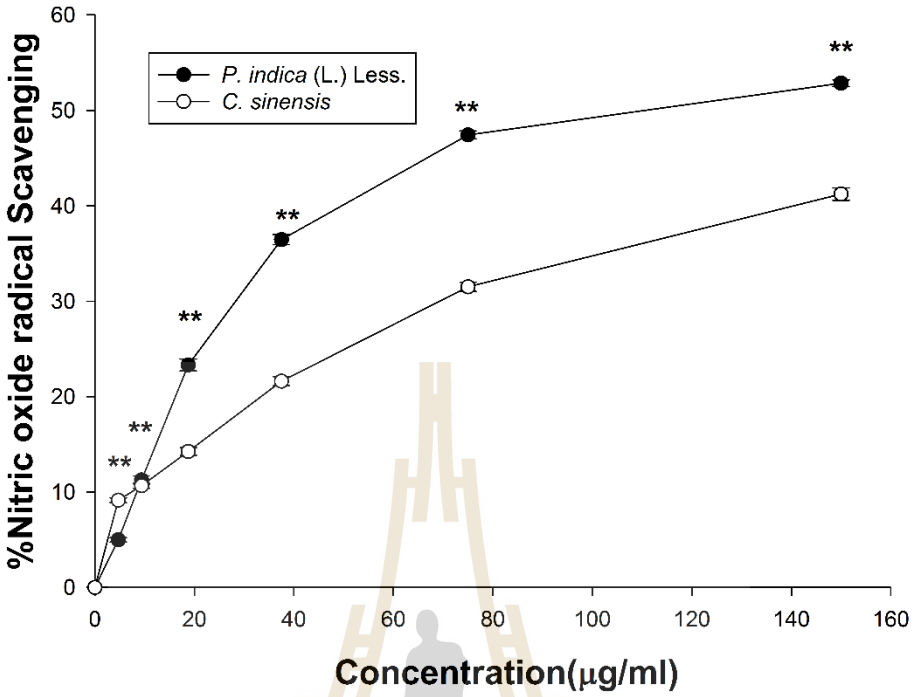


Figure 4.5 The nitric oxide radical scavenging activity of *P. indica* (L.) Less. tea and *C. sinensis* tea in various concentrations. The data represent the percentage of nitric oxide inhibition. Each value represents mean ± S.D. (n = 6). ** indicates a statistical difference between the group at $p < 0.01$.

4.2.5 Peroxynitrite Scavenging Assay

The peroxynitrite scavenging effect of the teas at various times was measured by Evan's blue assay. The results are shown in Figure 4.6. High concentration of *P. indica* (L.) Less. tea (75 µg/ml) almost fully prevented the peroxynitrite oxidation over 5 h. Whereas at 37.5 µg/ml, the %peroxynitrite scavenging was continuously reduced to 64.50 ± 8.07 at 2 h and gradual rise until 5 h. The peroxynitrite scavenging activity of *C. sinensis* tea at high concentration (75 µg/ml) was significantly lower peroxynitrite scavenging activity than *P. indica* (L.)

Less. tea at the same concentration ($p < 0.05$) and continuously reduced to 51.55 ± 2.15% at 3h and stable until 5 h. Besides, *C. sinensis* tea at 37.5 µg/ml continuously reduced the peroxynitrite scavenging to 38.73 ± 3.12% at 2 h and maintain this effect until the end of the experiment.

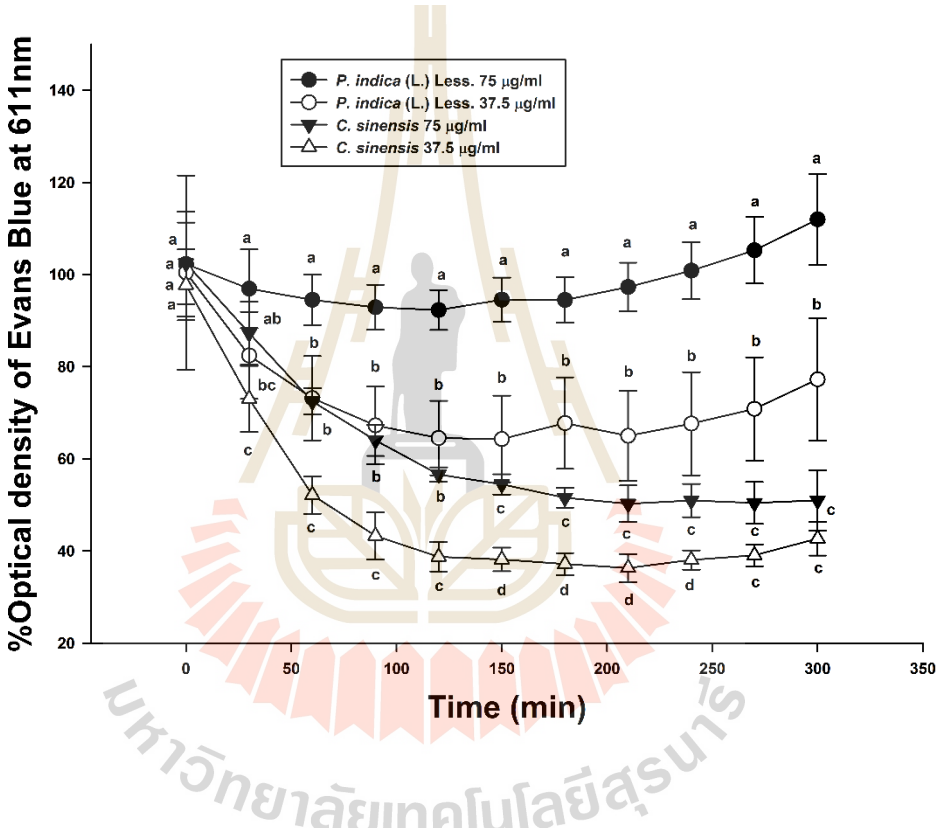


Figure 4.6 The peroxynitrite radical scavenging activity of *P. indica* (L.) Less. tea and *C. sinensis* tea at various times at the doses of 37.5 and 75 µg/ml. The data represent the percentage of peroxynitrite inhibition. Means ± SD is illustrated for six replicates. Means with the same superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

4.2.6 Copper induces LDL Oxidation Assay

Isolated human LDL was challenged by Cu^{2+} and various teas in each test sample at 15 µg/ml. The result was presented by MDA equivalence (nmol/mg Protein), which calculated from the MDA calibration standard curve. Figure 4.7 showed that Cu^{2+}-treated group continuously increased the MDA equivalence to 222.73 ± 2.22 nmol/mg Protein at 210 min. Interestingly, *P. indica* (L.) tea and *C. sinensis* tea treated groups could almost entirely prevent Cu^{2+} from oxidized human LDL.

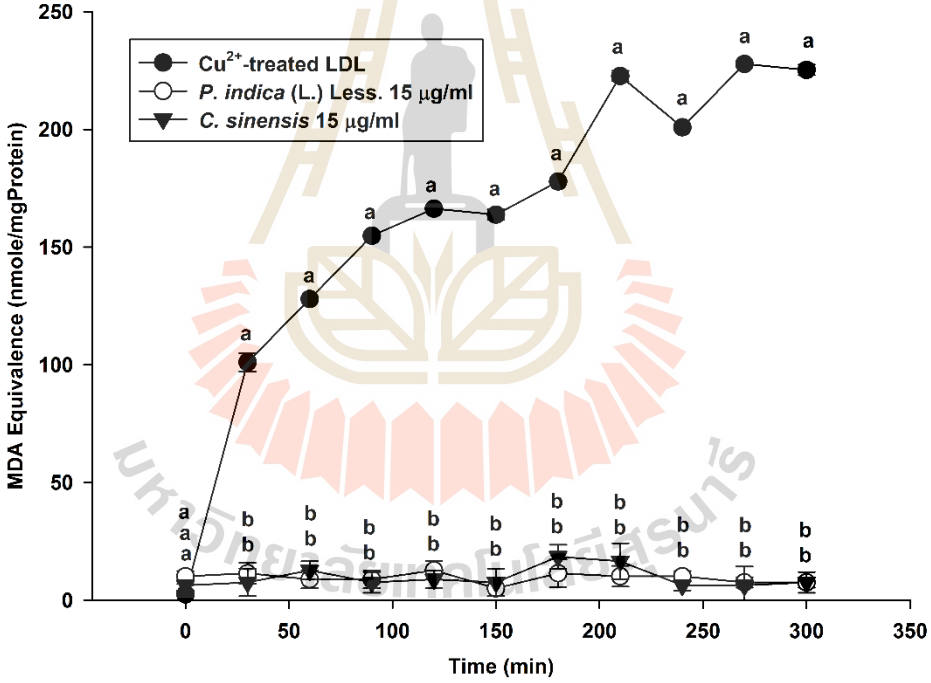


Figure 4.7 The copper induces LDL oxidation scavenging activity of *P. indica* (L.) Less. tea and *C. sinensis* tea at 15 µg/ml at various times. The data represent the MDA equivalence. Means ± SD is illustrated for three replicates. Means with the same

superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

4.2.7 AAPH induce LDL Oxidation Assay

The reactive oxygen species induce LDL oxidation scavenging effect has been investigated by measuring TBARS after challenged isolated human LDL with AAPH, which is reactive oxygen species. *P.indica* (L.) Less. tea and *C. sinensis* tea at 50 and 75 µg/ml were used for the experiment. The samples were incubated for 3h and were kept in -80 °C at various times to stop the reaction before measuring TBARS. The result showed that the anti-oxidative effects of both teas were significantly lower MDA equivalence initiating from 60 min until the end of the experiment compared to AAPH-treated group ($p < 0.05$) (Figure 4.8). Furthermore, a higher concentration of both teas showed stronger LDL oxidation inhibition than lower concentrations. A lag time of the oxidation was observed, the increasing of TBARS in every concentration of both teas were increased lag-time from 40 min to 70 min approximately.

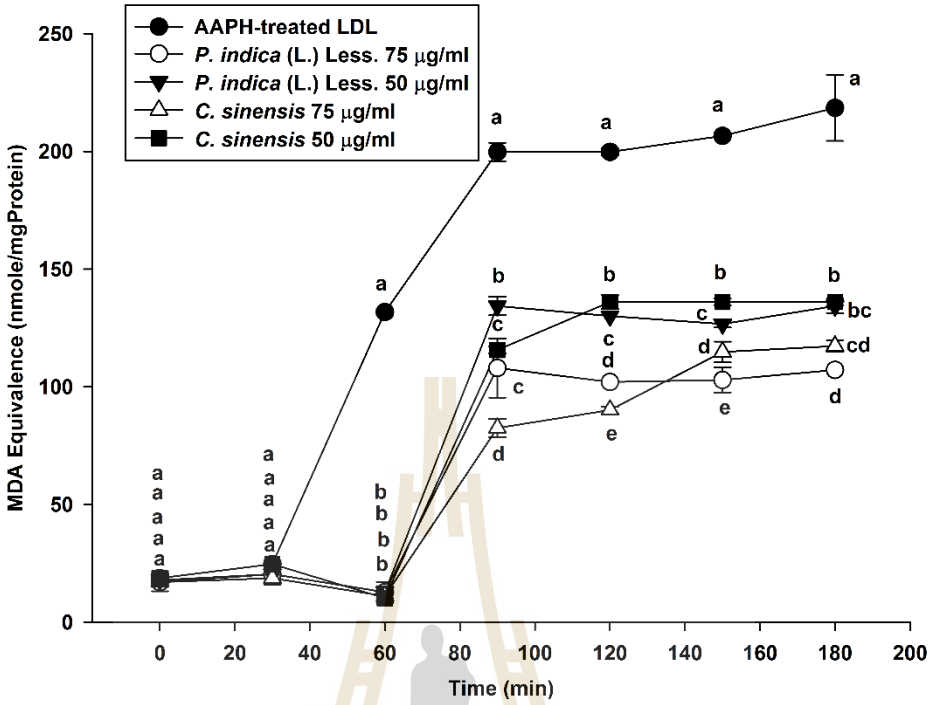


Figure 4.8 The AAPH induce LDL oxidation scavenging activity of *P. indica* (L.) Less. tea and *C. sinensis* tea at 50 and 75 µg/ml at various times. The data represent the MDA equivalence. Means ± SD is illustrated for three replicates. Means with the same superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

4.2.8 SIN-1 induce LDL Oxidation Assay

SIN-1 which is reactive nitrogen species generator was used for investigating the scavenging effect of RNS induced LDL oxidation. After incubating, isolated human LDL with SIN-1 and tea extracts were added. The result showed that the RNS scavenging effect of both *P.indica* (L.) Less. tea and *C. sinensis* tea at 15 µg/ml were significantly lower MDA equivalence compared to SIN-1 group ($p < 0.05$) (Figure 4.9).

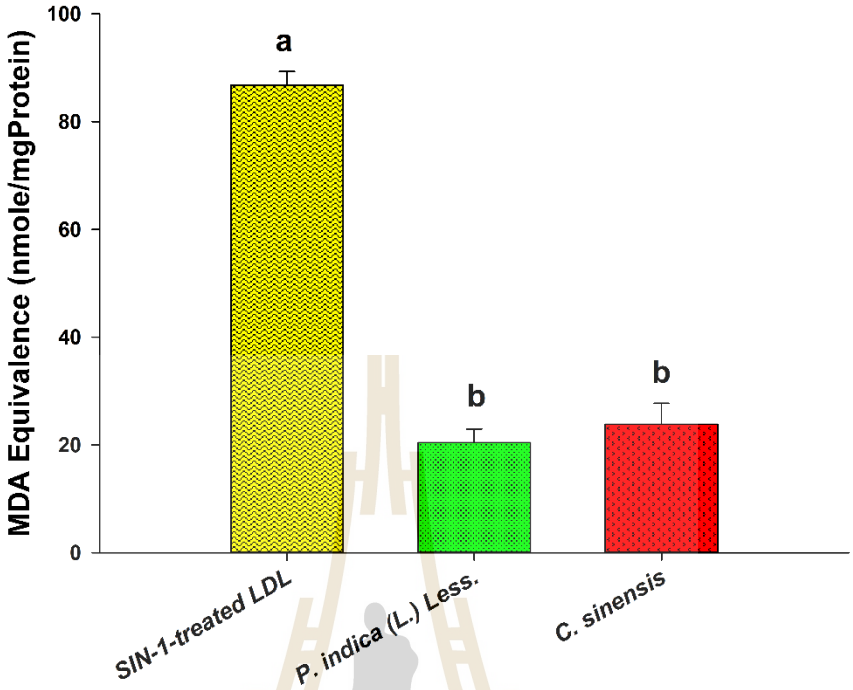


Figure 4.9 The SIN-1 induce LDL oxidation scavenging activity of *P. indica* (L.) Less. tea and *C. sinensis* tea at 15 µg/ml. The data represent the MDA equivalence. Means ± SD is illustrated for three replicates. Means with the same superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

4.3 3T3-L1 preadipocytes

4.3.1 *In vitro* 3T3-L1 cytotoxic test (MTT Assay)

Concentrations of the PIT from 250 – 1000 µg/ml did not significantly affect the viability of 3T3-L1 preadipocytes viability compared to control untreated cells (Figure 4.10), as assessed by the MTT assay ($p > 0.05$). In all subsequent experiments, doses of 1000 µg/ml or less were used.

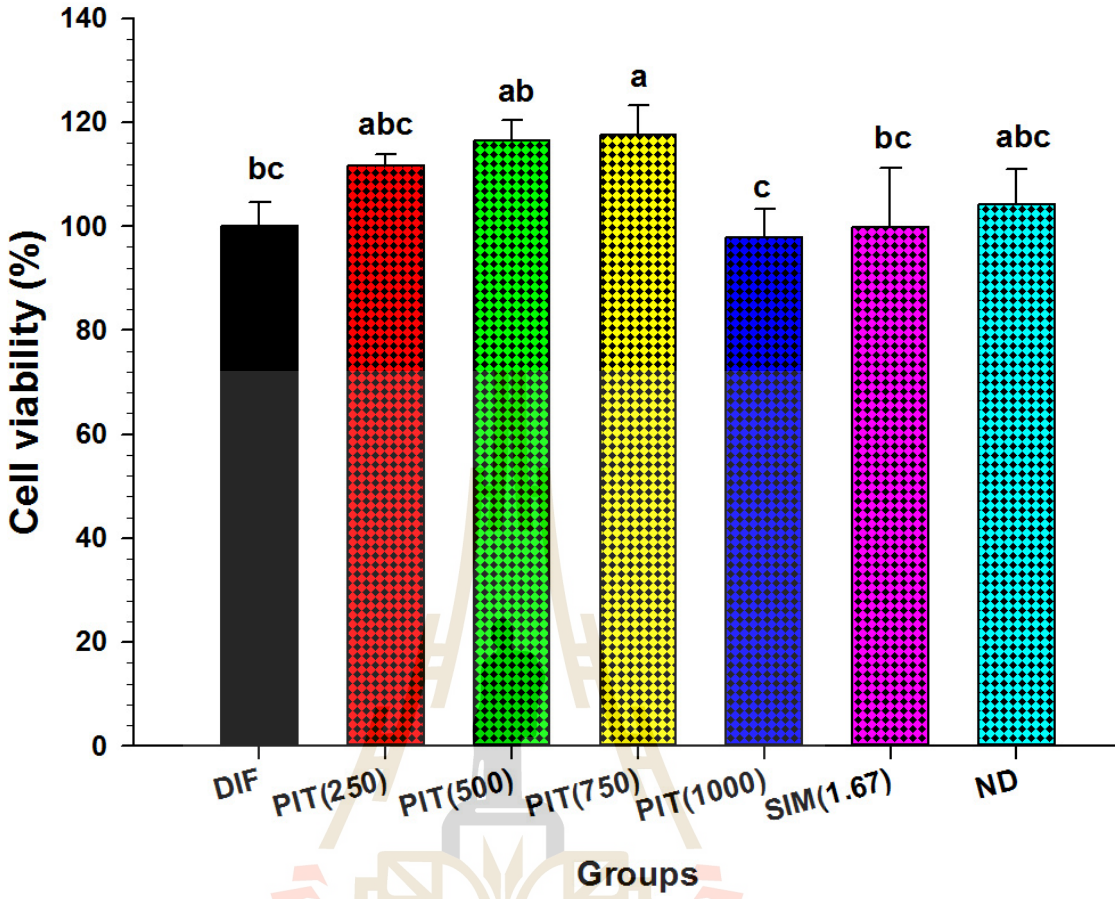


Figure 4.10 The effect of PIT on the viability of 3T3-L1 preadipocytes. DIF = Differentiate 3T3-L1 adipocytes; PIT(250) = PIT at 250 µg/ml treated-3T3 adipocytes; SIM(1.67) = Simvastatin at 1.67 µg/ml treated-3T3 adipocytes; ND = 3T3-L1 preadipocytes (non-differentiated cells). Means ± SD is illustrated for three replicates. Means with the same superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

4.3.2 Effect of the PIT on 3T3-L1 preadipocyte differentiation and lipid accumulation

Cells were treated with the PIT at various concentrations (250, 500, 750, and 1000 µg/ml) during the differentiation of 3T3-L1 preadipocytes to adipocytes. After treated, the intracellular lipid level was quantified using an Oil Red O staining method. The 3T3-L1 preadipocytes exposure to differentiation medium resulted in a significant increase of lipid accumulation in comparison to untreated-adipocytes (DIF) ($p < 0.05$) (Figure 4.11). Microscopic observation of Oil Red O and hematoxylin-stained cells exhibited that PIT decreased Oil Red O stained droplets of mature adipocytes in a dose-dependent manner (Figure 4.12). The intracellular lipid accumulation showed that PIT at 750 and 1000 µg/ml significantly decreased the intracellular lipid accumulation to 76.87 ± 3.99% and 71.93 ± 2.05% respectively, compared to untreated 3T3-L1 adipocytes (DIF) ($p < 0.05$). The 33% and 50% inhibitory effect (IC_{33} and IC_{50}) of the PIT on lipid accumulation were determined to be 1085.5 ± 129.40 and 1841.07 ± 272.60 µg/ml, respectively. In addition, simvastatin at 1.67 µg/ml exhibited a 33% lipid accumulation reduction (IC_{33}). The effect of simvastatin is therefore 650 times more effective than a PIT. Accordingly, the PIT concentration at 750 µg/ml was chosen to study FTIR microspectroscopy.

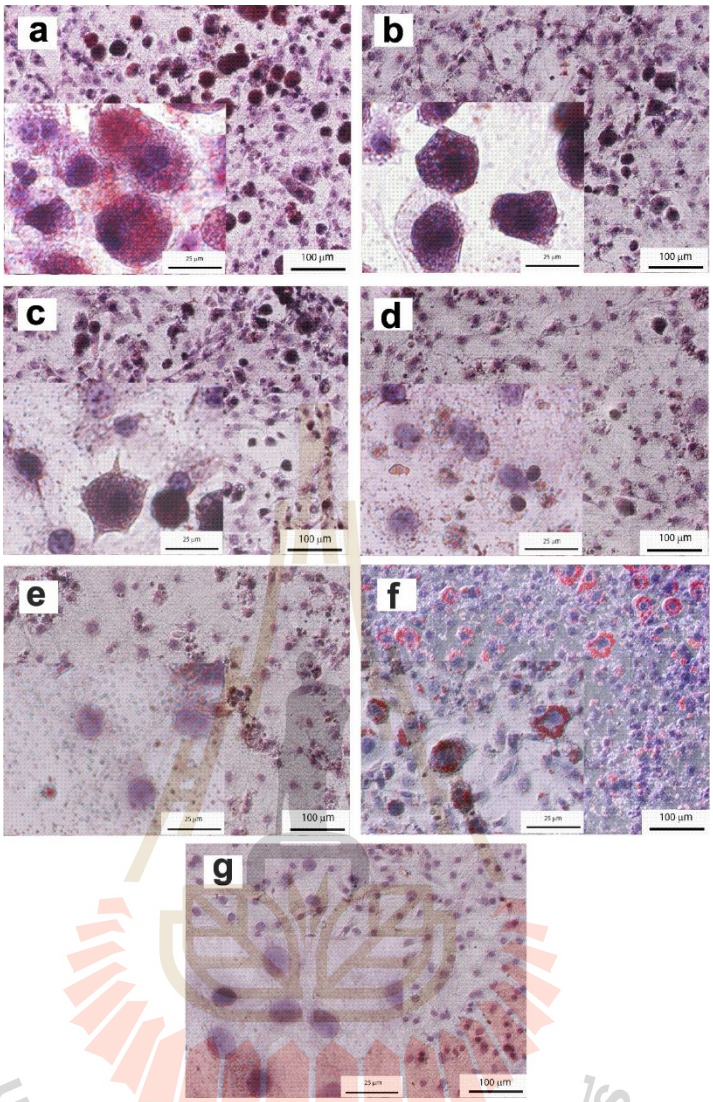


Figure 4.11 Microscopic imaging of intracellular lipid after Oil Red O and hematoxylin staining of samples. (**a**) = Differentiate 3T3-L1 adipocytes (untreated adipocytes); (**b**) = PIT at 250 µg/ml treated adipocytes; (**c**) = PIT at 500 µg/ml treated adipocytes; (**d**) = PIT at 750 µg/ml treated adipocytes; (**e**) = PIT at 1000 µg/ml treated adipocytes; (**f**) = Simvastatin at 1.67 µg/ml treated adipocytes; (**g**) = 3T3-L1 preadipocytes (non-differentiated cells) (original magnification at x100, scale bar; 100 µm and inset view at x600, scale bar; 25 µm).

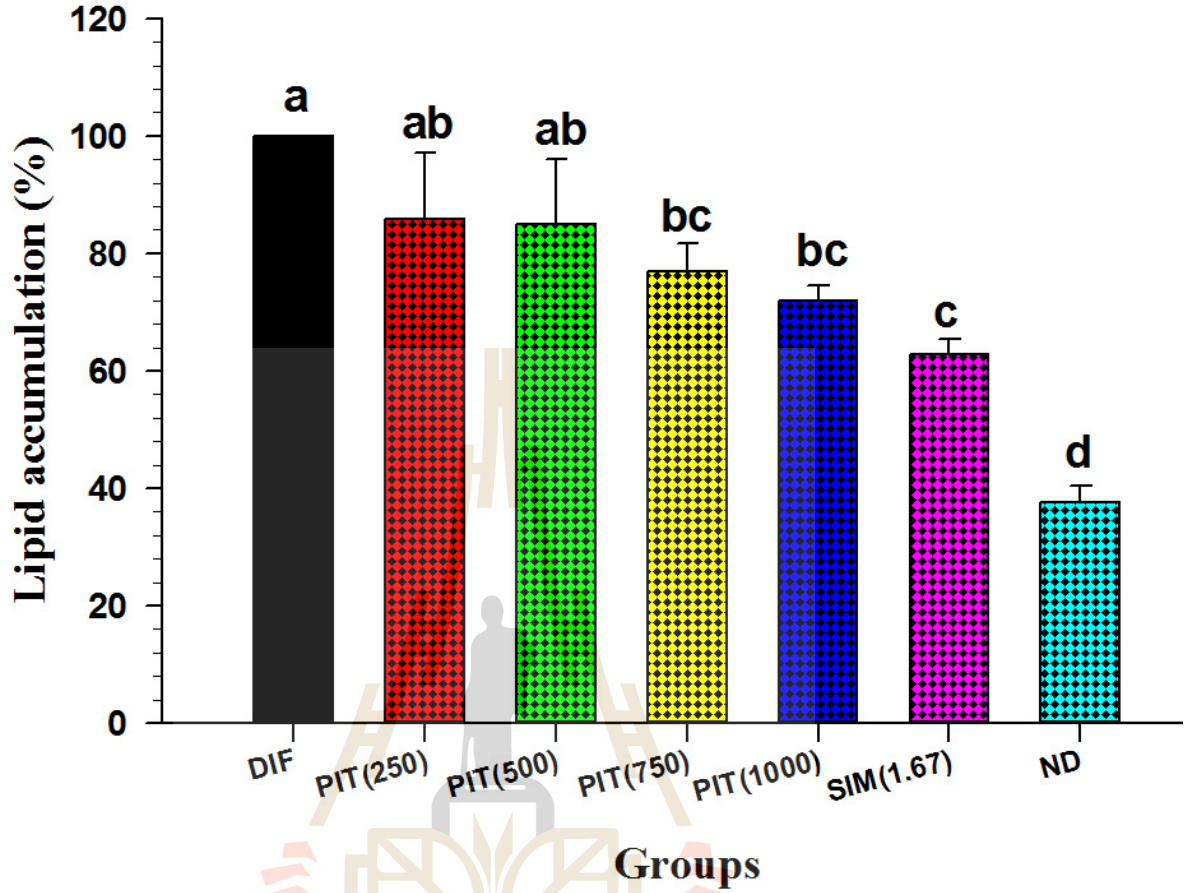


Figure 4.12 Graphically represents the effect of the PIT on the percentage of intracellular lipid in 3T3-L1 differentiated cells after Oil Red O staining. DIF = Differentiate 3T3-L1 adipocytes (untreated adipocytes); PIT(250) = PIT at 250 µg/ml treated adipocytes; SIM(1.67) = Simvastatin at 1.67 µg/ml treated adipocytes; ND = 3T3-L1 preadipocytes (non-differentiated cells). Means ± SD is illustrated for three replicates. Means with the same superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

4.3.3 Biomolecule changing detected by FTIR microspectroscopy

FTIR microspectroscopy was used to determine the biochemical composition of pre-adipocytes (ND), untreated adipocytes (DIF), Simvastatin- and PIT-treated adipocytes. The FTIR absorption spectrum of the sample at wavelengths between 4000-950 cm^{-1} is shown in Figure 4.13. The three distinct areas of the lipid region (3000-2800 cm^{-1}), the protein regions (1700-1500 cm^{-1}), carbohydrate and nucleic acid regions (1300-950 cm^{-1}) were investigated. Hence, the spectrum was very difficult to analyze due to the different spectral between the groups. In order to overcome this problem second derivative of the spectral range at 3000-2800 cm^{-1} and 1800-950 cm^{-1} were analyzed (Figure 4.14a and 4.14b).

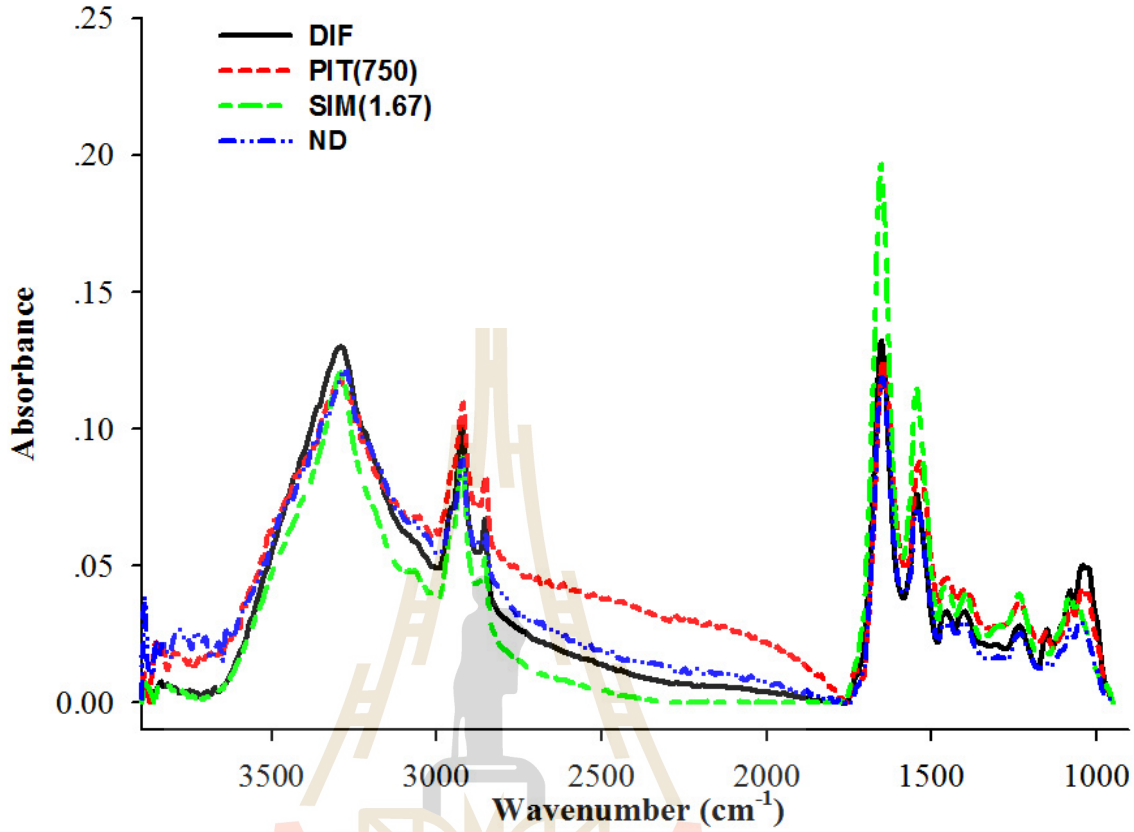


Figure 4.13 Average original FTIR spectra (4000–950 cm^{-1}) obtained from 3T3-L1 cells. DIF = Differentiate 3T3-L1 adipocytes (untreated adipocytes); PIT(750) = PIT at 750 µg/ml treated adipocytes; SIM(1.67) = Simvastatin at 1.67 µg/ml treated adipocytes; ND = 3T3-L1 preadipocytes (non-differentiated cells).

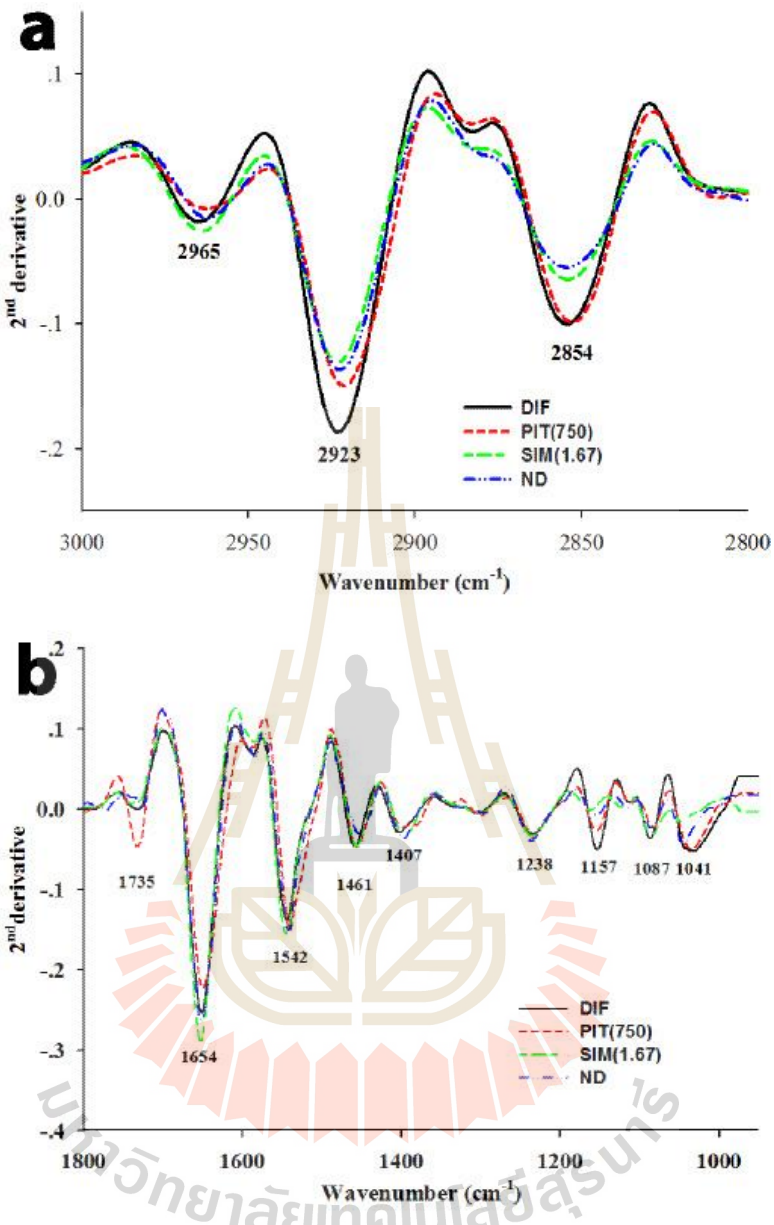


Figure 4.14 Average the secondary derivative spectra of 3T3-L1 cells. DIF = Differentiate 3T3-L1 adipocytes (untreated adipocytes); PIT(750) = PIT at 750 µg/ml treated adipocytes; SIM(1.67) = Simvastatin at 1.67 µg/ml treated adipocytes; ND = 3T3-L1 preadipocytes (non-differentiated cells). The data were represented in two regions: **(a)** lipid regions (3000-2800 cm^{-1}) and **(b)** protein, nucleic acid, glycogen and other carbohydrate regions (1800-950 cm^{-1}).

The strong peak at 2923 cm^{-1} and 2854 cm^{-1} correspond to the CH_2 asymmetric and symmetric stretching frequency (mainly lipids, with the little from proteins, carbohydrates, nucleic acids), respectively (Aksoy and Severcan, 2012). The decrease in signal intensity and area of the peaks (at 2954 cm^{-1} and 2923 cm^{-1}), which reflected an absorption peak of lipids of the PIT-treated adipocytes, presented less than untreated adipocytes (Figure 4.15a). Then, we calculated the ratio of the integrated area of several functional groups, including CH_2 (2938-2906 cm^{-1}, centered at 2923 cm^{-1})/CH_3 (2973-2954 cm^{-1}, centered at 2965 cm^{-1}) asymmetric stretching that belongs to lipids. The results showed that the ratio of the integrated area of the lipids region in the PIT- and Simvastatin-treated adipocytes displayed significantly less than the untreated adipocytes group ($p < 0.05$) (Figure 4.15b). The major contributor in the spectrum ranges from 1700-1500 cm^{-1}, which are attributed to an absorption peak of proteins amide I and II. The ratio of integrated area of several functional groups, including CH_2 asymmetric stretching (2938-2906 cm^{-1}, centered at 2923 cm^{-1})/amide I (1674-1624 cm^{-1}, centered at 1654 cm^{-1}) that belong to proteins of the PIT- and simvastatin-treated adipocytes displayed significantly less integral area ratio than the untreated adipocytes group ($p < 0.05$) (Figure 4.15b).

The signal intensity and area of the peaks of the PIT- and simvastatin-treated adipocytes at 1157 cm^{-1} and 1041 cm^{-1} which are attributed to an absorption peak of C–O vibrations from glycogen and other carbohydrates (Cao et al., 2013) undoubtedly exhibited significantly less than untreated 3T3-L1 adipocytes ($p < 0.05$) (Figure 4.15a). The functional group of PO_2 stretching mode from mainly nucleic acids at 1238 cm^{-1} and 1087 cm^{-1} regions of the PIT-treated adipocytes displayed signal intensity and area less than the untreated adipocytes and demonstrated significantly

less integrated area than that of the untreated adipocytes group ($p < 0.05$) (Figure 4.15a) (Garip et al., 2009).

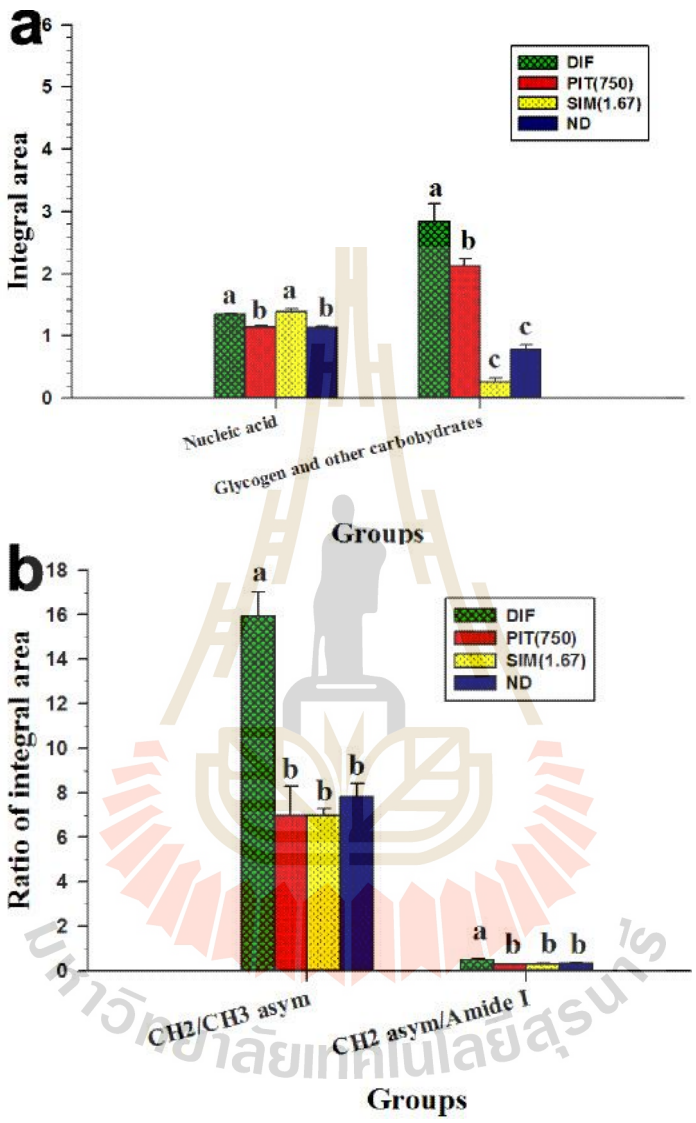


Figure 4.15 The histogram of integrated areas of 3T3-L1 cells. DIF = Differentiated 3T3-L1 adipocytes (untreated adipocytes); PIT(750) = PIT at 750 µg/ml treated adipocytes; SIM(1.67) = Simvastatin at 1.67 µg/ml treated adipocytes; ND = 3T3-L1 preadipocytes (non-differentiated cells). **(a)** The integral area of nucleic acids, glycogen, and other carbohydrates. **(b)** The ratio of the integral area of lipids (CH_2/CH_3 asymmetric stretching) and proteins (CH_2 asymmetric stretching/Amide I).

Means ± SD is illustrated for three replicates. Means with the same superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

We further investigated the second derivative spectra using the principal component analysis (PCA). The PCA score plot showed that PIT (750) and DIF cluster were isolated from SIM (1.67), and ND cluster with PC-1 (42%) and the cluster of DIF group was separated from PIT (750), SIM (1.67) and the ND group with PC-2 (14%) (Figure 4.16a). The PCA loading plot was used to detect the wavelength of the spectrum that discriminated the clustering (Figure 4.16b). PC-1 is discriminated by negative loading in the C-H stretching region (centered at 2919 cm^{-1} and 2854 cm^{-1}), negative loading of C-O vibrations from glycogen and other carbohydrates at 1153 cm^{-1}, positive loading of C-O vibrations from glycogen and other carbohydrates at 1064 cm^{-1}, protein amide I and amide II (positive loading centred at 1658 cm^{-1} and 1548 cm^{-1}, respectively) and negative loading from C=O stretching vibrations of lipids ester (centered at 1734 cm^{-1}). These results suggest that DIF and PIT (750) groups possess higher lipids, glycogen and other carbohydrates, and proteins than SIM (1.67) and ND groups.

The discrimination along PC-2 that expressed the separation between DIF group and other groups which demonstrated positive loading in C-H stretching region (centred at 2911 cm^{-1} and 2846 cm^{-1}), negative loading of C-O vibrations from glycogen and other carbohydrates at 1153 cm^{-1} and 1022 cm^{-1}, positive loading of C-O vibrations from glycogen and other carbohydrates at 1064 cm^{-1}, protein amide I (positive loading centered at 1627 cm^{-1}), PO_2-symmetric stretching vibrations of nucleic acid (negative loading centered at 1083 cm^{-1}), and positive loading from C=O

stretching vibrations of lipids ester (centered at 1728 cm^{-1}). These results seem consistent with PC-1 that DIF group expresses higher lipids, glycogen and other carbohydrates, proteins, and nucleic acid than other groups.

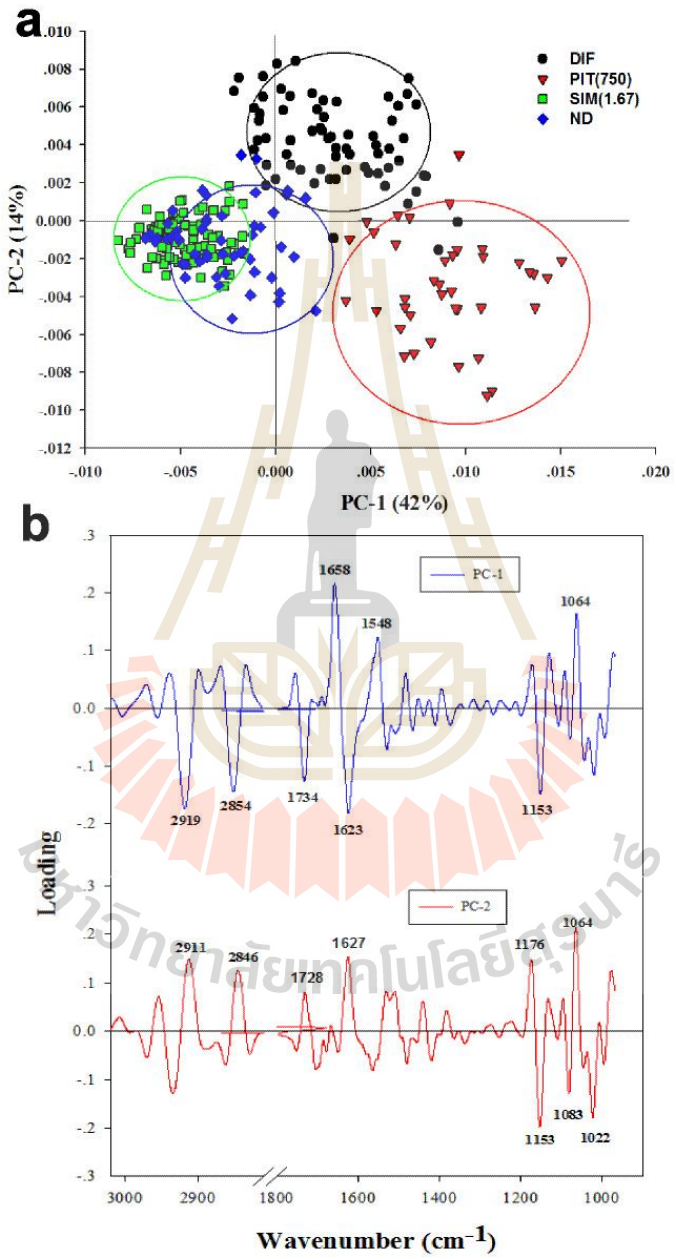


Figure 4.16 PCA analysis of FTIR spectral ranges 3000-2800 cm^{-1} and 1800-950 cm^{-1} giving PCA score plot (**a**) and PCA loading plot (**b**). The 2D PCA score plots showed the clustering separation spectra between groups. DIF = Differentiated 3T3-

L1 adipocytes (untreated adipocytes); PIT(750) = PIT at 750 µg/ml treated adipocytes; SIM(1.67) = Simvastatin at 1.67 µg/ml treated adipocytes; ND = 3T3-L1 preadipocytes (non-differentiated cells) at day 10 after differentiation. The biomarker differences over a spectral range of samples are identified by PC1 and PC2 loading plots.

4.4 Effect of the PIT on Pancreatic Lipase Activity

Pancreatic lipase is an enzyme responsible for the hydrolysis of lipid into free fatty acid and glycerol. The PIT concentrations between 250 to 1000 µg/ml showed significantly higher inhibitory lipase activity than those of the controls ($p < 0.05$) (Figure 4.17). Furthermore, the IC_{50} of the PIT for the inhibition of pancreatic lipase was 1708.35 ± 335.85 µg/ml. While the inhibitory effect of the positive control orlistat at 6.25 to 50 µg/ml, demonstrated an IC_{50} at 68.23 ± 6.67 µg/ml. Under those circumstances, the potential strength of the PIT on lipase activity inhibition is approximately 25 times lower than orlistat. These results suggest that the inhibitory implications of the PIT on pancreatic lipase activity increased in a dose-dependent manner.

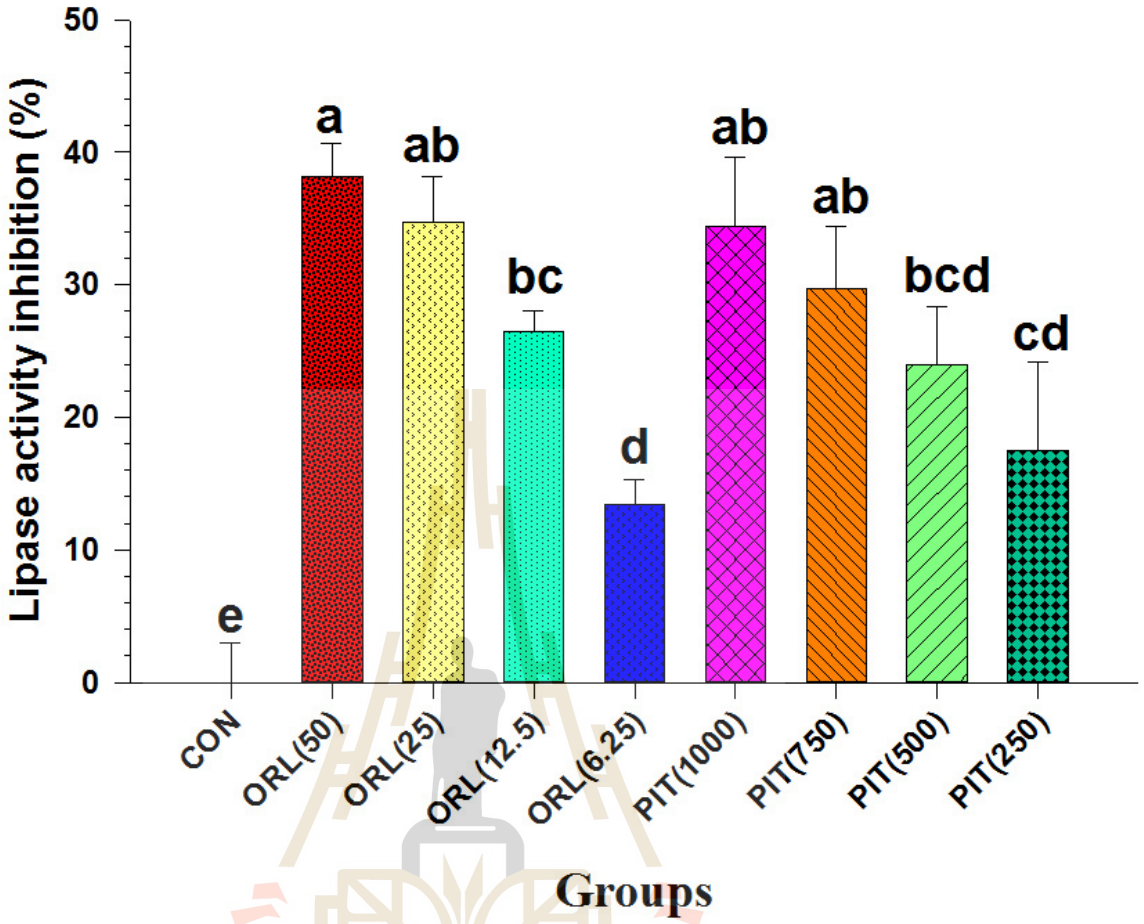


Figure 4.17 Inhibitory effects of PIT (%) at various concentrations on lipase activity. CON = Control; ORL(12.5) = Orlistat at 12.5 µg/ml; PIT(250) = PIT at 250 µg/ml. Orlistat was used as a positive control. Means ± SD is illustrated for three replicates. Means with the same superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

4.5 Effects of *P. indica* (L.) Less. tea on mice

4.5.1 The effect of *P. indica* (L.) Less. tea on oral glucose tolerance test (OGTT)

The effect on oral glucose tolerance test of *P. indica* (L.) Less. tea was evaluated in normal mice. *P. indica* (L.) Less. tea at 400 and 600 mg/kg were compared with Glibenclamide 10 mg/kg, which used as a positive control. The blood samples were measured for glucose content at 0, 15, 30, 60 and 120 min after receiving dextrose, respectively. Figure 4.18 displayed that PIL, PIH and GLI groups exhibited significantly lower blood glucose level than the untreated group initiated since 15, 30, and 60 min after oral administration of the extracts ($p < 0.05$). At 30 min after oral administration of the extracts, the high dose of *P. indica* (L.) Less. tea (PIH) expressed significantly lower blood glucose level compared with GLI and CON ($p < 0.05$). At 120 min after oral administration of the extracts, a blood sugar of all groups was reduced, and blood sugar of PIL showed no significant difference with CON whereas PIH and GLI were significantly lower than CON and PIL ($p < 0.05$).

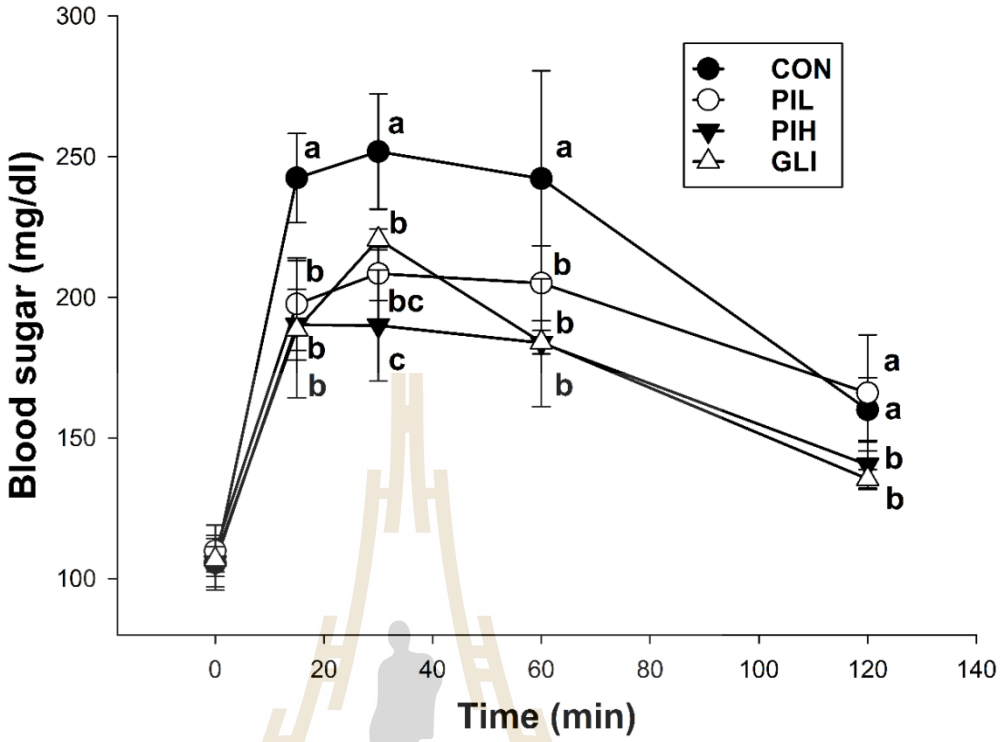


Figure 4.18 Effect of PIT on the oral glucose tolerance test. CON = Control; PIL = *P. indica* (L.) Less. tea at 400 mg/kg; PIH = *P. indica* (L.) Less. tea at 600 mg/kg; GLI = Glibenclamide at 10 mg/kg. Data are expressed as means ± SD (n = 10). Means with the same superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

4.5.2 Effect of *P. indica* (L.) Less. tea on serum lipid profile

The effect of *P. indica* (L.) Less. tea on serum cholesterol, LDL, HDL, and Triglyceride have been investigated. PIL and PIH groups were fed with HFD and *P. indica* (L.) Less. tea at 400 and 600 mg/kg/d, respectively. CON group was fed with HFD and water, and the SIM group was fed with HFD and simvastatin 20 mg/kg/d which was used as positive control. The serum total cholesterol level in the PIL, PIH and SIM groups were 235.29 ± 39.43 mg/dl, 231.14 ± 29.39 mg/dl and 231.8 ± 22.44 mg/dl, respectively, that significantly lower than the CON which was 293.42 ± 55.63 mg/dl at week 4 ($p < 0.05$; Figure 4.19a). Furthermore, Figure 4.19b showed the serum LDL-C level of PIL (48.17 ± 21.47 mg/dl), PIH (37.7 ± 14.42 mg/dl) and SIM (28.34 ± 4.81 mg/dl) at week 4, were significantly lower than the CON (86.96 ± 12.92 mg/dl) ($p < 0.05$). Interestingly, the serum triglyceride level of PIT treated group with PIH (149 ± 35.05 mg/dl) was significantly lower than the positive control (SIM, 229.8 ± 35.28 mg/dl) and CON (229.8 ± 35.28 mg/dl) ($p < 0.05$). However, the reduction of the serum triglyceride level of PIH treated group was not a significant difference from the PIL group ($p > 0.05$; Figure 4.19c). The result of serum HDL-C level is shown in Figure 4.19d. The result displayed that the increase in serum HDL-C level at week 4 of PIL, PIH, and SIM treated groups were not a significant difference ($p > 0.05$).

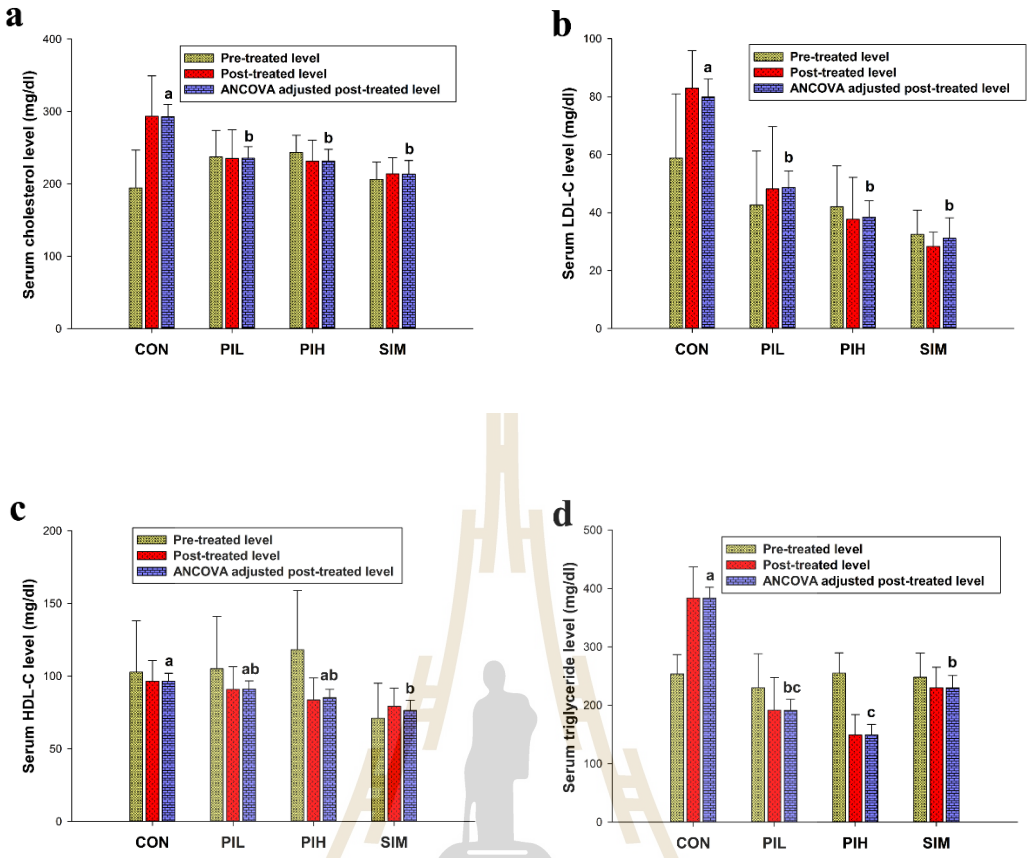


Figure 4.19 Effect of PIT on serum lipid profile. CON = Control; PIL = PIT at 400 mg/kg/d; PIH = PIT at 600 mg/kg/d; SIM = Simvastatin at 20 mg/kg/d. **(a)** Serum cholesterol **(b)** Serum LDL-C **(c)** Serum HDL-C and **(d)** Serum TG. Data are expressed as means ± SD (n = 10). Means with the same superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

4.5.3 Effect of *P. indica* (L.) Less. tea on food intake and body weight

The average food intake of mice had been investigated. Figure 4.20 shows the effect of PIT at low (PIL) and high (PIH) doses on food intake compared with CON and SIM groups. PIH showed significantly lower food intake than the CON group start at week one until the end of the experiment ($p < 0.05$). Furthermore, the

food intake of PIL and SIM groups were significantly lower than the CON group from week 2 to week 4.

The body weight of mice in all groups had been measured every week. The result was consistent with the average food intake result (Figure 4.21). At week one, PIH showed significantly lower body weight than the CON group, but not a significant difference from PIL and SIM. The body weight of PIH group was significantly lower than the CON group throughout the end of the experiment ($p < 0.05$). Moreover, the body weight of PIL group was significantly lower than the CON group from week 3 to week 4. Interestingly, at a final week, the body weight of the PIH group was significantly lower than CON, SIM, and PIL groups ($p < 0.05$).

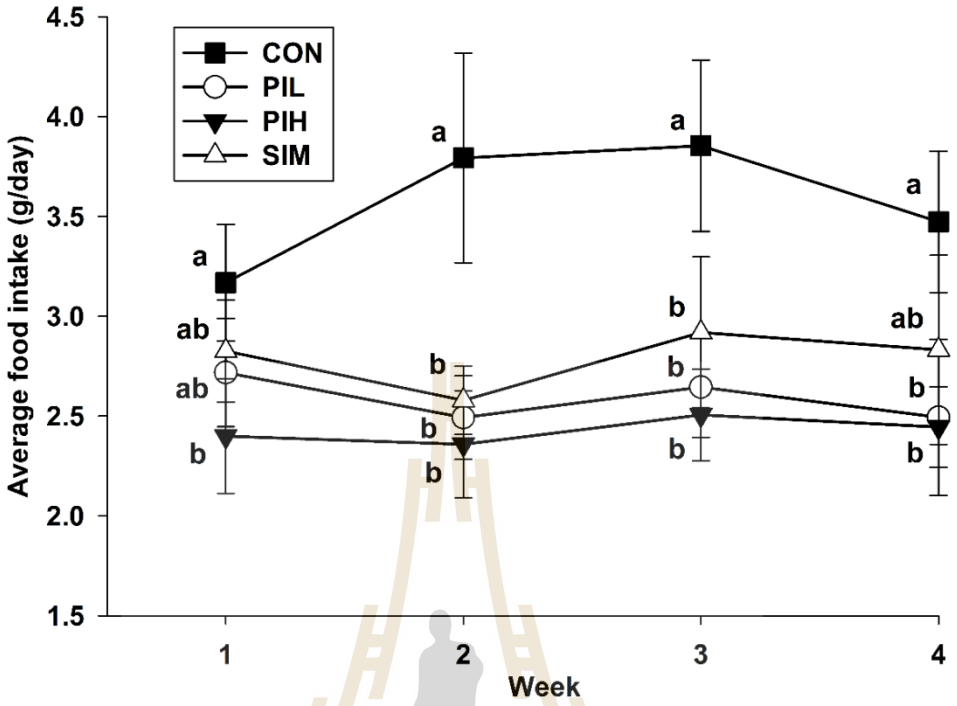


Figure 4.20 Effect of PIT on average food intake. CON = Control; PIL = PIT at 400 mg/kg/d; PIH = PIT at 600 mg/kg/d; SIM = Simvastatin at 20 mg/kg/d. Data are expressed as means ± SD (n = 10). Means with the same superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

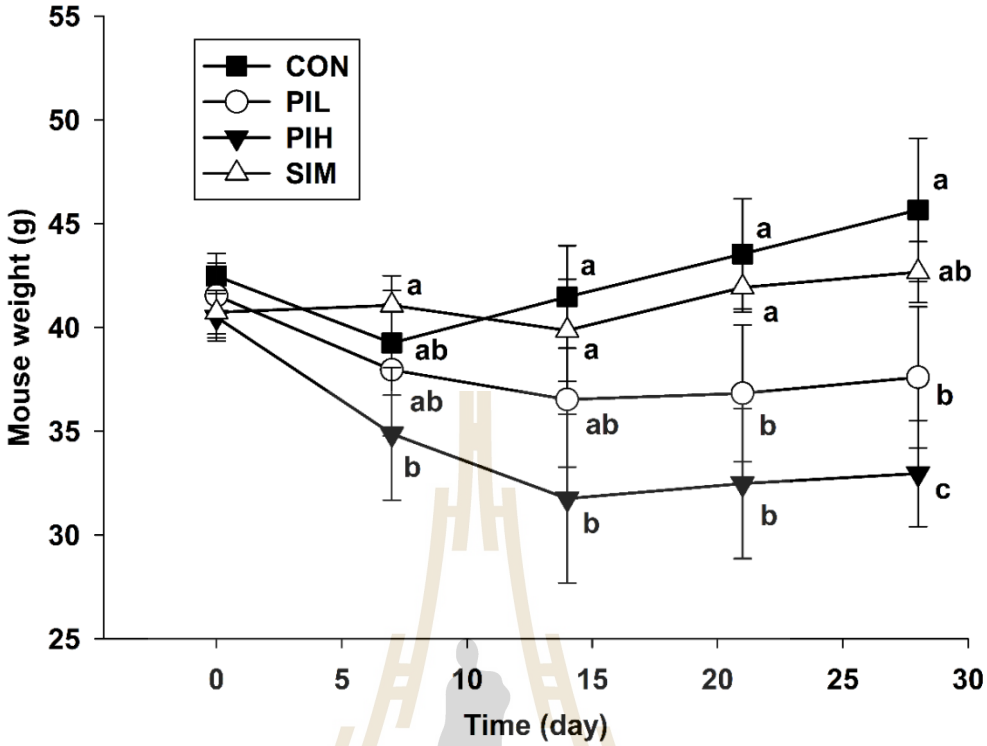


Figure 4.21 Effect of PIT on mouse weight. CON = Control; PIL = PIT at 400 mg/kg/d; PIH = PIT at 600 mg/kg/d; SIM = Simvastatin at 20 mg/kg/d. Data are expressed as means ± SD (n = 10). Means with the same superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

4.5.4 Effect of *P. indica* (L.) Less. tea on relative organ weight

Liver, Heart, Kidney, Lung, and Spleen weights of mice after fed with HFD, HFD plus PIT at 400 and 600 mg/kg/d and HFD plus simvastatin at 10 mg/kg/d are shown in Table 1. The relative weight of the liver, heart, kidney, lung, and spleen of PIL, PIH, and SIM were not significant differences from the CON ($p > 0.05$).

Table 4.1 Effect of PIT on relative organ weight. CON = Control; PIL = PIT at 400 mg/kg/d; PIH = PIT at 600 mg/kg/d; SIM = Simvastatin at 20 mg/kg/d. Data are expressed as means ± SD (n = 10). Means with the same superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

Groups	Relative weight (g/100 g body weight)				
	Liver	**Heart**	**Kidney**	**Lung**	**Spleen**
HFD	3.80 ± 0.33^a	0.45 ± 0.01^a	1.43 ± 0.23^a	0.51 ± 0.03^a	0.27 ± 0.12^a
PIL	3.96 ± 0.23^a	0.48 ± 0.03^a	1.67 ± 0.25^a	0.57 ± 0.15^a	0.30 ± 0.05^a
PIH	3.93 ± 0.08^a	0.46 ± 0.01^a	1.72 ± 0.21^a	0.63 ± 0.06^a	0.33 ± 0.09^a
SIM	3.63 ± 0.35^a	0.44 ± 0.04^a	1.44 ± 0.11^a	0.54 ± 0.04^a	0.28 ± 0.05^a

4.5.5 Effect of *P. indica* (L.) Less. tea on biochemical parameters in serum

The serum creatinine level, serum alanine transaminase levels (ALT), serum alkaline phosphatase levels (ALP) and complete blood count (CBC) had been measured to investigate the toxicity of *P. indica* (L.) Less. tea on the liver, kidney, and blood. Figure 4.22a showed that the serum creatinine level of PIL, PIH, and SIM were not significant difference compared to the CON ($p > 0.05$). These results suggest that PIL, PIH, and SIM may not be toxic to the kidney. In addition, the serum alanine transaminase levels (ALT) and serum alkaline phosphatase levels (ALP) of the PIL, PIH, and SIM were not significantly different from the CON ($p > 0.05$, Figure 4.22b and 4.22c). These results imply that PIL, PIH, and SIM should not be toxic to the liver. The toxicity of blood had been evaluated by measuring the complete blood

count (CBC). Generally, the CBC test provides important information regarding three major types of cells in the blood, RBC count, WBC count, and platelets. Figure 4.23a to 4.23c display RBC count, WBC count, and platelets, respectively. These results presented that all treated groups were not significantly different compared to the CON ($p > 0.05$).

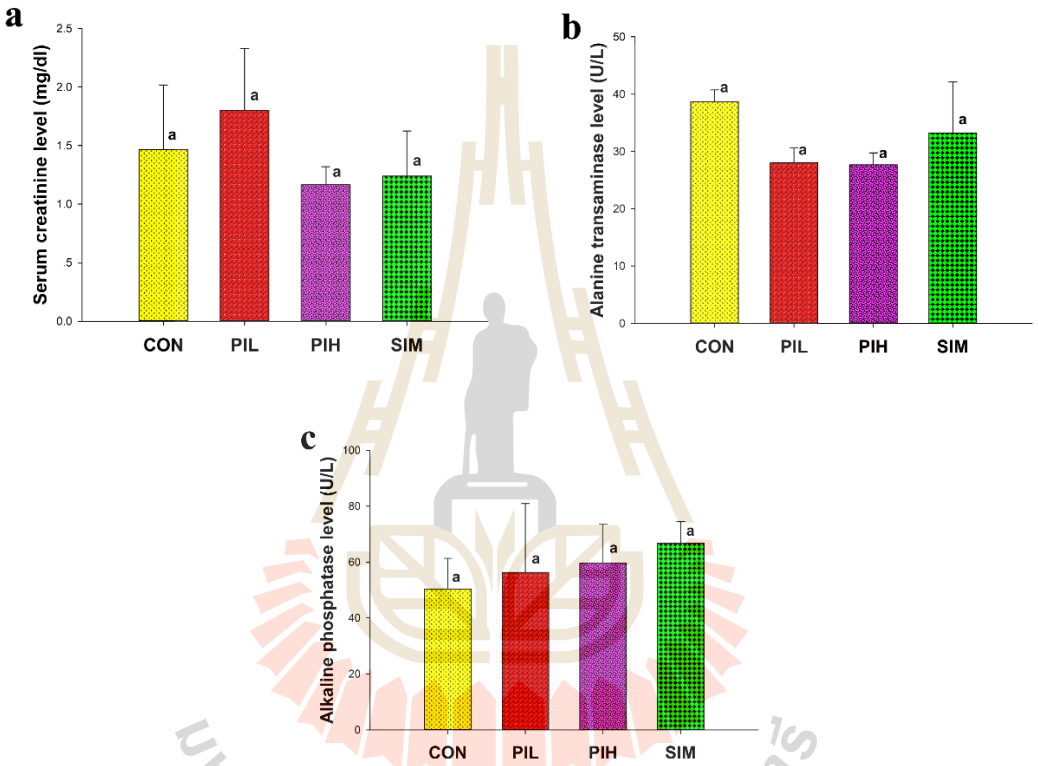


Figure 4.22 Effect of PIT on biochemical parameters in serum. CON = Control; PIL = PIT at 400 mg/kg/d; PIH = PIT at 600 mg/kg/d; SIM = Simvastatin at 20 mg/kg/d. **(a)** Serum creatinine **(b)** Serum ALT and **(c)** Serum ALP. Data are expressed as means ± SD (n = 10). Means with the same superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

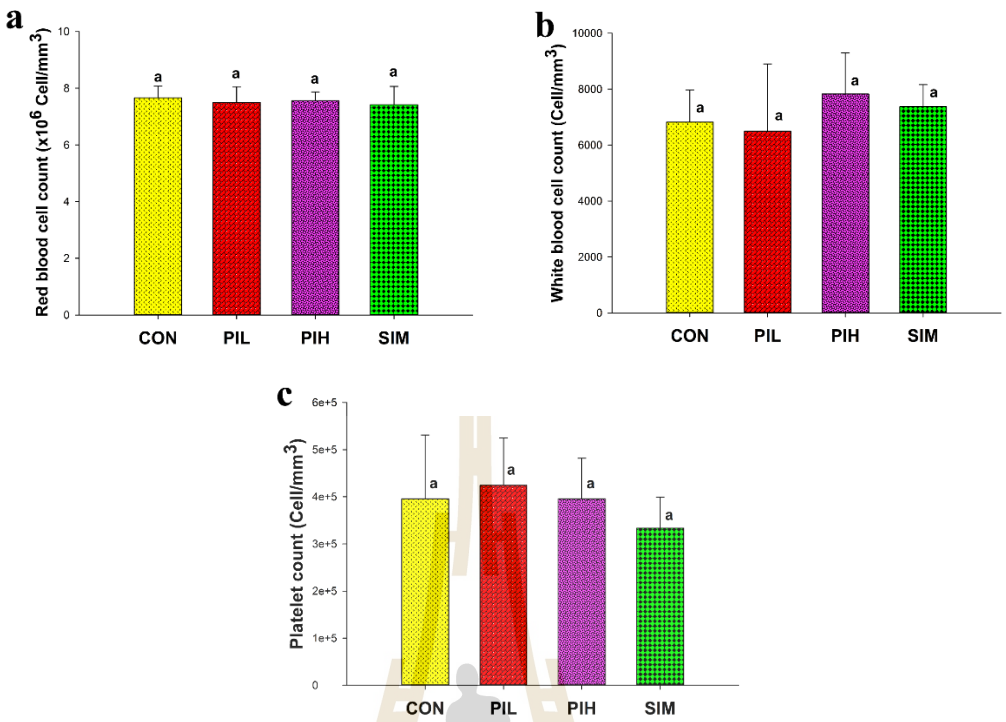


Figure 4.23 Effect of PIT on complete blood count. CON = Control; PIL = PIT at 400 mg/kg/d; PIH = PIT at 600 mg/kg/d; SIM = Simvastatin at 20 mg/kg/d. **(a)** Red blood cell count **(b)** White blood cell count and **(c)** Platelet count. Data are expressed as means ± SD (n = 10). Means with the same superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

4.5.6 Effect of *P. indica* (L.) Less. tea on perigonadal fat

The perigonadal fat of all groups had been collected after the end of the experiment, and perigonadal fat weight was investigated. The result is expressed in Figure 4.24. The perigonadal fat weight of PIL and PIH were significantly lower than the CON group ($p < 0.05$), but not a significant difference from SIM. Moreover, the histology of perigonadal fat was examined by using histology microscopy. The H&E staining of perigonadal fat photographs is shown in Figure 4.25. The adipocytes size (area) and a number of adipocytes per field are demonstrated in Figure 4.26a and 4.26b, respectively. The mean area of adipocytes of PIL and PIH were significantly lower than the CON group ($p < 0.05$), but not a significant difference from SIM which is correspondence with the result of adipocytes number per field that PIL and PIH were significantly higher this parameter than the CON group ($p < 0.05$).

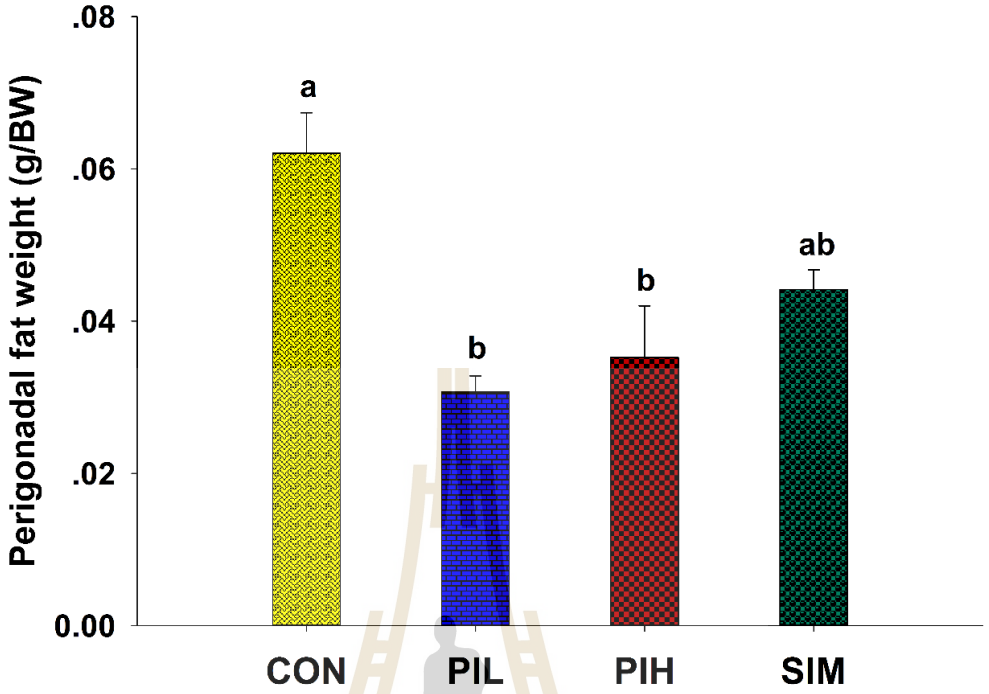


Figure 4.24 Effect of PIT on perigonadal fat weight. CON = Control; PIL = PIT at 400 mg/kg/d; PIH = PIT at 600 mg/kg/d; SIM = Simvastatin at 20 mg/kg/d. Data are expressed as means ± SD (n = 10). Means with the same superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

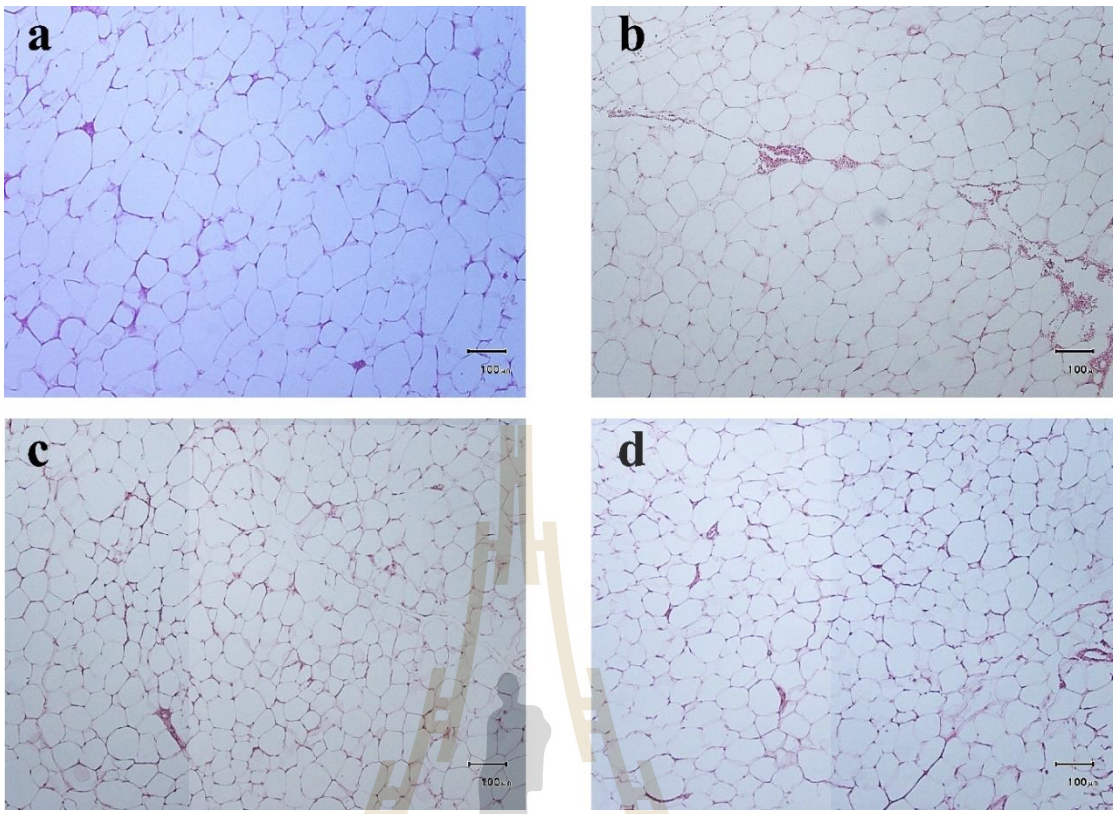


Figure 4.25 Microscopic imaging of perigonadal fat tissue after Hematoxylin & Eosin staining of samples. **(a)** = Control; **(b)** = PIT at 400 mg/kg/d; **(c)** = PIT at 600 mg/kg/d; **(d)** = Simvastatin at 20 mg/kg/d. Original magnification 10× (Scale bars = 100 µm).

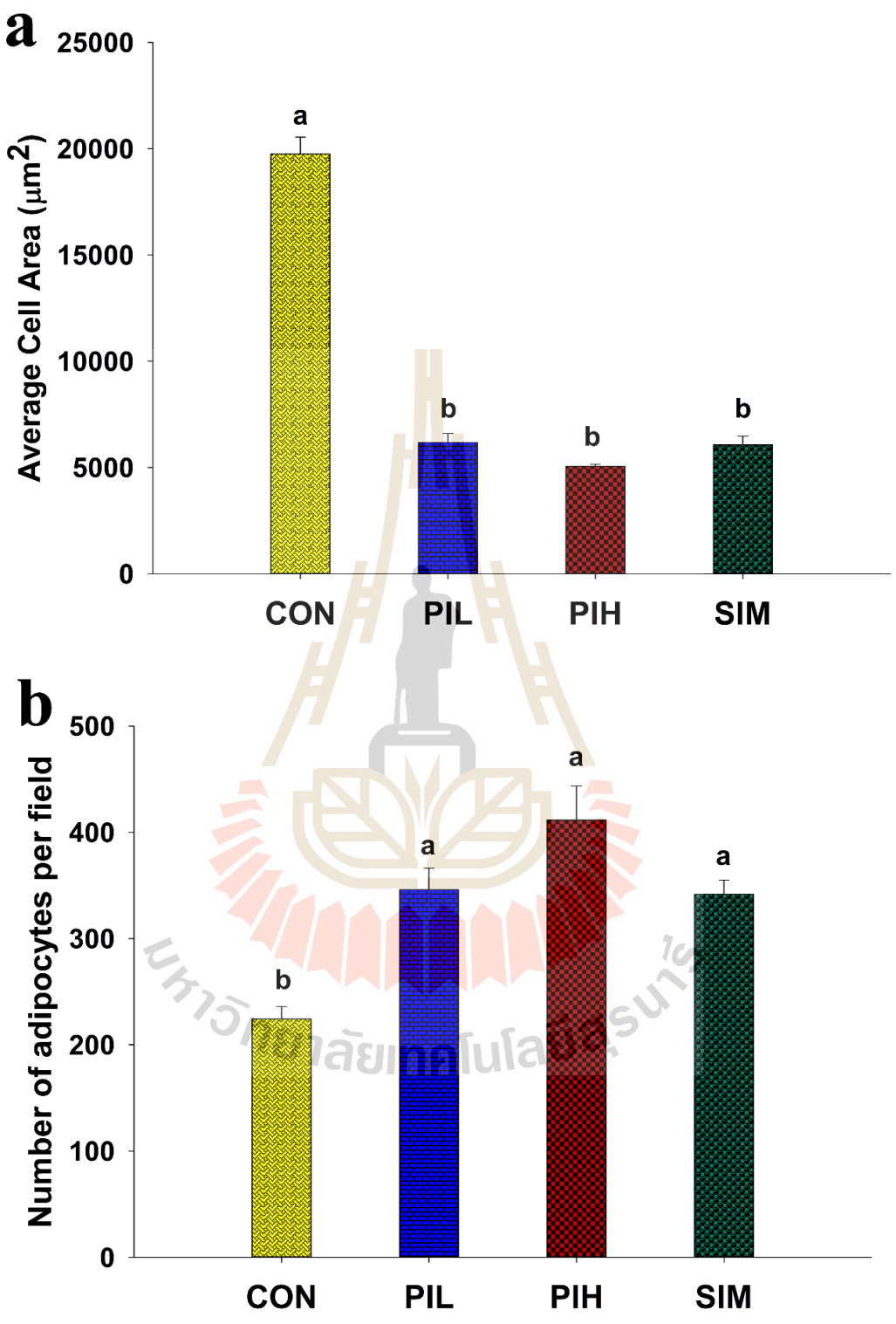


Figure 4.26 Effect of PIT on perigonadal fat tissues. CON = Control; PIL = PIT at 400 mg/kg/d; PIH = PIT at 600 mg/kg/d; SIM = Simvastatin at 20 mg/kg/d. **(a)**

adipocytes size (area) and **(b)** number of adipocytes per field. Data are expressed as means ± SD (n = 10). Means with the same superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

CHAPTER V

DISCUSSION AND CONCLUSION

5.1 Discussion

Obesity and hyperlipidemia are caused by an imbalance between energy consumption and energy expenditure which can cause some of the health problems such as type 2 diabetes, ischemic heart disease, stroke and cancer (Artham et al., 2008; Guo et al., 2015). Nowadays, we have conventional medicine for treat dyslipidemia, diabetes mellitus and obesity, such as Simvastatin for dyslipidemia and Orlistat for obesity, but the medication has limited efficacies and has some side effects such as rhabdomyolysis (Van Puijenbroek et al., 1996), nausea, vomiting (Filippatos et al., 2008). Therefore, traditional medicine is an essential alternative medicine to alleviate these diseases and may have minor side effects and easily consumable when compared with modern medicine. Furthermore, if traditional medicine can use alone or combine with modern medicine, it will decrease the using of modern medicine or reduce the dose and side effects of modern medicine as well (Liu et al., 2004; Liu et al., 2002).

P. indica (L.) Less. was reported that it had many therapeutic indications such as decrease blood sugar (Pramanik et al., 2006), reduce blood pressure. In addition, from folk medicine, it can help lower the hyperlipidemia, but this indication has not been proven the efficacy by the researcher. Nevertheless, *P. indica* (L.) Less. has the

potential for prevention and treatment of metabolic syndrome because it may have effects on both lower blood glucose and blood lipid. Moreover, no work has been done on its efficacy and safety.

5.1.1 The radical scavenging of *P. indica* (L.) Less. tea

Free radical including Reactive oxygen species (ROS) and Reactive nitrogen species (RNS) are derived from both endogenous sources (mitochondria, peroxisomes, endoplasmic reticulum, phagocytic cells, etc.) and exogenous sources (pollution, alcohol, tobacco smoke, heavy metals, transition metals, industrial solvents, pesticides, certain drugs like halothane, paracetamol, and radiation) (Phaniendra et al., 2015). The imbalance between free radical and antioxidant system can cause extensive damage to tissues and biomolecules (Halliwell and Gutteridge, 2015) leading to various diseases, especially degenerative diseases of aging such as cancer, immune-system decline, brain dysfunction and cardiovascular (Ames et al., 1993). The preventions of free radical are using the synthetic drug or using antioxidant from natural sources such as herbal beverage, especially teas which are widely used for an alternative solution to prevent free radical (Fukushima et al., 2009; Wojcik et al., 2010).

In this study, *P. indica* (L.) Less. tea had been investigated the antioxidant activities compared to well-known and popular antioxidant tea, *C. sinensis* tea, with several antioxidant assays, including scavenging of DPPH, ABTS, Hypochlorous acid, Nitric oxide, Peroxynitrite and the scavenging of ROS, RNS, and copper induces LDL oxidation assay.

The DPPH radical scavenging assay has been widely used in the determination of antioxidant activity of natural antioxidants from plant sources

(Brand-Williams et al., 1995; Yen and Duh, 1994). This assay determines the reduction of DPPH radical by measuring the color changing from the violet color of DPPH radical to yellow of non-radical DPPH by spectrophotometer at 515 nm. *C. sinensis* tea or green tea is a well-studied source of polyphenols antioxidants, and it can also scavenge the DPPH radical (Bastos et al., 2007; Manian et al., 2008). Interestingly, *P. indica* (L.) Less. tea showed significantly stronger antioxidant activity than *C. sinensis* tea in all concentrations. These results are in substantial agreement with Srisook et al. (Srisook et al., 2012) that hot water extract of *P. indica* tea leaves (HWEP) have the DPPH radical scavenging activity (EC_{50} value = 23.8 ± 1.0 µg/ml).

$ABTS^+$ radical cation decolorization assay has been used for measuring the antioxidant capacity of *P. indica* (L.) Less. tea compared with *C. sinensis* tea. The result showed that *C. sinensis* tea displayed significantly inhibit $ABTS^+$ radical better than *P. indica* (L.) Less. tea. This result may from *C. sinensis* tea contains various classes of polyphenols which have been linked to being a potent antioxidant to $ABTS^+$ radical (Lee et al., 2014; Tsao, 2010).

Hypochlorous acid is a weak acid that could generate harmful ROS. Hypochlorous acid could inactivate the antioxidant enzyme catalase by which breaks down the heme prosthetic group (Sarkar et al., 2009). The results suggest that both *P. indica* (L.) and *C. sinensis* tea is a potent scavenger to hypochlorous acid, which was reported by Valentão et al. and Cabrera et al. that the hypochlorous acid scavenged activity of *C. sinensis* tea (Cabrera et al., 2006; Valentão et al., 2003).

Nitric oxide has an important role in several physiological processes like neural signal transmission, immune response, control vasodilation and control of

blood pressure (Palmer et al., 1987; Rees et al., 1989). Nevertheless, the elevation of the nitric oxide causes inflammation and sustains levels of nitric oxide results in tissue toxicity and several pathological including in vascular disease (Li and Förstermann, 2000). The present study expressed that *P. indica* (L.) Less. tea indicated significantly higher nitric oxide scavenging effect than a reference *C. sinensis* tea. Tsai et al. reported that *C. sinensis* tea had IC_{50} values of nitric oxide scavenging less than 500 µg/ml and was proven to be good nitric oxide suppressor (Tsai et al., 2007). Therefore, *P. indica* (L.) Less. tea was also proven to be good nitric oxide suppressor.

Peroxynitrite (ONOO-) is one of the nitrogen-containing species that indicates as reactive nitrogen species (RNS). Generation of excess peroxynitrite represents a crucial pathogenic mechanism in conditions such as stroke, myocardial infarction, chronic heart failure, diabetes, circulatory shock, chronic inflammatory diseases, cancer, and neurodegenerative disorders (Pacher et al., 2007). According to the results, *P. indica* (L.) Less. tea could almost fully inhibit peroxynitrite oxidation at 75 µg/ml that better than *C. sinensis* tea at the same concentration. These results are in substantial agreement with those of Chung et al. that catechins with a galloyl group in green tea can inhibit peroxynitrite formation by SIN-1 and scavenge peroxynitrite itself (Chung et al., 1998).

LDL lipid oxidation is considered to be essential in the pathogenesis of atherosclerotic vascular diseases (Yoshida and Kisugi, 2010). Several lines of evidence suggest the important mechanisms of LDL lipid oxidation occurs by ROS, RNS *in vivo* (Govindarajan et al., 2005) and Cu^{2+} *in vitro* (Witztum, 1994). The natural compounds with anti-LDL oxidation activity could have some beneficial effects in the prevention of the disease (Craig, 1999; Fuhrman and Aviram, 2001).

The investigation of anti-LDL oxidation activity *in vivo* can be measured in vitro by using whole plasma/serum (Spranger et al., 1998). In this study, we measured the LDL oxidation in AAPH, Cu^{2+} and SIN-1 challenged isolated human LDL by using TBARS assay and presented the results by MDA equivalence. AAPH which is ROS generator was scavenged and lag time phase was also increased by both *P. indica* (L.) Less. tea and *C. sinensis* tea. These results are correspondence with those of Liu et al. that polyphenolic component extracted from green tea leaves is effective antioxidants against AAPH-initiated photosensitized LDL oxidation (Liu et al., 2000). As one might expect, this is the first reported that *P. indica* (L.) Less. tea had AAPH scavenging activity compared to the reference tea. In addition, the Cu^{2+} scavenging activity effect of both *P. indica* (L.) Less. tea and *C. sinensis* tea could inhibit Cu^{2+} induced LDL oxidation. This finding is also the first reported of Cu^{2+} scavenging activity of *P. indica* (L.) Less. tea compared with *C. sinensis* tea. These findings are correspondent with Yokozawa et al. that green tea extract markedly delays Cu^{2+} induced LDL oxidation with a dose-dependent pattern (Yokozawa and Dong, 1997). In the same way, SIN-1, which is RNS generator, was used to investigate SIN-1 induced LDL oxidation scavenging activity. The RNS, which is produced from SIN-1 was scavenged by both teas. These results are consistent with a peroxynitrite scavenging assay that *P. indica* (L.) Less. tea and *C. sinensis* tea could inhibit peroxynitrite, which was generated by SIN-1.

P. indica (L.) Less. tea has been identified the main chemical constituents by using LC-MS/MS technique. The results presented that caffeoylquinic acid derivatives were the main chemical compositions of *P. indica* (L.) Less. tea. This result is correspondence with Kongkiatpaiboon *et al.* (Kongkiatpaiboon et al., 2018)

that *P. indica* (L.) Less. tea contains six caffeoylquinic acid derivatives. Interestingly, caffeoylquinic acid derivatives were reported that it was a potent antioxidant substance. Caffeoylquinic acid derivatives from *Dipsacus asper* Wall (Dipsacaceae) demonstrated antioxidant activity against free radical, Cu^{2+}-mediated LDL oxidation and may have a role to play in preventing the development and progression of atherosclerotic disease (Hung et al., 2006). Furthermore, caffeoylquinic acid derivatives also showed high DPPH-radical and peroxynitrite scavenging activity (Islam et al., 2003; Park et al., 2009). These findings provide evidence that caffeoylquinic acid derivative from *P. indica* (L.) Less. tea plays an important role in free radical scavenging activities and anti-LDL oxidation effects, whereas, polyphenol catechins which are the main active ingredients of *C. sinensis* tea may act these effects. (Folch-Cano et al., 2010).

5.1.2 The *in vitro* study of *P. indica* (L.) Less. tea on 3T3-L1 cells

The inhibition of adipogenesis using 3T3-L1 cells can predict the efficacy of *P. indica* (L.) Less. on lipid formation inhibition (Naowaboot et al., 2012). In addition, cell viability can be investigated by MTT assay, which has been widely used to evaluate the cell viability, that can investigate the preliminary safety and acute toxicity of the compound.

The MTT assay can detect the cytotoxicity of the compound on the cells. Viable cells can generate MTT to formazan which has a purple color. The MTT results showed that the viability of cells treated with a PIT at 250 – 1000 μg/ml was not significantly different compared to the controls (DIF) ($p > 0.05$). These findings provide evidence that PIT at ≤ 1000 μg/ml is safe for 3T3-L1 cells. Our results are in substantial agreement with those of Srisook et al. and Pramanik et al. that *P. indica*

(L.) Less. alone at 25 to 400 µg/ml showed no cytotoxic effect in RAW 264.7 macrophage cells (Srisook et al., 2012) and at 400 mg/kg did not cause any side effects to rat (Pramanik et al., 2006).

Oil Red O staining is a technique that can be used for investigation of the adipogenesis of 3T3-L1 cell. The microscopic observation results of Oil Red O and hematoxylin-stained cells displayed that the PIT decreased Oil Red O stained droplets of mature adipocytes in a dose-dependent manner. Also, we can detect the quantity of lipid by eluting Oil Red O dye by 100% isopropanol and measuring lipid accumulation by microplate spectrophotometer. Interestingly, the effect of the PIT at 750 and 1000 µg/ml did not show significantly different in lipid accumulation compared to 1.67 µg /ml of simvastatin, which is a leading lipid-lowering drug.

The main active ingredients of PIT had been investigated by using LC-MS/MS technique. The result showed that the main chemical compositions of PIT were caffeoylquinic acid derivatives, which correlated with Kongkiatpaiboon et al. (Kongkiatpaiboon et al., 2018). Interestingly, caffeoylquinic acid has been reported about the effect on hyperlipidemia that the enriching of the caffeoylquinic acid derivative in *Pandanus tectorius* Fruit Extract moderated hyperlipidemia and improved the liver lipid profile in hamsters fed with high fat diet. These effects may be caused by increasing the expression of PPARα and its downstream genes and by upregulation of LPL and AMPK activities (Zhang et al., 2013). Moreover, the flavonoid ingredient of PIT had been investigated by using the HPLC technique (data not shown). Luteolin, apigenin, kaempferol, catechin, and quercetin have been used as reference standard. The results showed that the PIT contained quercetin that is consistent with those of Srisook et al. (Srisook et al., 2012).

The total phenolic and total flavonoid content of PIT might be reduced from the production processes. The *P. indica* leaves were dried with high temperature that might affect phenolic and flavonoid content. However, the total phenolic and total flavonoid content results are in substantial agreement with those of Susetyarini et al. (Susetyarini and Ariesandyb, 2016) that tannin which is phenolic compound was found in *P. indica* (L.) Less. Furthermore, these results are consistent with Andarwulan et al. that the active flavonoid and phenolic compounds were discovered in *P. indica* (L.) Less. (Andarwulan et al., 2010). Likewise, *P. indica* (L.) Less. appeared to possess eudesmane derivatives, terpene glycosides, benzenoids, phenylpropanoids, lignan glycosides, stigmasterol glucoside, quercetin and chlorogenic acid (Srisook et al., 2012).

Moreover, the data are consistent with those of Hsu et al. that flavonoids and phenolic acids caused 3T3-L1 cell cycle arrest in the G1 phase that may play a role in the control adipogenesis of 3T3-L1 cell and might have the further implication of *in vivo* anti-obesity effects (Hsu et al., 2006). Apart from this, the previous study reported that quercetin, which is one of the active ingredients in *P. indica* (L.) Less., could inhibit adipogenesis in 3T3-L1 adipocyte cell (Park et al., 2008). In addition, quercetin was reported that it could improve hypertriglyceridemia, alleviate hypercholesterolemia and elevate HDL-cholesterol in db/db mice by decreasing the expression of peroxisome proliferator-activated receptor-α (PPAR-α), sterol regulatory element-binding protein-1c (SREBP-1c) and reducing acetyl-CoA carboxylase (ACC) activity (Jeong et al., 2012). These results provide evidence that PIT may decrease the adipogenesis of the cells and have the potential to develop to be an herbal supplement that can prevent hyperlipidemia.

FTIR microspectroscopy has previously been used to characterize the spectral properties of biological change in various samples (Dunkhunthod et al., 2017; Manoharan et al., 1993). These results showed that the integrated area of lipids, proteins, nucleic acids, glycogen and carbohydrates of the PIT-treated adipocytes group were significantly lower than the untreated adipocytes group (DIF) ($p < 0.05$). The results are correspondence with those of Dunkhuntod et al. that baicalein which is one of the ingredients in PIT can reduce lipids, proteins, glycogen, and other carbohydrates in baicalein-treated 3T3-L1 adipocytes compared to untreated 3T3-L1adipocytes. Moreover, the decreasing of integrated area of lipids of FTIR is consistent with the Oil Red O staining results. PCA analysis exhibited discriminate of four clusters of the FTIR spectra of pre-adipocyte (ND), untreated adipocyte (DIF), simvastatin- and PIT-treated adipocytes.

5.1.3 Effect of the *P. indica* (L.) Less. tea on Pancreatic Lipase Activity

The pancreatic lipase activity investigation can suggest the efficacy of PIT on lipid absorption inhibition from the gastrointestinal tract to the blood results in blood lipid reduction.

Lipase is the enzyme responsible for the digestion of the lipid before it is absorbed into the bloodstream. The inhibition of the enzyme can reduce the absorption of lipid which can minimize the risk of obesity and hyperlipidemia disease. The results indicated that PIT could inhibit the enzyme lipase activity in a dose-dependent manner and had the potency less than orlistat for 25.04 times. These results are correspondence with those of Zhang et al. and Birari that a polyphenolic compound like flavonoid could inhibit the enzyme lipase activity and reduce lipid absorption in the intestine (Birari and Bhutani, 2007; Zhang et al., 2015). Besides,

Dunkhunthod et al. reported that baicalein could inhibit pancreatic lipase activity (Dunkhunthod et al., 2017). As well as, the polyphenol-rich plants extract derived from grape seed extract, fermented oats, berry or strawberry had been reported to inhibit lipases activity, and TPC enhanced the pancreatic lipase inhibitory effect (Cai et al., 2012; McDougall et al., 2009). Thus, the inhibitory effect of the PIT may depend on the amount of TPC and TFC, which vary with the PIT concentrations.

5.1.4 The *in vivo* study of *P. indica* (L.) Less. tea on anti-hyperglycemia, anti-dyslipidemia, and toxicity

High fat diet feeding model in mice has been widely used as a model for dyslipidemia studies for evaluation of anti-dyslipidemia because it can induce obesity and dyslipidemia. *P. indica* (L.) Less. tea has been used for a health-promoting drink (Kongkiatpaiboon et al., 2018) that has the potential to prevent hyperglycemia, obesity, and dyslipidemia. In this study, *P. indica* (L.) Less. tea had been investigated its anti-hyperglycemia compared with glibenclamide which is conventional medicine for treating hyperglycemia. The anti-obesity and anti-dyslipidemia were also studied compared to simvastatin which is HMG-CoA reductase inhibitor for dyslipidemia treatment.

The anti-hyperglycemia of *P. indica* (L.) Less. tea was investigated by using an oral glucose tolerance test which used to measure the response of insulin receptors to elevate exogenous glucose (Megalli et al., 2006). The results showed that *P. indica* (L.) Less. tea at 400 and 600 mg/kg showed a significant hypoglycemic effect in mice after feeding with glucose. The PIT showed a hypoglycemic effect initiated from 15 min and maintained the duration of action similar to the standard oral hypoglycemic agent, glibenclamide. Interestingly, at 30 min *P. indica* (L.) Less.

tea at a high dose reduced the plasma glucose level to 190 ± 19.76 mg/dl, which significantly lower than the standard drug, glibenclamide. These results are in substantial agreement with Pramanik et al. (Pramanik et al., 2006) that the methanolic extract of *P. indica* Less. leaves treat normal rats, and streptozotocin-induced diabetic rats exhibit hypoglycemic and anti-hyperglycemic activity. Apart from this, *P. indica* (L.) Less. tea had been reported that the main chemical constituents were caffeoylquinic acid derivatives (Arsiningtyas et al., 2014; Kongkiatpaiboon et al., 2018; Sirichaiwetchakoon et al., 2018). The isolated caffeoylquinic acid derivatives from *P. indica* (L.) Less. could inhibit intestinal maltase which might delay postprandial hyperglycemia (Arsiningtyas et al., 2014). Likewise, the caffeoylquinic acid derivative from *Pandanus tectorius* fruit extract had been reported that it might be beneficial for the treatment of diabetes by alleviating hyperglycemia, increasing insulin sensitivity via activation of AMPK–AS160–GLUT4 pathway in skeletal muscles, and inhibiting of gluconeogenesis in the liver (Wu et al., 2014). These findings lead us to believe that caffeoylquinic acid derivatives from *P. indica* (L.) Less. tea can reduce hyperglycemia by increasing insulin sensitivity via activation of AMPK–AS160–GLUT4 pathway and inhibiting the gluconeogenesis in the liver.

Furthermore, the HFD-fed mice were fed with *P. indica* (L.) Less. tea (400 and 600 mg/kg daily) resulted in lower TG, TC, and LDL-cholesterol compared to the HFD group. Several studies reported that improving serum lipid parameters were related to anti-obesity of the extracts (Cho et al., 2010; Choi et al., 2007; Poirier et al., 2005). In this study, *P. indica* (L.) Less. tea at low and high doses could prevent obesity in HFD-fed mice with the dose-dependent manner that corresponds to food intake result of the PIT. As well as, the PIT could decrease perigonadal fat pad

weight, and H&E staining of perigonadal fat showed the reducing of adipocyte size and increasing the number of adipocyte per field after receiving the PIT. Our data are consistent with Sirichaiwetchakoon et al. (Sirichaiwetchakoon et al., 2018) that *P. indica* (L.) Less. tea inhibits lipids and carbohydrate accumulation in adipocytes and also inhibits pancreatic lipase activity that has the potential to reduce obesity and serum lipid parameters. Likewise, caffeoylquinic acid derivatives in coffee and polyphenols, which were reported that appeared in *P. indica* (L.) Less. tea (Arsiningtyas et al., 2014; Kongkiatpaiboon et al., 2018; Sirichaiwetchakoon et al., 2018), suppressed diet-induced body fat accumulation by downregulating SREBP-1c and related molecules in C57BL/6J mice (Murase et al., 2010). Furthermore, several studies had reported about caffeoylquinic acid derivatives containing herbal expressed anti-hyperlipidemia and anti-obesity effects. Nugroho et al. (Nugroho et al., 2010) reported that oral administration of caffeoylquinic acid-rich, *L. stenocephala* in BuOH fraction form, decreased the rat body weight to the level of the untreated group and decreased abdominal fat pad weight. It could be used for the treatment or prevention of obesity. These results are correspondence with the study of Zhang et al. (Zhang et al., 2013) that caffeoylquinic acid-rich, *Pandanus tectorius* fruit extract, has anti-hyperlipidemia effect in high fat-diet hamsters and the effects might be caused by increasing the expression of PPARα, its downstream genes, and upregulation of LPL and AMPK activities. Our findings lead us to believe that *P. indica* (L.) Less. tea acts the anti-dyslipidemia by increasing the expression of PPARα and upregulation of LPL and AMPK activities.

The toxicity of *P. indica* (L.) Less. tea on relative organ and biochemical parameters in serum had been investigated. The PIT at high and low doses did not

change the liver, heart, kidney, lung, and spleen weight after feeding the PIT for 4 weeks compared with the control group. Also, the PIT at high and low doses did not change ALP, ALT, creatinine, RBC, WBC and platelet which could be implied that the PIT was safe for liver, kidney, and blood after feeding for 4 weeks. Our results are in substantial agreement with those of Pramanik et al. (Pramanik et al., 2006) that the toxicity study of a methanolic extract of *Pluchea indica* (L.) Less. in the rat was safe to use even at the doses of 3.2 gm/kg of body weight orally. The doses of *P. indica* (L.) Less. tea in these experiments could be converted to human dose by a simple practice guide for dose conversion between animals and human (Nair and Jacob, 2016). From the formula, *P. indica* (L.) Less. tea at 400 and 600 mg/kg/d in mice are approximately 35.52 and 48.78 mg/kg/d in human, respectively. From these calculated dosages, we can imply that *P. indica* (L.) Less. tea drinking at 35 - 45 mg/kg/d once daily should be effective for anti-dyslipidemia, anti-hyperglycemic and safe for a human to drink once daily for 4 weeks.

5.2 Conclusion

These results provide evidence that the *P. indica* (L.) Less. tea can decrease lipid accumulation in 3T3-L1 adipocytes, primarily inhibit adipogenesis. The *P. indica* (L.) Less. tea also modifies the lipid, carbohydrate, protein, nucleic acid and glycogen concentrations within the cells. This study also demonstrates that FTIR microspectroscopy can provide valuable information on the biochemical changes within the 3T3-L1 adipocytes. Furthermore, the PIT could inhibit lipase activity *in vitro*. Moreover, *in vivo* study provides evidence that the *P. indica* (L.) Less. tea could protect hyperglycemia and dyslipidemia. In addition, the *P. indica* (L.) Less. tea could

reduce appetite and weight gain from the high-fat diet-induced dyslipidemia mice. In this study, *P. indica* (L.) Less. tea also showed the scavenging effects by preventing DPPH, ABTS, HOCl, Nitric oxide and Peroxynitrite radical. Besides, *P. indica* (L.) Less. tea acted as anti-LDL oxidation by scavenging Copper, AAPH and SIN-1 induced LDL oxidation.

Moreover, the safety of *P. indica* (L.) Less. tea was also investigated, and the MTT assay investigation displayed safety of this tea at 1000 μg/ml in the 3T3-L1 adipocytes. Apart from this, *in vivo* study demonstrated that the PIT at 600 mg/kg/day was safe for the tested major organs of mice.

From this study, *P. indica* (L.) Less. tea has potentials to develop to be an antioxidant beverage for preventing atherosclerotic disease, diseases that occur by free radical, anti-hyperglycemic, anti-dyslipidemia, and anti-obesity beverage or medicine. However, the results need to be further pharmacological activity investigation and safety in human.

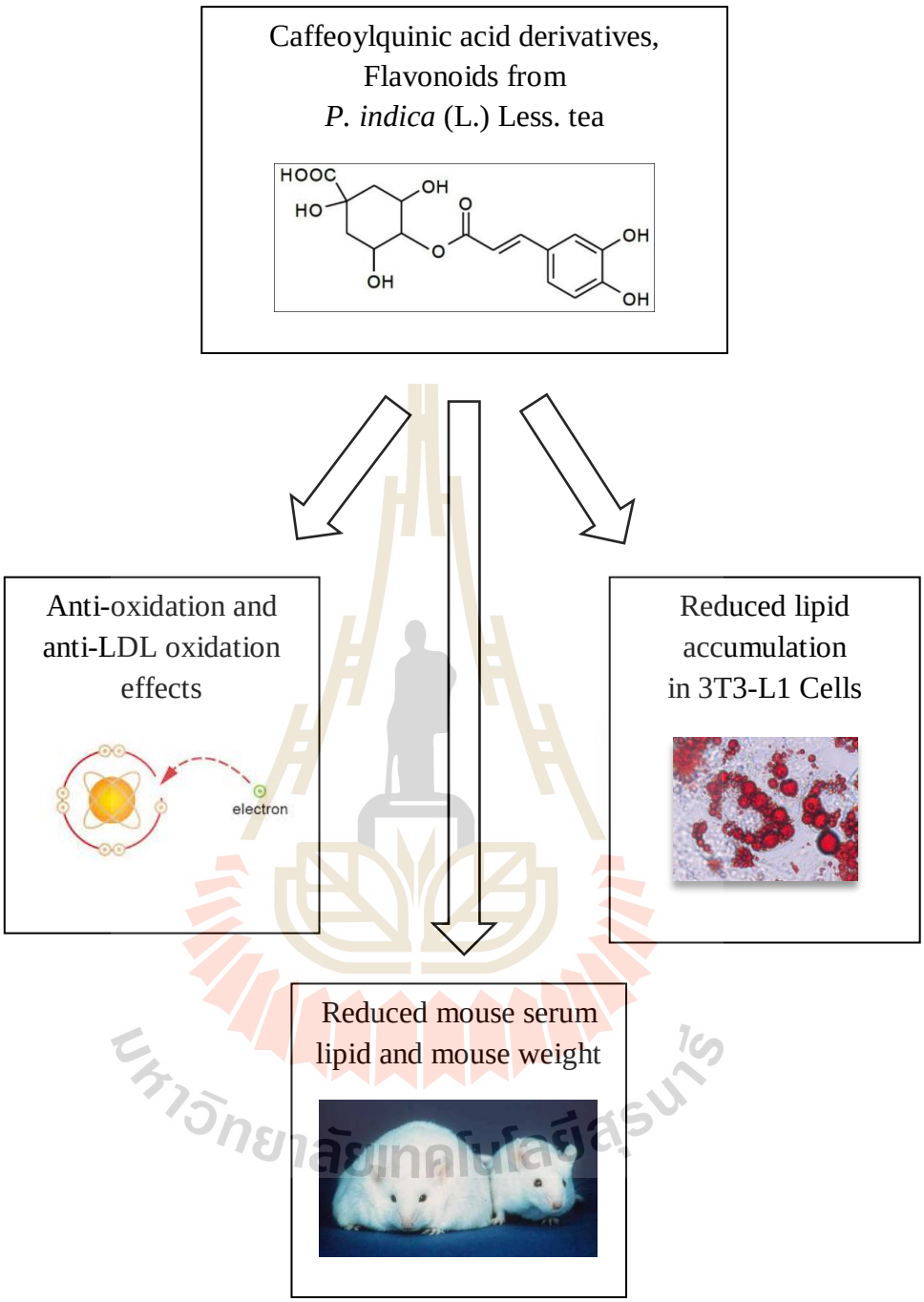


Figure 5.1 The summarized diagram of *P. indica* (L.) Less. tea effects.

REFERENCES

REFERENCES

Abdesselem, H., Madani, A., Hani, A., Al-Noubi, M., Goswami, N., Hamidane, H. B., Billing, A. M., Pasquier, J., Bonkowski, M. S., and Halabi, N. (2016). SIRT1 limits adipocyte hyperplasia through c-Myc inhibition. **Journal of Biological Chemistry**. 291(5): 2119-2135.

Ailhaud, G., Grimaldi, P., and Negrel, R. (1992). Cellular and molecular aspects of adipose tissue development. **Annual Review of Nutrition**. 12(1): 207-233.

AK, E., and Ahmad, Z. (2008). Anti-inflammatory and antinociceptive activities of the ethanolic extract of *Pluchea indica* (L) less leaf. **Pharmacologyonline**. 2: 349-360.

Aksoy, C., and Severcan, F. (2012). Role of vibrational spectroscopy in stem cell research. **Journal of Spectroscopy**. 27(3): 167-184.

Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. **Proceedings of the National Academy of Sciences**. 90(17): 7915-7922.

Amin, K. A., and Nagy, M. A. (2009). Effect of carnitine and herbal mixture extract on obesity induced by high fat diet in rats. **Diabetology & Metabolic Syndrome**. 1(1): 17.

An, L., Pang, Y.-W., Gao, H.-M., Tao, L., Miao, K., Wu, Z.-H., and Tian, J.-H. (2012). Heterologous expression of *C. elegans* fat-1 decreases the n-6/n-3 fatty

acid ratio and inhibits adipogenesis in 3T3-L1 cells. **Biochemical and Biophysical Research Communications**. 428(3): 405-410.

Andarwulan, N., Batari, R., Sandrasari, D. A., Bolling, B., and Wijaya, H. (2010). Flavonoid content and antioxidant activity of vegetables from Indonesia. **Food Chemistry**. 121(4): 1231-1235.

Arsiningtyas, I. S., Gunawan-Puteri, M. D., Kato, E., and Kawabata, J. (2014). Identification of α-glucosidase inhibitors from the leaves of *Pluchea indica* (L.) Less., a traditional Indonesian herb: promotion of natural product use. **Natural Product Research**. 28(17): 1350-1353.

Artham, S. M., Lavie, C. J., Milani, R. V., and Ventura, H. O. (2008). The obesity paradox: impact of obesity on the prevalence and prognosis of cardiovascular diseases. **Postgraduate Medicine**. 120(2): 34-41.

Aruoma, O. I., and Halliwell, B. (1987). Action of hypochlorous acid on the antioxidant protective enzymes superoxide dismutase, catalase and glutathione peroxidase. **Biochemical Journal**. 248(3): 973-976.

Ascaso, J., Santos, P. G., Mijares, A. H., Rojas, A. M., Masana, L., Millan, J., Pallardo, L. F., Pedro-Botet, J., Jimenez, F. P., and Pintó, X. (2007). Management of dyslipidemia in the metabolic Syndrome. **American Journal of Cardiovascular Drugs**. 7(1): 39-58.

Babu, A., Pon, V., and Liu, D. (2008). Green tea catechins and cardiovascular health: an update. **Current Medicinal Chemistry**. 15(18): 1840-1850.

Bahorun, T., Soobrattee, M., Luximon-Ramma, V., and Aruoma, O. (2006). Free radicals and antioxidants in cardiovascular health and disease. **Internet Journal of Medical Update**. 1(2): 25-41.

Bastos, D. H., Saldanha, L. A., Catharino, R. R., Sawaya, A., Cunha, I. B., Carvalho, P. O., and Eberlin, M. N. (2007). Phenolic antioxidants identified by ESI-MS from yerba maté (*Ilex paraguariensis*) and green tea (*Camelia sinensis*) extracts. **Molecules**. 12(3): 423-432.

Beisson, F., Tiss, A., Rivière, C., and Verger, R. (2000). Methods for lipase detection and assay: a critical review. **European Journal of Lipid Science and Technology**. 102(2): 133-153.

Birari, R. B., and Bhutani, K. K. (2007). Pancreatic lipase inhibitors from natural sources: unexplored potential. **Drug Discovery Today**. 12(19): 879-889.

Bodmer, M., Meier, C., Krähenbühl, S., Jick, S. S., and Meier, C. R. (2008). Metformin, sulfonylureas or other antidiabetic drugs and the risk of lactic acidosis or hypoglycemia: a nested case-control analysis. **Diabetes Care**. 15(16): 668-679.

Bolton, J. L., Trush, M. A., Penning, T. M., Dryhurst, G., and Monks, T. J. (2000). Role of quinones in toxicology. **Chemical Research in Toxicology**. 13(3): 135-160.

Brand-Williams, W., Cuvelier, M.-E., and Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. **LWT-Food Science and Technology**. 28(1): 25-30.

Buapool, D., Mongkol, N., Chantimal, J., Roytrakul, S., Srisook, E., and Srisook, K. (2013). Molecular mechanism of anti-inflammatory activity of *Pluchea indica* leaves in macrophages RAW 264.7 and its action in animal models of inflammation. **Journal of Ethnopharmacology**. 146(2): 495-504.

Burdi, A., Poissonnet, C., Garn, S., Lavelle, M., Sabet, M., and Bridges, P. (1984). Adipose tissue growth patterns during human gestation: a histometric comparison of buccal and gluteal fat depots. **International Journal of Obesity**. 9(4): 247-256.

Cabrera, C., Artacho, R., and Giménez, R. (2006). Beneficial effects of green tea-a review. **Journal of the American College of Nutrition**. 25(2): 79-99.

Cai, S., Wang, O., Wang, M., He, J., Wang, Y., Zhang, D., Zhou, F., and Ji, B. (2012). *In vitro* inhibitory effect on pancreatic lipase activity of subfractions from ethanol extracts of fermented oats (*Avena sativa* L.) and synergistic effect of three phenolic acids. **Journal of Agricultural and Food Chemistry**. 60(29): 7245-7251.

Calzadilla, P., Gómez-Serrano, M., García-Santos, E., Schiappacasse, A., Abalde, Y., Calvo, J. C., Peral, B., and Guerra, L. N. (2013). N-Acetylcysteine affects obesity-related protein expression in 3T3-L1 adipocytes. **Redox Report**. 18(6): 210-218.

Canaan, S., Dupuis, L., Rivière, M., Faessel, K., Romette, J.-L., Verger, R., and Wicker-Planquart, C. (1998). Purification and interfacial behavior of recombinant human gastric lipase produced from insect cells in a bioreactor. **Protein Expression and Purification**. 14(1): 23-30.

Cao, J., Ng, E. S., McNaughton, D., Stanley, E. G., Elefanty, A. G., Tobin, M. J., and Heraud, P. (2013). The characterisation of pluripotent and multipotent stem cells using Fourier transform infrared microspectroscopy. **International Journal of Molecular Sciences**. 14(9): 17453-17476.

Chang, C.-C., Lin, K.-Y., Peng, K.-Y., Day, Y.-J., and Hung, L.-M. (2015). Resveratrol exerts anti-obesity effects in high-fat diet obese mice and displays differential dosage effects on cytotoxicity, differentiation, and lipolysis in 3T3-L1 cells. **Endocrine Journal**. 11(2): 22-29.

Chapman, A. E., Kiroff, G., Game, P., Foster, B., O'brien, P., Ham, J., and Maddern, G. J. (2004). Laparoscopic adjustable gastric banding in the treatment of obesity: a systematic literature review. **Surgery**. 135(3): 326-351.

Charan, J., and Kantharia, N. (2013). How to calculate sample size in animal studies? **Journal of Pharmacology and Pharmacotherapeutics**. 4(4): 303.

Chen, J.-J., and Li, X.-R. (2007). Hypolipidemic effect of flavonoids from mulberry leaves in triton WR-1339 induced hyperlipidemic mice. **Asia Pacific Journal of Clinical Nutrition**. 16(S1): 290-294.

Cheung, B. M. Y., Cheung, T. T., and Samaranayake, N. R. (2013). Safety of antiobesity drugs. **Therapeutic Advances in Drug Safety**. 4(4): 171-181.

Cho, A.-S., Jeon, S.-M., Kim, M.-J., Yeo, J., Seo, K.-I., Choi, M.-S., and Lee, M.-K. (2010). Chlorogenic acid exhibits anti-obesity property and improves lipid metabolism in high-fat diet-induced-obese mice. **Food and Chemical Toxicology**. 48(3): 937-943.

Choi, H., Eo, H., Park, K., Jin, M., Park, E.-J., Kim, S.-H., Park, J. E., and Kim, S. (2007). A water-soluble extract from *Cucurbita moschata* shows anti-obesity effects by controlling lipid metabolism in a high fat diet-induced obesity mouse model. **Biochemical and Biophysical Research Communications**. 359(3): 419-425.

Choi, H., Han, B. S., Kim, W. K., Lee, S. C., Oh, K.-J., and Bae, K.-H. (2016). c-Jun regulates adipocyte differentiation via the KLF15-mediated mode. **Biochemical and Biophysical Research Communications**. 469(3): 552-558.

Chung, H. Y., Yokozawa, T., Soung, D. Y., Kye, I. S., No, J. K., and Baek, B. S. (1998). Peroxynitrite-scavenging activity of green tea tannin. **Journal of Agricultural and Food Chemistry**. 46(11): 4484-4486.

Cornelius, P., MacDougald, O. A., and Lane, M. D. (1994). Regulation of adipocyte development. **Annual Review of Nutrition**. 14(1): 99-129.

Couillard, C., Mauriege, P., Imbeault, P., Prud'homme, D., Nadeau, A., Tremblay, A., Bouchard, C., and Despres, J. (2000). Hyperleptinemia is more closely associated with adipose cell hypertrophy than with adipose tissue hyperplasia. **International Journal of Obesity**. 24(6): 782-788.

Crabbe, T., Weir, A. N., Walton, E. F., Brown, M. E., Sutton, C. W., Tretout, N., Bonnerjea, J., Lowe, P. A., and Yarranton, G. T. (1996). The secretion of active recombinant human gastric lipase by *Saccharomyces cerevisiae*. **Protein Expression and Purification**. 7(3): 229-236.

Craig, W. J. (1999). Health-promoting properties of common herbs. **The American Journal of Clinical Nutrition**. 70(3): 491s-499s.

Dellamora-Ortiz, G. M., Martins, R. C., Rocha, W. L., and Dias, A. P. (1997). Activity and stability of a *Rhizomucor miehei* lipase in hydrophobic media. **Biotechnology and Applied Biochemistry**. 26(1): 31-37.

Denizot, F., and Lang, R. (1986). Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved

sensitivity and reliability. **Journal of Immunological Methods**. 89(2): 271-277.

Dhawan, V. (2014). Reactive Oxygen and Nitrogen Species: General Considerations. **Humana Press**. 27-47.

Dunkhunthod, B., Thumanu, K., and Eumkeb, G. (2017). Application of FTIR microspectroscopy for monitoring and discrimination of the anti-adipogenesis activity of baicalein in 3T3-L1 adipocytes. **Vibrational Spectroscopy**. 89: 92-101.

Eseberri, I., Miranda, J., Lasa, A., Churruca, I., and Portillo, M. P. (2015). Doses of quercetin in the range of serum concentrations exert delipidating effects in 3T3-L1 preadipocytes by acting on different stages of adipogenesis, but not in mature adipocytes. **Oxidative Medicine and Cellular Longevity**. 2015.

Eumkeb, G., Siriwong, S., and Thumanu, K. (2012). Synergistic activity of luteolin and amoxicillin combination against amoxicillin-resistant *Escherichia coli* and mode of action. **Journal of Photochemistry and Photobiology B: Biology**. 117: 247-253.

Filippatos, T. D., Derdemezis, C. S., Gazi, I. F., Nakou, E. S., Mikhailidis, D. P., and Elisaf, M. S. (2008). Orlistat-associated adverse effects and drug interactions. **Drug Safety**. 31(1): 53-65.

Folch-Cano, C., Jullian, C., Speisky, H., and Olea-Azar, C. (2010). Antioxidant activity of inclusion complexes of tea catechins with β-cyclodextrins by ORAC assays. **Food Research International**. 43(8): 2039-2044.

Fuhrman, B., and Aviram, M. (2001). Flavonoids protect LDL from oxidation and attenuate atherosclerosis. **Current Opinion in Lipidology**. 12(1): 41-48.

Fukushima, Y., Ohie, T., Yonekawa, Y., Yonemoto, K., Aizawa, H., Mori, Y., Watanabe, M., Takeuchi, M., Hasegawa, M., and Taguchi, C. (2009). Coffee and green tea as a large source of antioxidant polyphenols in the Japanese population. **Journal of Agricultural and Food Chemistry**. 57(4): 1253-1259.

Garip, S., Gozen, A. C., and Severcan, F. (2009). Use of Fourier transform infrared spectroscopy for rapid comparative analysis of *Bacillus* and *Micrococcus* isolates. **Food Chemistry**. 113(4): 1301-1307.

Gomes, A., Saha, A., Chatterjee, I., and Chakravarty, A. (2007). Viper and cobra venom neutralization by β-sitosterol and stigmasterol isolated from the root extract of *Pluchea indica* Less.(Asteraceae). **Phytomedicine**. 14(9): 637-643.

Govindarajan, R., Vijayakumar, M., and Pushpangadan, P. (2005). Antioxidant approach to disease management and the role of 'Rasayana' herbs of Ayurveda. **Journal of Ethnopharmacology**. 99(2): 165-178.

Graham, J., Higgins, J. A., Gillott, T., Taylor, T., Wilkinson, J., Ford, T., and Billington, D. (1996). A novel method for the rapid separation of plasma lipoproteins using self-generating gradients of iodixanol. **Atherosclerosis**. 124(1): 125-135.

Green, H., and Kehinde, O. (1974). Sublines of mouse 3T3 cells that accumulate lipid. **Cell**. 1(3): 113-116.

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

Grundy, S. (2001). Third report of the national cholesterol education program (NCEP). **National Heart, Lung, and Blood Institute, NIH Publication**. (01-3670).

Guidi, I., Galimberti, D., Lonati, S., Novembrino, C., Bamonti, F., Tiriticco, M., Fenoglio, C., Venturelli, E., Baron, P., and Bresolin, N. (2006). Oxidative imbalance in patients with mild cognitive impairment and Alzheimer's disease. **Neurobiology of Aging**. 27(2): 262-269.

Guo, X., Liu, J., Cai, S., Wang, O., and Ji, B. (2015). Synergistic interactions of apigenin, naringin, quercetin and emodin on inhibition of 3T3-L1 preadipocyte differentiation and pancreas lipase activity. **Obesity Research & Clinical Practice**.

Gupta, R., Gupta, N., and Rathi, P. (2004). Bacterial lipases: an overview of production, purification and biochemical properties. **Applied Microbiology and Biotechnology**. 64(6): 763-781.

Haffner, S. M., Valdez, R. A., Hazuda, H. P., Mitchell, B. D., Morales, P. A., and Stern, M. P. (1992). Prospective analysis of the insulin-resistance syndrome (syndrome X). **Diabetes**. 41(6): 715-722.

Halliwell, B., and Gutteridge, J. M. (2015). **Free Radicals in Biology and Medicine.** Oxford University Press, USA.

Hausman, G., Basu, U., Wei, S., Hausman, D., and Dodson, M. (2014). Preadipocyte and adipose tissue differentiation in meat animals: Influence of species and anatomical location. **Annual Review of Animal Biosciences.** 2(1): 323-351.

Hsu, C.-L., Huang, S.-L., and Yen, G.-C. (2006). Inhibitory effect of phenolic acids on the proliferation of 3T3-L1 preadipocytes in relation to their antioxidant activity. **Journal of Agricultural and Food Chemistry**. 54(12): 4191-4197.

Hung, T. M., Na, M., Thuong, P. T., Su, N. D., Sok, D., Song, K. S., Seong, Y. H., and Bae, K. (2006). Antioxidant activity of caffeoyl quinic acid derivatives

from the roots of *Dipsacus asper* Wall. **Journal of Ethnopharmacology**. 108(2): 188-192.

Iordache, N., Vizeteu, R., Iorgulescu, A., Zmeu, B., and Iordache, M. (2002). Laparoscopic adjustable gastric-banding treatment for morbid obesity our first year experience. **Chirurgia (Bucharest, Romania: 1990)**. 98(2): 135-142.

Islam, M. S., Yoshimoto, M., and Yamakawa, O. (2003). Distribution and physiological functions of caffeoylquinic acid derivatives in leaves of sweet potato genotypes. **Journal of Food Science**. 68(1): 111-116.

Isomaa, B., Almgren, P., Tuomi, T., Forsén, B., Lahti, K., Nissén, M., Taskinen, M.-R., and Groop, L. (2001). Cardiovascular morbidity and mortality associated with the metabolic syndrome. **Diabetes Care**. 24(4): 683-689.

Jensen, R. G. (1983). Detection and determination of lipase (acylglycerol hydrolase) activity from various sources. **Lipids**. 18(9): 650-657.

Jeong, S.-M., Kang, M.-J., Choi, H.-N., Kim, J.-H., and Kim, J.-I. (2012). Quercetin ameliorates hyperglycemia and dyslipidemia and improves antioxidant status in type 2 diabetic db/db mice. **Nutrition Research and Practice**. 6(3): 201-207.

Kang, M.-C., Kang, N., Ko, S.-C., Kim, Y.-B., and Jeon, Y.-J. (2016). Anti-obesity effects of seaweeds of Jeju Island on the differentiation of 3T3-L1 preadipocytes and obese mice fed a high-fat diet. **Food and Chemical Toxicology**. 90: 36-44.

Kao, C.-L., Cho, J., Lee, Y.-Z., Cheng, Y.-B., Chien, C.-Y., Hwang, C.-F., Hong, Y.-R., Tseng, C.-N., and Cho, C.-L. (2015). Ethanolic extracts of *Pluchea indica*

induce apoptosis and antiproliferation effects in human nasopharyngeal carcinoma Cells. **Molecules**. 20(6): 11508-11523.

Kataoka, S., Satoh, J., Fujiya, H., Toyota, T., Suzuki, R., Itoh, K., and Kumagai, K. (1983). Immunologic aspects of the nonobese diabetic (NOD) mouse: abnormalities of cellular immunity. **Diabetes**. 32(3): 247-253.

Kato, H., Tanaka, G., Masuda, S., Ogasawara, J., Sakurai, T., Kizaki, T., Ohno, H., and Izawa, T. (2015). Melatonin promotes adipogenesis and mitochondrial biogenesis in 3T3-L1 preadipocytes. **Journal of Pineal Research**. 59(2): 267-275.

Kelly, J. P., Kaufman, D. W., Kelley, K., Rosenberg, L., Anderson, T. E., and Mitchell, A. A. (2005). Recent trends in use of herbal and other natural products. **Archives of Internal Medicine**. 165(3): 281-286.

Kinnula, V. L., and Crapo, J. D. (2004). Superoxide dismutases in malignant cells and human tumors. **Free Radical Biology and Medicine**. 36(6): 718-744.

Kitabchi, A. E., Umpierrez, G. E., Miles, J. M., and Fisher, J. N. (2009). Hyperglycemic crises in adult patients with diabetes. **Diabetes Care**. 32(7): 1335-1343.

Kongkiatpaiboon, S., Chewchinda, S., and Vongsak, B. (2018a). Optimization of extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea indica* leaves from different provenances in Thailand. **Revista Brasileira de Farmacognosia**. 28(2): 145-150.

Kongkiatpaiboon, S., Chewchinda, S., and Vongsak, B. (2018b). Optimization of extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea*

indica leaves from different provenances in Thailand. **Revista Brasileira de Farmacognosia**. 19(3): 129-143.

Koo, S. I., and Noh, S. K. (2007). Green tea as inhibitor of the intestinal absorption of lipids: potential mechanism for its lipid-lowering effect. **The Journal of Nutritional Biochemistry**. 18(3): 179-183.

Lai, C.-S., Chen, Y.-Y., Lee, P.-S., Kalyanam, N., Ho, C.-T., Liou, W.-S., Yu, R.-C., and Pan, M.-H. (2016). Bisdemethoxycurcumin inhibits adipogenesis in 3T3-L1 preadipocytes and suppresses obesity in High-Fat Diet-Fed C57BL/6 mice. **Journal of Agricultural and Food Chemistry**. 64(4): 821-830.

Lee, L.-S., Kim, S.-H., Kim, Y.-B., and Kim, Y.-C. (2014). Quantitative analysis of major constituents in green tea with different plucking periods and their antioxidant activity. **Molecules**. 19(7): 9173-9186.

Lee, M.-J., and Fried, S. K. (2014). Optimal protocol for the differentiation and metabolic analysis of human adipose stromal cells. **Methods in Enzymology**. 538: 49.

Li, H., and Förstermann, U. (2000). Nitric oxide in the pathogenesis of vascular disease. **The Journal of Pathology**. 190(3): 244-254.

Li, S., Li, S.-K., Li, H.-B., Xu, X.-R., Deng, G.-F., and Xu, D.-P. (2014). Antioxidant capacities of herbal infusions. In: **Processing and Impact on Antioxidants in Beverages**. Elsevier, pp. 41-50

Lichtenstein, A. H., Appel, L. J., Brands, M., Carnethon, M., Daniels, S., Franch, H. A., Franklin, B., Kris-Etherton, P., Harris, W. S., and Howard, B. (2006). Diet and lifestyle recommendations revision 2006: a scientific statement from the

American Heart Association Nutrition Committee. **Circulation**. 114(1): 82-96.

Lien, C. C., Jiang, J. L., Jian, D. Y., Kwok, C. F., Ho, L. T., and Juan, C. C. (2016). Chronic endothelin-1 infusion causes adipocyte hyperplasia in rats. **Obesity**. 24(3): 643-653.

Like, A. A., Rossini, A. A., Guberski, D. L., Appel, M. C., and Williams, R. M. (1979). Spontaneous diabetes mellitus: reversal and prevention in the BB/W rat with antiserum to rat lymphocytes. **Science**. 206(4425): 1421-1423.

Liu, J., Yang, M., and Du, X. (2004). Herbal medicines for viral myocarditis. **The Cochrane Library**.

Liu, J. P., Zhang, M., Wang, W., and Grimsgaard, S. (2002). Chinese herbal medicines for type 2 diabetes mellitus. **The Cochrane Library**.

Liu, Z.-Q., Ma, L.-P., Zhou, B., Yang, L., and Liu, Z.-L. (2000). Antioxidative effects of green tea polyphenols on free radical initiated and photosensitized peroxidation of human low density lipoprotein. **Chemistry and Physics of Lipids**. 106(1): 53-63.

Ma, X., Ding, W., Wang, J., Wu, G., Zhang, H., Yin, J., Zhou, L., and Li, D. (2012). LOC66273 isoform 2, a novel protein highly expressed in white adipose tissue, induces adipogenesis in 3T3-L1 cells. **The Journal of Nutrition**. 142(3): 448-455.

Mammi, C., Marzolla, V., Armani, A., Feraco, A., Antelmi, A., Maslak, E., Chlopicki, S., Cinti, F., Hunt, H., and Fabbri, A. (2016). A novel combined glucocorticoid-mineralocorticoid receptor selective modulator markedly

prevents weight gain and fat mass expansion in mice fed a high-fat diet. **International Journal of Obesity**. 40(6): 964-972.

Manian, R., Anusuya, N., Siddhuraju, P., and Manian, S. (2008). The antioxidant activity and free radical scavenging potential of two different solvent extracts of *Camellia sinensis* (L.) O. Kuntz, *Ficus bengalensis* L. and *Ficus racemosa* L. **Food Chemistry**. 107(3): 1000-1007.

Manoharan, R., Baraga, J. J., Rava, R. P., Dasari, R. R., Fitzmaurice, M., and Feld, M. S. (1993). Biochemical analysis and mapping of atherosclerotic human artery using FT-IR microspectroscopy. **Atherosclerosis**. 103(2): 181-193.

Maritim, A., Sanders, a., and Watkins Iii, J. (2003). Diabetes, oxidative stress, and antioxidants: a review. **Journal of Biochemical and Molecular Toxicology**. 17(1): 24-38.

Matsuo, H., Kondo, Y., Kawasaki, T., and Imamura, N. (2015). Cineromycin B isolated from *Streptomyces cinerochromogenes* inhibits adipocyte differentiation of 3T3-L1 cells via Krüppel-like factors 2 and 3. **Life Sciences**. 135: 35-42.

McDougall, G. J., Kulkarni, N. N., and Stewart, D. (2009). Berry polyphenols inhibit pancreatic lipase activity in vitro. **Food Chemistry**. 115(1): 193-199.

Megalli, S., Davies, N. M., and Roufogalis, B. D. (2006). Anti-hyperlipidemic and hypoglycemic effects of *Gynostemma pentaphyllum* in the Zucker fatty rat. **Journal of Pharmacy and Pharmaceutical Sciences**. 9(3): 281-291.

Mensink, R. P., Aro, A., Den Hond, E., German, J. B., Griffin, B. A., ten Meer, H.-U., Mutanen, M., Pannemans, D., and Stahl, W. (2003). PASSCLAIM–Diet-related cardiovascular disease. **European Journal of Nutrition**. 42(1): i6-i27.

Miller, M. (2009). Dyslipidemia and cardiovascular risk: the importance of early prevention. **QJM : Monthly Journal of The Association of Physicians**. 102(9): 657-667.

Miura, Y., Chiba, T., Tomita, I., Koizumi, H., Miura, S., Umegaki, K., Hara, Y., and Ikeda, M. (2001). Tea catechins prevent the development of atherosclerosis in apoprotein E–deficient mice. **The Journal of Nutrition**. 131(1): 27-32.

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

Moller, D. E., and Flier, J. S. (1991). Insulin resistance-mechanisms, syndromes, and implications. **New England Journal of Medicine**. 325(13): 938-948.

Moreno, D. A., Ilic, N., Poulev, A., Brasaemle, D. L., Fried, S. K., and Raskin, I. (2003). Inhibitory effects of grape seed extract on lipases. **Nutrition**. 19(10): 876-879.

Murase, T., Misawa, K., Minegishi, Y., Aoki, M., Ominami, H., Suzuki, Y., Shibuya, Y., and Hase, T. (2010). Coffee polyphenols suppress diet-induced body fat accumulation by downregulating SREBP-1c and related molecules in C57BL/6J mice. **American Journal of Physiology-Endocrinology and Metabolism**. 300(1): E122-E133.

Nair, A. B., and Jacob, S. (2016). A simple practice guide for dose conversion between animals and human. **Journal of Basic and Clinical Pharmacy**. 7(2): 27-31.

Naowaboot, J., Chung, C. H., Pannangpetch, P., Choi, R., Kim, B. H., Lee, M. Y., and Kukongviriyapan, U. (2012). Mulberry leaf extract increases adiponectin in murine 3T3-L1 adipocytes. **Nutrition Research**. 32(1): 39-44.

Nugroho, A., Bachri, M. S., Choi, J. W., Choi, J. S., Kim, W. B., Lee, B. I., Dai Kim, J., and Park, H. J. (2010). The inhibitory effect of the caffeoylquinic acid-rich extract of *Ligularia stenocephala* leaves on obesity in the high fat diet-induced rat. **Natural Product Sciences**. 16(2): 80-87.

Okabe, Y., Shimada, T., Horikawa, T., Kinoshita, K., Koyama, K., Ichinose, K., Aburada, M., and Takahashi, K. (2014). Suppression of adipocyte hypertrophy by polymethoxyflavonoids isolated from *Kaempferia parviflora*. **Phytomedicine**. 21(6): 800-806.

Oliveira, M. d., Luvizotto, R. d. A. M., Olimpio, R. M. C., Sibio, M. T. d., Silva, C. B. R., Conde, S. J., Padovani, C. R., and Nogueira, C. R. (2013). Modulation of thyroid hormone receptors, TRα and TRβ, by using different doses of triiodothyronine (T3) at different times. **Arquivos Brasileiros de Endocrinologia & Metabologia**. 57(5): 368-374.

Omar, M. A., and Wilson, J. P. (2002). FDA adverse event reports on statin-associated rhabdomyolysis. **Annals of Pharmacotherapy**. 36(2): 288-295.

Pacher, P., Beckman, J. S., and Liaudet, L. (2007). Nitric oxide and peroxynitrite in health and disease. **Physiological Reviews**. 87(1): 315-424.

Paik, S.-G., Blue, M. L., Fleischer, N., and Shin, S.-I. (1982). Diabetes susceptibility of BALB/cBOM mice treated with streptozotocin: inhibition by lethal irradiation and restoration by splenic lymphocytes. **Diabetes**. 31(9): 808-815.

Palmer, R. M., Ferrige, A., and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. **Nature**. 327(6122): 524.

Park, H.-J., Nugroho, A., Lee, J.-H., Kim, J.-D., Kim, W.-B., Lee, K.-R., and Choi, J.-S. (2009). HPLC analysis of caffeoylquinic acids in the extract of *Cacalia firma* and peroxynitrite scavenging effect. **Korean Journal of Pharmacognosy**.

Park, H. J., Yang, J.-Y., Ambati, S., Della-Fera, M. A., Hausman, D. B., Rayalam, S., and Baile, C. A. (2008). Combined effects of genistein, quercetin, and resveratrol in human and 3T3-L1 adipocytes. **Journal of Medicinal Food**. 11(4): 773-783.

Patel, R., Apostolatos, A., Carter, G., Ajmo, J., Gali, M., Cooper, D. R., You, M., Bisht, K. S., and Patel, N. A. (2013). Protein kinase C δ (PKCδ) splice variants modulate apoptosis pathway in 3T3L1 cells during adipogenesis. **Journal of Biological Chemistry**. 288(37): 26834-26846.

Pham-Huy, L. A., He, H., and Pham-Huy, C. (2008). Free radicals, antioxidants in disease and health. **International Journal of Biomedical Science**. 4(2): 89.

Phaniendra, A., Jestadi, D. B., and Periyasamy, L. (2015). Free Radicals: properties, sources, targets, and their implication in various diseases. **Indian Journal of Clinical Biochemistry**. 30(1): 11-26.

Pi-Sunyer, X. (2009). The medical risks of obesity. **Postgraduate Medicine**. 121(6): 21-33.

Poirier, B., Bidouard, J. P., Cadrouvele, C., Marniquet, X., Staels, B., O'connor, S., Janiak, P., and Herbert, J. M. (2005). The anti-obesity effect of rimonabant is

associated with an improved serum lipid profile. **Diabetes, Obesity and Metabolism**. 7(1): 65-72.

Poulos, S. P., Dodson, M. V., and Hausman, G. J. (2010). Cell line models for differentiation: preadipocytes and adipocytes. **Experimental Biology and Medicine**. 235(10): 1185-1193.

Pramanik, K. C., Bhattacharya, P., Biswas, R., Bandyopadhyay, D., Mishra, M., and Chatterjee, T. (2006). Hypoglycemic and antihyperglycemic activity of leaf extract of *Pluchea indica* Less. **Oriental Pharmacy and Experimental Medicine**. 6: 232-236.

Pramanik, K. C., Biswas, R., Mitra, A., Bandyopadhyay, D., Mishra, M., and Chatterjee, T. K. (2007). Tissue culture of the plant *Pluchea indica* (L.) Less. and evaluation of diuretic potential of its leaves. **Oriental Pharmacy and Experimental Medicine**. 7(2): 197-204.

Pramanik, K. C., Ghosh, S., Midya, D. K., and Chatterjee, T. K. (2008). Antitumor activity and antioxidant role of tissue-cultured *Pluchea indica* root against Herlich Ascites carcinoma in swiss albino mice. **International Journal of Biomedical and Pharmaceutical Sciences**. 2(1): 47-50.

Rees, D., Palmer, R., and Moncada, S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. **Proceedings of the National Academy of Sciences**. 86(9): 3375-3378.

Regnier, S. M., El-Hashani, E., Kamau, W., Zhang, X., Massad, N. L., and Sargis, R. M. (2015). Tributyltin differentially promotes development of a phenotypically distinct adipocyte. **Obesity**. 23(9): 1864-1871.

Roussel, A., Miled, N., Berti-Dupuis, L., Rivière, M., Spinelli, S., Berna, P., Gruber, V., Verger, R., and Cambillau, C. (2002). Crystal structure of the open form of dog gastric lipase in complex with a phosphonate inhibitor. **Journal of Biological Chemistry**. 277(3): 2266-2274.

Rupasinghe, H. V., Wang, L., Huber, G. M., and Pitts, N. L. (2008). Effect of baking on dietary fibre and phenolics of muffins incorporated with apple skin powder. **Food Chemistry**. 107(3): 1217-1224.

Russell, T. R., and Ho, R. (1976). Conversion of 3T3 fibroblasts into adipose cells: triggering of differentiation by prostaglandin F2alpha and 1-methyl-3-isobutyl xanthine. **Proceedings of the National Academy of Sciences**. 73(12): 4516-4520.

Sarkar, R., Hazra, B., Mandal, S., Biswas, S., and Mandal, N. (2009). Assessment of *in vitro* antioxidant and free radical scavenging activity of *Cajanus cajan*. **Journal of Complementary and Integrative Medicine**. 6(1).

Sas, K., Robotka, H., Toldi, J., and Vécsei, L. (2007). Mitochondria, metabolic disturbances, oxidative stress and the kynurenine system, with focus on neurodegenerative disorders. **Journal of The Neurological Sciences**. 257(1-2): 221-239.

Sen, T., Dhara, A., Bhattacharjee, S., Pal, S., and Nag Chaudhuri, A. (2002). Antioxidant activity of the methanol fraction of *Pluchea indica* root extract. **Phytotherapy Research**. 16(4): 331-335.

Sen, T., Ghosh, T., Bhattacharjee, S., and Nag Chaudhuri, A. (1996). Action of *Pluchea indica* methanol extract as a dual inhibitor on PAF-induced paw oedema and gastric damage. **Phytotherapy Research**. 10(1): 74-76.

Settharaksa, S., Madaka, F., Sueree, L., Kittiwisut, S., Sakunpak, A., Moton, C., and Charoenchai, L. (2014). Effect of solvent types on phenolic, flavonoid contents and antioxidant activities of *Syzygium gratum* (Wight) SN. **International Journal of Pharmacy and Pharmaceutical Sciences**. 6(2): 114-116.

Shenoy, R., and Shirwaikar, A. (2002). Anti inflammatory and free radical scavenging studies of *Hyptis suaveolens* (Labiatae). **Indian Drugs**. 39(11): 574-577.

Shorr, R. I., Ray, W. A., Daugherty, J. R., and Griffin, M. R. (1997). Incidence and risk factors for serious hypoglycemia in older persons using insulin or sulfonylureas. **Archives of Internal Medicine**. 157(15): 1681-1686.

Singh, U., and Jialal, I. (2006). Oxidative stress and atherosclerosis. **Pathophysiology**. 13(3): 129-142.

Singleton, V. L., Orthofer, R., and Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. **Methods in Enzymology**. 299: 152-178.

Sirichaiwetchakoon, K., Lowe, G. M., Thumanu, K., and Eumkeb, G. (2018). The effect of *Pluchea indica* (L.) Less. tea on adipogenesis in 3T3-L1 adipocytes and lipase Activity. **Evidence-Based Complementary and Alternative Medicine**. 2018.

Slavin, J. L., and Lloyd, B. (2012). Health benefits of fruits and vegetables. **Advances in Nutrition**. 3(4): 506-516.

Smith, M. A., Rottkamp, C. A., Nunomura, A., Raina, A. K., and Perry, G. (2000). Oxidative stress in Alzheimer's disease. **Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease**. 1502(1): 139-144.

Spiegelman, B., Choy, L., Hotamisligil, G., Graves, R., and Tontonoz, P. (1993). Regulation of adipocyte gene expresión in differentiation and syndromes of obesity/diabetes. **Journal of Biological Chemistry.** 268.

Spranger, T., Finckh, B., Fingerhut, R., Kohlschütter, A., Beisiegel, U., and Kontush, A. (1998). How different constituents of human plasma and low density lipoprotein determine plasma oxidizability by copper. **Chemistry and Physics of Lipids**. 91(1): 39-52.

Srisook, K., Buapool, D., Boonbai, R., Simmasut, P., Charoensuk, Y., and Srisook, E. (2012). Antioxidant and anti-inflammatory activities of hot water extract from *Pluchea indica* Less. herbal tea. **Journal of Medicinal Plants Research**. 6(23): 4077-4408.

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

Susetyarini, R. E., and Ariesandyb, M. (2016). Fructose level of male white rats' semen after the treatment of *Pluchea indica* within various observations. **International Journal of Applied Environmental Sciences**. 11(6): 1427-1434.

Tietz, N. W., and Shuey, D. F. (1993). Lipase in serum-the elusive enzyme: an overview. **Clinical Chemistry**. 39(5): 746-756.

Toyokuni, S. (1999). Reactive oxygen species-induced molecular damage and its application in pathology. **Pathology International**. 49(2): 91-102.

Trevisan, M., Liu, J., Bahsas, F. B., Menotti, A., Factor, R., and Group, L. E. R. (1998). Syndrome X and mortality: a population-based study. **American Journal of Epidemiology**. 148(10): 958-966.

Tsai, P.-J., Tsai, T.-H., Yu, C.-H., and Ho, S.-C. (2007). Comparison of NO-scavenging and NO-suppressing activities of different herbal teas with those of green tea. **Food Chemistry**. 103(1): 181-187.

Tsao, R. (2010). Chemistry and biochemistry of dietary polyphenols. **Nutrients**. 2(12): 1231-1246.

Tutino, V., Orlando, A., Russo, F., and Notarnicola, M. (2016). Hydroxytyrosol inhibits cannabinoid CB1 receptor gene expression in 3T3-L1 preadipocyte cell line. **Journal of Cellular Physiology**. 231(2): 483-489.

Upston, J. M., Kritharides, L., and Stocker, R. (2003). The role of vitamin E in atherosclerosis. **Progress in Lipid Research**. 42(5): 405-422.

Valentão, P., Fernandes, E., Carvalho, F., Andrade, P., Seabra, R., and Bastos, M. (2003). Hydroxyl radical and hypochlorous acid scavenging activity of small centaury (*Centaurium erythraea*) infusion. A comparative study with green tea (*Camellia sinensis*). **Phytomedicine**. 10(6-7): 517-522.

Van Puijenbroek, E., Du Buf-Vereijken, P., Spooren, P., and Van Doormaal, J. (1996). Possible increased risk of rhabdomyolysis during concomitant use of simvastatin and gemfibrozil. **Journal of Internal Medicine**. 240(6): 403-404.

Vishwanath, D., Srinivasan, H., Patil, M. S., Seetarama, S., Agrawal, S. K., Dixit, M., and Dhar, K. (2013). Novel method to differentiate 3T3 L1 cells in vitro to produce highly sensitive adipocytes for a GLUT4 mediated glucose uptake

using fluorescent glucose analog. **Journal of Cell Communication and Signaling**. 7(2): 129-140.

Wicker-Planquart, C., Canaan, S., Riviere, M., Dupuis, L., and Verger, R. (1996). Expression in insect cells and purification of a catalytically active recombinant human gastric lipase. **Protein Engineering**. 9(12): 1225-1232.

Widyawati, P. S. (2016). Determination of antioxidant capacity in *Pluchea indica* Less leaves extract and it fractions. **International Journal of Pharmacy and Pharmaceutical Sciences**. 8(9).

Widyawati, P. S., Budianta, T. D. W., Gunawan, D. I., and Wongso, R. S. (2015). Evaluation antidiabetic activity of various leaf extracts of *Pluchea indica* Less. **International Journal of Pharmacognosy and Phytochemical Research**. 7(3): 597-603.

Widyawati, P. S., Wijaya, C. H., Hardjosworo, P. S., and Sajuthi, D. (2013). Volatile compounds of *Pluchea indica* Less and O*cimum basillicum* Linn essential oil and potency as antioxidant. **HAYATI Journal of Biosciences**. 20(3): 117-126.

Witztum, J. L. (1994). The oxidation hypothesis of atherosclerosis. **The Lancet**. 344(8925): 793-795.

Wojcik, M., Burzynska-Pedziwiatr, I., and Wozniak, L. (2010). A review of natural and synthetic antioxidants important for health and longevity. **Current Medicinal Chemistry**. 17(28): 3262-3288.

Wolins, N. E., Quaynor, B. K., Skinner, J. R., Tzekov, A., Park, C., Choi, K., and Bickel, P. E. (2006). OP9 mouse stromal cells rapidly differentiate into

adipocytes: characterization of a useful new model of adipogenesis. **Journal of Lipid Research**. 47(2): 450-460.

World Health Organization. (2000). **Obesity: Preventing and Managing The Global Epidemic.** World Health Organization.

Wu, C., Zhang, X., Zhang, X., Luan, H., Sun, G., Sun, X., Wang, X., Guo, P., and Xu, X. (2014). The caffeoylquinic acid-rich *Pandanus tectorius* fruit extract increases insulin sensitivity and regulates hepatic glucose and lipid metabolism in diabetic db/db mice. **The Journal of Nutritional Biochemistry**. 25(4): 412-419.

Yen, G. C., and Duh, P. D. (1994). Scavenging effect of methanolic extracts of peanut hulls on free-radical and active-oxygen species. **Journal of Agricultural and Food Chemistry**. 42(3): 629-632.

Yokozawa, T., and Dong, E. (1997). Influence of green tea and its three major components upon low-density lipoprotein oxidation. **Experimental and Toxicologic Pathology**. 49(5): 329-335.

Yoshida, H., and Kisugi, R. (2010). Mechanisms of LDL oxidation. **Clinica Chimica Acta**. 411(23-24): 1875-1882.

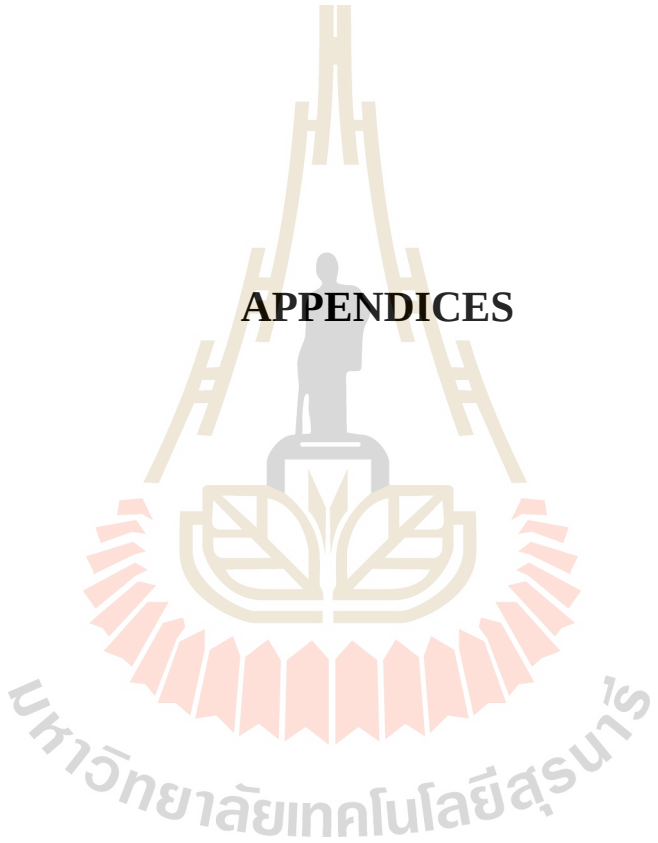
Zebisch, K., Voigt, V., Wabitsch, M., and Brandsch, M. (2012). Protocol for effective differentiation of 3T3-L1 cells to adipocytes. **Analytical Biochemistry**. 425(1): 88-90.

Zhang, B., Deng, Z., Ramdath, D. D., Tang, Y., Chen, P. X., Liu, R., Liu, Q., and Tsao, R. (2015). Phenolic profiles of 20 *Canadian lentil* cultivars and their contribution to antioxidant activity and inhibitory effects on α-glucosidase and pancreatic lipase. **Food Chemistry**. 172: 862-872.

Zhang, X., Wu, C., Wu, H., Sheng, L., Su, Y., Zhang, X., Luan, H., Sun, G., Sun, X., and Tian, Y. (2013). Anti-hyperlipidemic effects and potential mechanisms of action of the caffeoylquinic acid-rich *Pandanus tectorius* fruit extract in hamsters fed a high fat-diet. **Plos One**. 8(4): e61922.

Zimmet, P. Z. (2017). Diabetes and its drivers: the largest epidemic in human history? **Clinical Diabetes and Endocrinology**. 3: 1.

APPENDICES



APPENDIX A

CULTURE MEDIUM FOR 3T3-L1 CELLS

B.1 Phosphate buffer saline (PBS), 1X, pH 7.4

- KH_2PO_4 0.144 g
- $Na_2HPO_4.7H_2O$ 0.795 g
- NaCl 9.0 g
- DI water 1 l

  Adjust pH to 7.2 ± 0.1 and filters sterile (store at 4 ℃).

B.2 Trypsin/EDTA preparation

- Trypsin 0.25 g
- EDTA 0.04 g
- PBS, 1X 100 ml

  Filter, sterile and aliquot (store at 4 ℃).

B.3 Culture media preparation

- FBS (heat inactivated)
  - Slowly thaw the frozen FBS in a beaker filled with water.
  - Put in a water bath at 37 ℃ till completely thaw.
  - Heat inactivate (56 ℃, 20 min), gentle mix every 10 min.
  - Aliquot 45 ml into conical tubes.

  (Store at -20 ℃).

- HEPES buffer, 1M
  - HEPES 23.83 g
  - DI water 100 ml

  Filter, sterile and aliquot (store at -20 °C).
- Penicillin/Streptomycin, 100X
  - Penicillin 0.6 g
  - Streptomycin 1.34 g
  - PBS, 1X 100 ml

  Filter, sterile and aliquot (store at -20 °C).
- DMEM, high glucose, 1X (incomplete medium)
  - DMEM, high glucose, 1X with L-glutamine and phenol red 1 pack
  - $NaHCO_3$ 3.7 g
  - DI water 1 l

  Adjust pH to 7.2-7.4 and filter sterile (store at 4 °C).
- DMEM, high glucose, 1X (complete medium)
  - Inactivated FBS 20 ml
  - Penicillin/Streptomycin 2 ml
  - HEPES buffer, 1M 3 ml

  Adjust volume to 200 ml with DMEM, high glucose, 1X (incomplete medium). (Store at 4 °C)

APPENDIX B

PROCEEDING AND PUBLICATIONS

Sirichaiwetchakoon, K., Lowe, G. M., Thumanu, K., and Eumkeb, G. (2018). The Effect of *Pluchea indica* (L.) Less. Tea on Adipogenesis in 3T3-L1 Adipocytes and Lipase Activity. **Evidence-Based Complementary and Alternative Medicine**. 2018.

Eumkeb, G., Tanphonkrang, S., **Sirichaiwetchakoon, K.**, Hengpratom, T., and Naknarong, W. (2017). The synergy effect of daidzein and genistein isolated from *Butea superba* Roxb. on the reproductive system of male mice. **Natural Product Research**. 31(6): 672-675.

Phitaktim, S., Chomnawang, M., **Sirichaiwetchakoon, K.**, Dunkhunthod, B., Hobbs, G., and Eumkeb, G. (2016). Synergism and the mechanism of action of the combination of α-mangostin isolated from *Garcinia mangostana* L. and oxacillin against an oxacillin-resistant *Staphylococcus saprophyticus*. **BMC Microbiology**. 16(1): 195.

Teethaisong, Y., Autarkool, N., **Sirichaiwetchakoon, K.**, Krubphachaya, P., Kupittayanant, S., and Eumkeb, G. (2014). Synergistic activity and mechanism of action of *Stephania suberosa* Forman extract and ampicillin combination against ampicillin-resistant *Staphylococcus aureus*. **Journal of Biomedical Science**. 21(1): 90.

Eumkeb, G., Naknarong, W., and **Sirichaiwetchakoon, K.** (2013). The effects of red kwao krue (*Butea superba* roxb.) extract on sperm quality and testosterone level in mice. **Thai Journal of Pharmaceutical Sciences**. 38.

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Research Article

The Effect of *Pluchea indica* (L.) Less. Tea on Adipogenesis in 3T3-L1 Adipocytes and Lipase Activity

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Obesity and hyperlipidemia are a major problem in the world. *Pluchea indica* (L.) Less. tea (PIT) is a beverage that has various indications. This study focused on the effect of the PIT on inhibiting adipogenesis of 3T3-L1 cells and pancreatic lipase enzyme activity. The viability of 3T3-L1 cells was not significantly decreased after exposure to 200 to 1000 μg mL^{-1} PIT compared to controls ($p > 0.05$). The PIT at 750 to 1000 μg mL^{-1} exhibited a significantly reduced lipid accumulation compared to the control ($p < 0.05$). The inhibitory effects of the PIT at 250 to 1000 μg mL^{-1} on lipase activity were significantly increased compared to control ($p < 0.05$). The FTIR results showed that the integrated areas of lipids, proteins, nucleic acids, glycogen, and carbohydrates of the PIT-treated 3T3-L1 adipocytes were significantly lower than the untreated 3T3-L1 adipocytes ($p < 0.05$). These findings may indicate that the PIT is not only capable of inhibiting lipids and carbohydrate accumulation in adipocytes but also has a potential to inhibit pancreatic lipase activity. So, the PIT may be further developed to the novel lipid-lowering herbal supplement for the management of overweight or obesity.

1. Introduction

The global prevalence of obesity is increasing worldwide rapidly [1]. Obesity is caused by an imbalance of energy intake and expenditure [2]. The World Health Organization estimates that over 1.5 billion adults are overweight based on Body Mass Index (BMI) ≥ 25 and over 400 million of them are obese based on BMI ≥ 30 [1]. The consequences of an obese population are that they are more prone to develop major health problems such as type 2 diabetes, ischemic heart disease, stroke, and cancer [3, 4]. It is necessary to treat obese individuals by encouraging a reduced calorific intake, inhibiting pancreatic lipase and adipocyte differentiation, and stimulating energy expenditure by increasing physical activity, but also by regulating lipid metabolism and the surgical option such as laparoscopic adjustable gastric banding (LABG) in morbid obesity [2, 5, 6]. Reducing fat digestion and absorption can be effective in treating obesity [2]. Pancreatic lipase is an important enzyme that can hydrolyse dietary triacylglycerol to glycerol and fatty acids in the intestine [7]. Glycerol and fatty acids are regarded as the end products of lipid digestion in the gut; the inhibition of pancreatic lipase may be considered as a fat reducing absorption therapy [7, 8]; that is one mechanism of obesity treatment [9].

The 3T3-L1 cell line is a preadipose cell line which is developed from mouse cells. The 3T3-L1 preadipocyte cells will differentiate into adipocyte cells under an appropriate condition [10]. The inhibition of adipogenesis in 3T3-L1 adipocyte cells by Oil Red O staining method can be implied to attenuate hyperlipidemia and obesity [11].

Fourier transform infrared microspectroscopy (FTIR) is a technique which is used to obtain an infrared spectrum

of absorption or emission with high sensitivity of different functional groups such as lipids, carbohydrates, proteins, and nucleic acids in biological structure. In addition, FTIR has also been used for analysis biomolecular changes in 3T3-L1 adipocytes [11].

Conventional antiobesity and antihyperlipidemic drugs have limited efficacies and critical adverse effects such as Orlistat, often associated with rebound weight gain after the cessation of drug and many patients cannot tolerate its gastrointestinal side effects [12]. Simvastatin, an antihyperlipidemic drug, can cause severe adverse event such as rhabdomyolysis [13]. At present, there is an increased demand for using plants in therapy instead of using synthetic drugs because it may have minor adverse effects and traditional medicinal plants are often cheaper and easily consumable [14].

The plant *Pluchea indica* (L.) Less. (*P. indica*) (family: Asteraceae) is a large evergreen shrub found abundantly in salt marshes. The plant is also known to be used in rheumatoid arthritis [16]. The plant has also been reported to possess diuretic effects. So far some chemical constituents have been isolated from different parts of the plant [17]. Two new thiophene derivatives and two pentacyclic triterpenes have been isolated from the root of this plant [17, 18]. The methanolic extract of *P. indica* leaves showed a reduction in blood glucose level in normal (35.12% and 36.01% for 200 and 400 mg kg^{-1}, respectively) and streptozotocin-induced diabetic rats (36.10% and 41.87% for 200 and 400 mg kg^{-1}, respectively) [19]. The methanol fraction of *P. indica* root extract has been reported to possess significant hepatoprotective properties [20]. In a separate study the extract displayed significant anti-inflammatory activity against glucose oxidase induced paw oedema *in vivo*, inhibited hydroxyl radical and lysis of erythrocytes induced by hydrogen peroxide, and significantly reduced serum enzyme levels (AST, ALT, LDH, and serum alkaline phosphatase), serum bilirubin content in acute liver injury, and total serum protein, albumin, and albumin/globulin ratio [21].

The purpose of this study was to investigate the inhibitory effect of the PIT on pancreatic lipase activity and adipogenesis. The biochemical profile in 3T3-L1 adipocytes was also investigated using the FTIR technique. The total phenolic and flavonoid contents in the PIT were also measured.

2. Materials and Methods

2.1. Plant Materials. Fresh herb of *Pluchea indica* (L.) was collected from Nakhon Ratchasima and Northeast region of Thailand. The plant specimen was authenticated by Dr. Paul J Grote. Identification was made in comparison with the voucher specimen (BKF 194428) and deposited at Forest Herbarium, National Park, Wildlife, and Plant Conservation Department, Ministry of Natural Resources and Environment, Thailand. This herb was washed thoroughly. The production process was performed by the Crystal Biotechnology Co., Ltd., and Suranaree University of Technology. PIT dry sample was added to boiling distilled water. The concentration of PIT sample was calculated from concentration of *Pluchea indica* (L.) dry weight in distilled water (μg mL^{-1}), further heated for 10 min, and then filtered through Whatman No.1 paper.

2.2. Chemicals and Reagents. 3T3-L1 mouse embryonic fibroblasts and bovine calf serum were purchased from the American Type Culture Collection (ATCC, USA). Dulbecco's Modified Eagle's medium (DMEM) with high glucose, Penicillin, Streptomycin, N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Gibco Invitrogen (Grand Island, NY). Bovine calf serum (BCS), fetal bovine serum (FBS), and Oil Red O were obtained from Hyclone (Logan, Utah). Insulin solution from bovine, 3-Isobutyl-1-methylxanthine (IBMX), lipase from porcine pancreas, 4-nitrophenyl dodecanoate (pNP), Orlistat, gallic acid, Folin–Ciocalteau reagent, and catechin were obtained from Sigma-Aldrich (St. Louis, USA). Dexamethasone (DEX) was obtained from G Bioscience (St. Louis, USA). Dimethyl sulfoxide (DMSO) was obtained from Carlo Erba Reagents S.r.l. (Chaussée du Vexin, Val de Reuil, USA). 4-*O*-caffeoylquinic acid (4-CQ), 5-*O*-caffeoylquinic acid (5-CQ), 3,4-*O*-dicaffeoylquinic acid (3,4-CQ), 3,5-*O*-dicaffeoylquinic acid (3,5-CQ), and 4,5-*O*-dicaffeoylquinic acid (4,5-CQ) were purchased from Chengdu Biopurify Phytochemicals Ltd., (Sichuan, China). Other reagents used were all analytical grade.

2.3. Cell Culture. The 3T3-L1 preadipocytes were seeded in a 6-well plate at a density of 5×10^5 cells/well and cultured in DMEM with high glucose, added with 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 10% bovine calf serum until confluent. The cells were maintained at 37°C in 5% CO_2 and 95% humidity.

2.4. Differentiation Procedures. Two days after confluence (day 0), the 3T3-L1 preadipocyte cells were induced to differentiate into adipocytes by adding differentiation medium containing 1.0 μg mL^{-1} insulin, 1.0 μM dexamethasone, 10% FBS, and 0.5 mM of IBMX in DMEM for 48 h (day 2). The differentiation medium was changed to maintain medium on day 2. The maintenance media consisted of 1.0 μg mL^{-1} insulin and 10% FBS in DMEM for 48 h (day 4). The medium was replaced every 48 h until day 10. The 3T3-L1 preadipocytes were treated with various concentrations of the PIT at final concentrations (250 - 1000 μg mL^{-1}) for 48 h during periods of the differentiation phase (at day 0, 2, 4, 6, and 8). At day 10, the differentiation of 3T3-L1 preadipocytes was observed.

2.5. In Vitro Cytotoxic Test (MTT Assay). The cytotoxic effect of the PIT on cell proliferation was determined using the MTT assay [22]. Briefly, the cells were seeded in a 96-well plate at a density of 5×10^3 cells/well. The cells were allowed to adhere for 48 h and then were induced to differentiate into adipocytes by adding differentiation medium and treated with PIT between 250 and 1000 μg mL^{-1}. The differentiation medium was changed to maintain medium and treated with a PIT in various concentration on day 2, and the maintenance

medium with a PIT in various concentrations was replaced every 48 h until day 10. At day 10, the cytotoxic effect of the PIT on cell proliferation was investigated. The culture medium was removed and 0.5 mg mL^{-1} MTT reagent was added, and the cells were incubated for 4 h at 37°C. The viable cells formed formazan crystal and were dissolved in DMSO. The absorbance was measured at 540 nm with a microplate spectrophotometer (Benchmark Plus, Bio-Rad, Japan).

2.6. Oil Red O and Hematoxylin Staining. Oil Red O staining can assess the increased amount of lipid accumulation normally associated with adipocyte differentiation [23]. Briefly, 3T3-L1 preadipocytes were induced to start adipogenesis by standard adipogenic medium and treated with a PIT at various concentrations (250, 500, 750, and 1000 μg mL^{-1}). After 48h, differentiation medium was changed to maintaining medium with different concentrations of the PIT. The medium with various concentrations of the PIT was replaced every 48 h with maintaining medium until day 10. The cells were washed with PBS twice and fixed with 10% formaldehyde in PBS for 1h. After that, cells were washed with distilled water twice and stained with 0.5% Oil Red O solution in 60:40 (v/v) isopropanol: distilled water for 30 min at room temperature. The Oil Red O stained cells were washed twice with distilled water and treated with hematoxylin solution for 10 min at room temperature. The triglyceride droplets were washed twice with 60% isopropanol, eluted with 100% isopropanol, and transferred to new 96 well plates. The lipid accumulation was quantified by measuring the absorbance at 490 nm with a microplate spectrophotometer.

2.7. FPA-FTIR Microspectroscopy. FT-IR microspectroscopy technique was performed to investigate the effect of the PIT on 3T3-L1 adipocyte cells following the method of Eumkeb et al. and Dunkhuntod et al. [11, 24] with minor modifications. In brief, 3T3-L1 cells were seeded at the density 5×10^5 cells/well in a 24-well plate. The samples were divided into 4 groups, including differentiated group (DIF), PIT at 750 μg mL^{-1} (PIT 750), Simvastatin at 1.67 μg mL^{-1} (SIM 1.67), and nondifferentiate group (ND, preadipocytes). The 3T3-L1 cells were collected after treatment for 10 days and centrifuged at 400 × *g* for 5 min. Cells were washed with 0.85% NaCl and recentrifuged at 400 × *g* for 5 min. Cell pellets were dropped onto Barium Fluoride (BaF_2) optical window 13 mm Ø x 2 mm (Crystran, Crystran Ltd) and air vacuum dried for 30 min in a desiccator to eliminate the excess water. The dropped cell slides were kept in a desiccator until analysis with FTIR.

FTIR spectra were performed by using a spectroscopy facility, at the Synchrotron Light Research Institute (Public Organization), Thailand. FTIR spectra were obtained on a Bruker Vertex 27 spectrometer coupled with a Bruker Hyperion 3000 microscope (Bruker Optics Inc., Ettlin-Gen, Germany). The microscope was equipped with nitrogen-cooled 64x64 element MCT, FPA detector, which allowed simultaneous acquisition of spectral data with a 15 x objective.

The spectra were obtained in the transmission mode with the wavenumber range of 4000-700 cm^{-1}. Each of the images used to construct 4x4 binning FTIR image mosaic, 4 cm^{-1} spectral resolution, and 64 scans. The area of the sample, from which single spectra were acquired, was approximately 20 μm x 20 μm. OPUS 7.2 software (Bruker Optics Ltd, Ettlingen, Germany) was used to acquire FTIR spectral data and control instrument system.

The spectra of DIF, PIT (750), SIM (1.67), and ND groups were identified by principal component analysis (PCA) using variability of the Unscrambler 10.1 software (CAMO Software AS, Oslo, Norway). The spectral range of 3000-2800 cm^{-1} and 1800-850 cm^{-1} was used for WEP-treated cells. The preprocessing of the spectra was performed by second derivative transformations using Savitzky-Golay algorithm (nine smoothing points) and normalised with extended multiplicative signal correction (EMSC) using the spectral regions from 3000-2800 cm^{-1} and 1800-950 cm^{-1}. This method is used for identifying the overlapping of absorption peaks, reducing variation between replicates spectra, and correcting for baseline shift. Score plots (2D) and loading plots were used to represent the different classes of data and relations among variables of the data set, respectively. The integrated peak areas of the all groups were analysed using OPUS 7.2 software (Bruker).

2.8. Pancreatic Lipase Assay. Lipase activity assay was based on the lipase cleaving pNP-laureate to produce a coloured product. The aim of this experiment was to determine if PIT could inhibit lipase activity. Any inhibitory activity was compared to Orlistat, a known inhibitor of lipase activity. The method was based on the method of Guo et al. [2]. In brief, porcine pancreas lipase type 2 was dissolved in distilled water at a concentration of 5 mg mL^{-1}. The solution was centrifuged at 10,000 × *g* for 5 min, and the supernatant was collected. Reaction substrate was prepared by pNP laurate in reaction buffer (100 mM Tris buffer pH 8.2). 0.1% (w/v) pNP laurate was mixed with 5 mM sodium acetate (pH 5.0) containing 1% Triton X-100 and was heated in boiling water for 2 min until all solid matters were dissolved. After that, the solution was mixed well and cooled to room temperature. All test samples were dissolved in 50% DMSO in reaction buffer. Then 20 μL of the sample and 30 μL of lipase were mixed and added to 40 μL reaction buffer, and the reaction was started by adding 30 μL substrate solution. 50% DMSO instead of the sample was performed as a negative control, and the solution of Orlistat was used as a positive control. Sample blank for each test sample was prepared by the reaction solutions without enzyme. The mixtures were incubated at 37°C for 6 h and measured at 409 nm using a microplate spectrophotometer. The inhibition rate (%) was described as

$$\text{Inhibitionrate (\%)} = \left(1 - \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{sample blank}}}{\text{OD}_{\text{negative control}}}\right) \times 100 \quad (1)$$

2.9. Determination of Total Phenolic Content (TPC). The total phenolic content was investigated using the Folin–Ciocalteu assay as previously described by Singleton and Rupasinghe et al. [15, 25]. In brief, 100 μL of 0.2 N of Folin-Ciocalteu

was pipetted into a 96-well microtitre plate. This was followed by the addition of either 20 μL of the PIT or various concentrations of gallic acid prepared in methanol (0-0.0625 mg mL^{-1}). Finally, 80 μL of 7.5% (W/V) sodium carbonate was added and the mixture was incubated at room temperature for 2 hours. The absorbance of the blue colour solution was measured at 765 nm by spectrophotometry, and the total phenolic content was determined using a gallic acid standard curve. The results were expressed as mg gallic acid equivalents (mg GAE/g) per gramme of dry weight.

2.10. Determination of Total Flavonoid Content (TFC). The total flavonoid content was measured using an aluminium chloride colourimetric assay [26, 27]. In brief, 125 μL deionised water was pipetted into a 96-well microtitre plate. This was followed by either the addition of 25 μL standard catechin at various concentrations (0-0.4 mg mL^{-1}) or the PIT. Upon completion of the addition of standards or PIT, 10 μL of 5% $NaNO_2$ was also added. The mixture was incubated at room temperature for 6 min. To initiate a colour change, 15 μL of 10% $AlCl_3$ solution was added. The solution was allowed to stand for 5 min at room temperature. To prevent a further reaction, 50 μL of 1 M NaOH was added and shaken in microplate reader spectrophotometry for 5 min before measuring absorbance at 595 nm. The total flavonoid content was determined using a catechin standard curve. The results were presented as mg catechin equivalents (mg CE/g) per gramme of dry weight.

2.11. LC-MS/MS Instrument and Conditions. The chemical characteristic of the PIT was investigated by using LC-MS/MS instrument. The LC-MS/MS system was made up of a combination of chromatographic separation Agilent HPLC 1290 Infinity and the mass analyzer 6490 Triple Quad LC/MS Agilent Technologies equipped with electrospray ionization (ESI) source system, consisting of an autosampler, a binary pump, and vacuum degasser. The chromatographic separation was set on Agilent ZORBAX Rapid Resolution High Definition (RRHD) SB-C18, 2.1 mm id x 150 mm (1.8 μm). Mobile phase system used solvent A and solvent B which consisted of 1% formic acid in water and 1% formic acid in acetonitrile, respectively. Combination of both solvents in LC system was set at a ratio of solvent A: solvent B, 100:0 with gradient elution: from 30% solvent B at 10 min and 100% solvent B at 30 min at a flow rate of 0.2 mL/min. The column temperature was maintained at 25°C, and the sample injection volume was set at 5 μL. The stock solutions of standards (4-CQ, 5-CQ, 3,4-CQ, 3,5-CQ, and 4,5-CQ) were prepared to a final concentration of 1000 μg mL^{-1} by dissolving in methanol, and working solutions were diluted by methanol to obtain the desired concentration and PIT sample working solution was prepared to a final concentration of 1500 μg mL^{-1}.

2.12. Statistical Analysis. All the data were expressed as a mean ± standard deviation (SD). The statistical significance difference between treatment and control groups of cell viability, the amount of lipid accumulation, biomolecular changes, and lipase activity was analysed by one-way analysis of variance (ANOVA) with a Turkey's HSD post hoc test. Values were considered statistically significant when $p < 0.05$ and data were representative of at least three independent experiments.

3. Results

3.1. In Vitro 3T3-L1 Cytotoxic Test (MTT Assay). Concentrations of the PIT from 250 – 1000 μg mL^{-1} did not significantly affect the viability of 3T3-L1 preadipocytes viability compared to control untreated cells (Figure 1), as assessed by the MTT assay ($p > 0.05$). In all subsequent experiments, doses of 1000 μg mL^{-1} or less were used.

3.2. Effect of the PIT on 3T3-L1 Preadipocyte Differentiation and Lipid Accumulation. During differentiation of 3T3-L1 preadipocytes to adipocytes, the cells were treated with the PIT at various concentrations (250, 500, 750, and 1000 μg mL^{-1}), and the intracellular lipid level was quantified using an Oil Red O staining method. The 3T3-L1 preadipocytes exposure to differentiation medium resulted in a significant increase of lipid accumulation in comparison to untreated adipocytes (DIF) ($p < 0.05$) (Figure 3). Microscopic observation of Oil Red O and hematoxylin-stained cells exhibited that PIT decreased Oil Red O stained droplets of mature adipocytes in a dose-dependent manner (Figure 2). The intracellular lipid accumulation showed that PIT at 750 and 1000 μg mL^{-1} significantly decreased the intracellular lipid accumulation to 76.87 ± 3.99 and 71.93 ± 2.05, respectively, compared to untreated 3T3-L1 adipocytes (DIF) ($p < 0.05$) (Figure 3). The 33 and 50% inhibitory effects (IC_{33} and IC_{50}) of the PIT on lipid accumulation were determined to be 1085.5 ± 129.40 and 1841.07 ± 272.60 μg mL^{-1}, respectively. In addition, Simvastatin at 1.67 μg mL^{-1} exhibited a 33% lipid accumulation reduction (IC_{33}). The effect of Simvastatin is therefore 650 times more effective than a PIT. Accordingly, the PIT concentration at 750 μg mL^{-1} was chosen to study FTIR microspectroscopy.

3.3. Biomolecule Changing Detected by FTIR Microspectroscopy. FTIR microspectroscopy was used to determine the biochemical composition of preadipocytes (ND), untreated adipocytes (DIF), Simvastatin- and PIT-treated adipocytes. The FTIR absorption spectrum of the sample at wavelengths between 4000 and 950 cm^{-1} is shown in Figure 4. The three distinct areas of the lipid region (3000-2800 cm^{-1}), the protein regions (1700-1500 cm^{-1}), carbohydrate, and nucleic acid regions (1300-950 cm^{-1}) were investigated. Hence, the spectrum was very difficult to analyse due to the spectral difference between the groups. In order to overcome this problem second derivative of the spectral range at 3000-2800 cm^{-1} and 1800-950 cm^{-1} was analysed (Figures 5(a) and 5(b)).

The strong peak at 2923 cm^{-1} and 2854 cm^{-1} corresponds to the CH_2 asymmetric and symmetric stretching frequency (mainly lipids, with the little from proteins, carbohydrates, and nucleic acids), respectively [28]. The decrease in signal

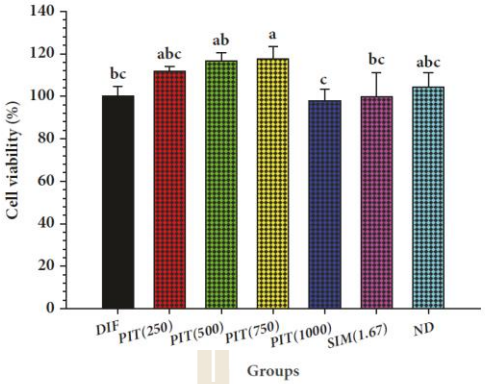

FIGURE 1: The effect of *Pluchea indica* (L.) tea [15] on the viability of 3T3-L1 preadipocytes. DIF = differentiate 3T3-L1 adipocytes; PIT(250) = PIT at 250 μg mL^{-1} treated-3T3 adipocytes; SIM(4) = Simvastatin at 1.67 μg mL^{-1} treated-3T3 adipocytes; ND = 3T3-L1 preadipocytes (nondifferentiated cells). Means ± SD are illustrated for three replicates. Means with the same superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

intensity and area of the peaks (at 2954 cm^{-1} and 2923 cm^{-1}), which reflected an absorption peak of lipids of the PIT-treated adipocytes, presented less than untreated adipocytes (Figure 5(a)). Then, we calculated the ratio of the integrated area of several functional groups, including CH_2 (2938-2906 cm^{-1}, centred at 2923 cm^{-1})/CH_3 (2973-2954 cm^{-1}, centred at 2965 cm^{-1}) asymmetric stretching that belongs to lipids. The results showed that the ratio of the integrated area of the lipids region in the PIT- and Simvastatin-treated adipocytes displayed significantly less than the untreated adipocytes group ($p < 0.05$) (Figure 6(b)). The major contributor in the spectrum ranges from 1700 to 1500 cm^{-1}, which is attributed to an absorption peak of proteins amide I and II. The ratio of integrated area of several functional groups, including CH_2 asymmetric stretching (2938-2906 cm^{-1}, centred at 2923 cm^{-1})/amide I (1674-1624 cm^{-1}, centred at 1654 cm^{-1}), that belong to proteins of the PIT- and simvastatin-treated adipocytes displayed significantly less integral area ratio than the untreated adipocytes group ($p < 0.05$) (Figure 6(b)).

The signal intensity and area of the peaks of the PIT- and simvastatin-treated adipocytes at 1157 cm^{-1} and 1041 cm^{-1} which are attributed to an absorption peak of C–O vibrations from glycogen and other carbohydrates [29] undoubtedly exhibited significantly less than untreated 3T3-L1 adipocytes ($p < 0.05$) (Figure 6(a)). The functional group of PO_2 stretching mode from mainly nucleic acids at 1238 cm^{-1} and 1087 cm^{-1} regions of the PIT-treated adipocytes displayed signal intensity and area less than the untreated adipocytes (Figure 5(b)) and demonstrated significantly less integrated area than that of untreated adipocytes group ($p < 0.05$) (Figure 6(a)) [30].

We further investigated the second derivative spectra using the principal component analysis (PCA). The PCA score plot showed that PIT (750) and DIF cluster were isolated from SIM (1.67) and ND cluster with PC-1 (42%) and the cluster of DIF group were separated from PIT (750), SIM (1.67), and the ND group with PC-2 (14%) (Figure 7(a)). The PCA loading plot was used to detect the wavelength of the spectrum that discriminated the clustering (Figure 7(b)). PC-1 is discriminated by negative loading in the C-H stretching region (centred at 2919 cm^{-1} and 2854 cm^{-1}), negative loading of C-O vibrations from glycogen and other carbohydrates at 1153 cm^{-1}, positive loading of C-O vibrations from glycogen and other carbohydrates at 1064 cm^{-1}, protein amide I, and amide II (positive loading centred at 1658 cm^{-1} and 1548 cm^{-1}, respectively), and negative loading from C=O stretching vibrations of lipids ester (centred at 1734 cm^{-1}). These results suggest that DIF and PIT (750) groups possess higher lipids, glycogen and other carbohydrates, and proteins than SIM (1.67) and ND groups.

The discrimination along PC-2 expressed the separation between DIF group and other groups which demonstrated positive loading in C-H stretching region (centred at 2911 cm^{-1} and 2846 cm^{-1}), negative loading of C-O vibrations from glycogen and other carbohydrates at 1153 cm^{-1} and 1022 cm^{-1}, positive loading of C-O vibrations from glycogen and other carbohydrates at 1064 cm^{-1}, protein amide I (positive loading centred at 1627 cm^{-1}), PO_2-symmetric stretching vibrations of nucleic acid (negative loading centred at 1083 cm^{-1}), and positive loading from C=O stretching vibrations of lipids ester (centred at 1728 cm^{-1}). These results seem consistent with PC-1 that DIF group expresses higher lipids, glycogen and other carbohydrates, proteins, and nucleic acid than other groups.

3.4. Effect of the PIT on Pancreatic Lipase Activity. Pancreatic lipase is an enzyme responsible for the hydrolysis of lipid into free fatty acid and glycerol. The PIT concentrations between 250 and 1000 μg mL^{-1} displayed significantly higher inhibitory lipase activity than those of the controls ($p < 0.05$) (Figure 8). Moreover, the IC_{50} of the PIT for the inhibition

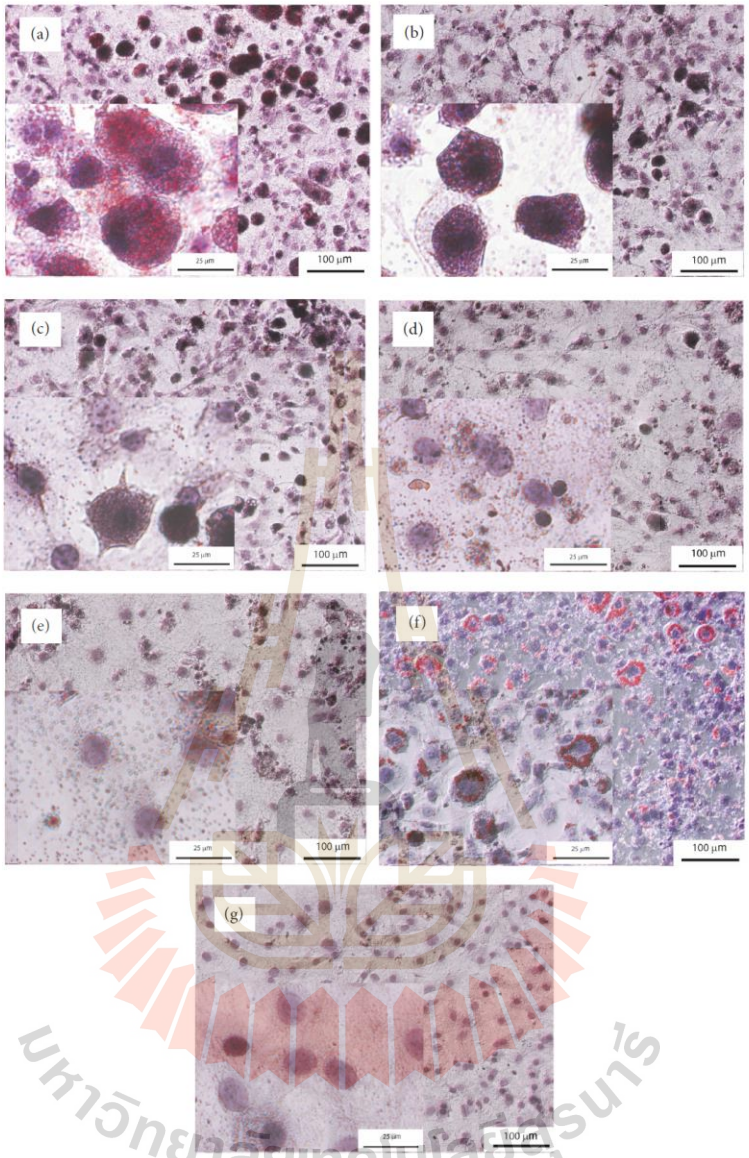

FIGURE 2: Microscopic imaging of intracellular lipid after Oil Red O and haematoxylin staining of samples. **(a)** = Differentiate 3T3-L1 adipocytes (untreated adipocytes); **(b)** = PIT at 250 μg mL^{-1} treated adipocytes; **(c)** = PIT at 500 μg mL^{-1} treated adipocytes; **(d)** = PIT at 750 μg mL^{-1} treated adipocytes; **(e)** = PIT at 1000 μg mL^{-1} treated adipocytes; **(f)** = Simvastatin at 1.67 μg mL^{-1} treated adipocytes; **(g)** = 3T3-L1 preadipocytes (nondifferentiated cells) (original magnification at x100, scale bar; 100 μm and *Inset* view at x600, scale bar; 25 μm).

of pancreatic lipase was 1708.35 ± 335.85 μg mL^{-1}, while the inhibitory effect of the positive control Orlistat at 6.25 to 50 μg mL^{-1} demonstrated an IC_{50} at 68.23 ± 6.67 μg mL^{-1}. Under those circumstances, the potential strength of Orlistat on lipase activity inhibition is approximately 25 times greater than the PIT. These results suggest that the inhibitory implications of the PIT on pancreatic lipase activity increased in a dose-dependent manner.

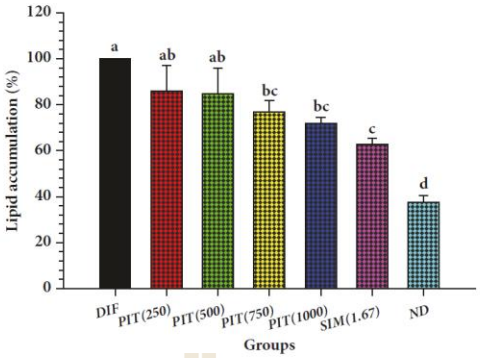

FIGURE 3: The figure graphically represents the effect of the PIT on the percentage of intracellular lipid in 3T3-L1 differentiated cells after Oil Red O staining. DIF = differentiate 3T3-L1 adipocytes (untreated adipocytes); PIT(250) = PIT at 250 μg mL^{-1} treated adipocytes; SIM(1.67) = Simvastatin at 1.67 μg mL^{-1} treated adipocytes; ND = 3T3-L1 preadipocytes (nondifferentiated cells). Means ± SD are illustrated for three replicates. Means with the same superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

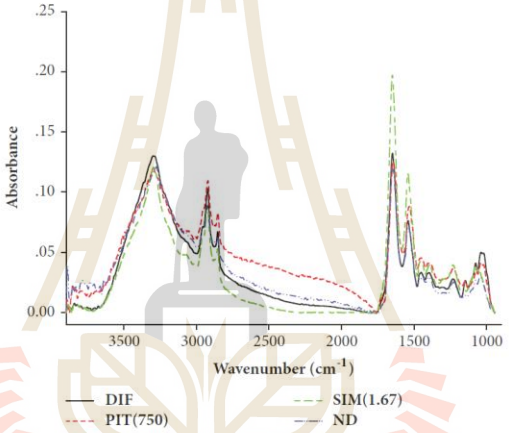

FIGURE 4: Average original FTIR spectra (4000–950 cm^{-1}) obtained from 3T3-L1 cells. DIF = differentiate 3T3-L1 adipocytes (untreated adipocytes); PIT(750) = PIT at 750 μg mL^{-1} treated adipocytes; SIM(1.67) = Simvastatin at 1.67 μg mL^{-1} treated adipocytes; ND = 3T3-L1 preadipocytes (nondifferentiated cells).

3.5. Total Phenolic Content (TPC) of PIT. The total phenolic content was investigated by using the Folin–Ciocalteu colourimetric assay. Gallic acid was used as a standard of the phenolic compound. A standard calibration curve of gallic acid had an R^2 value of 0.9991 and standard equation y = 51.696x + 0.0624. The total phenolic content was calculated and showed as gallic acid equivalents (GAE)/g of dry weight. The results indicated that total phenolic of the PIT was 107.95 ± 4.87 mg GAE/g of dry weight.

3.6. Total Flavonoid Content (TFC) of PIT. Total flavonoid content was investigated by using the aluminium chloride colourimetric assay and expressed regarding catechin equivalent (CE)/g of dry weight. The total flavonoid content was calculated by standard calibration curve of catechin with an R^2 value of 0.994 and the standard equation of y = 3.3315x + 0.0825. The results showed that the total flavonoid content of PIT was 95.33 ± 0.48 mg CE/g of dry weight.

3.7. Chemical Identification of PIT by LC-MS/MS. Negative ion mode was selected for ESI-MS analysis in this study, and the Multiple Reaction Monitoring (MRM) mode has been used for identification. Two pairs of MRM transition were selected at m/z 353.1⟶191.0 and 515⟶353. MRM chromatograms of PIT and standards (4-CQ, 5-CQ, 3,4-CQ, 3,5-CQ, and 4,5-CQ) of MRM transition at m/z 353⟶191.0 are shown in Figure 9(a). The result showed that PIT had been detected 4-CQ and 5-CQ in the extract. Figure 9(b) revealed

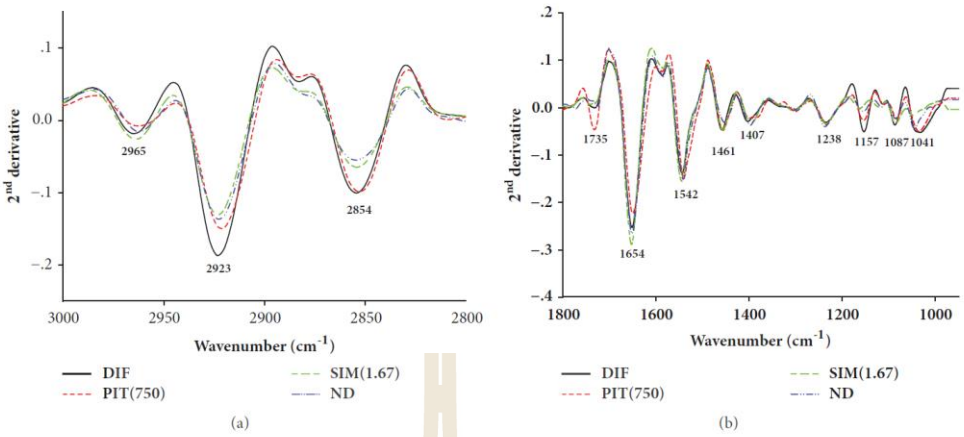

Figure 5: Average the secondary derivative spectra of 3T3-L1 cells. DIF = differentiate 3T3-L1 adipocytes (untreated adipocytes); PIT(750) = PIT at 750 μg mL^{-1} treated adipocytes; SIM(1.67) = Simvastatin at 1.67 μg mL^{-1} treated adipocytes; ND = 3T3-L1 preadipocytes (nondifferentiated cells). The data were represented in two regions: **(a)** lipid regions (3000-2800 cm^{-1}) and **(b)** protein, nucleic acid, glycogen, and other carbohydrate regions (1800-950 cm^{-1}).

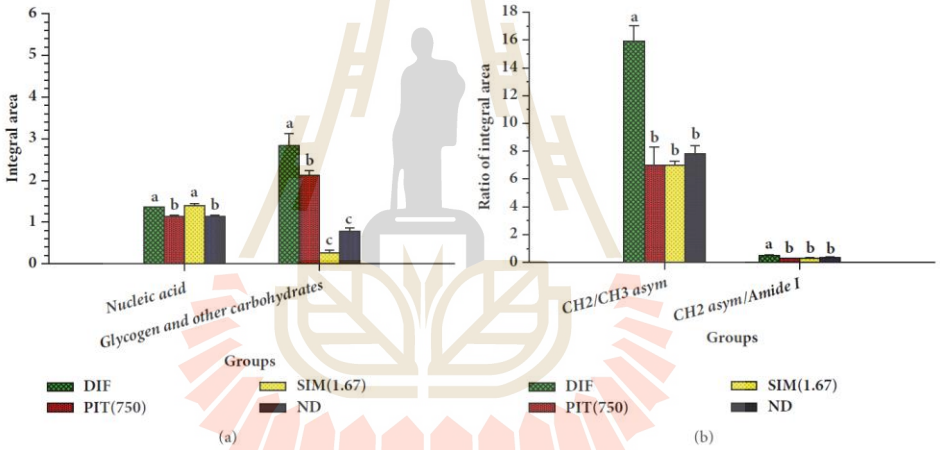

Figure 6: The histogram of integrated areas of 3T3-L1 cells. DIF = differentiate 3T3-L1 adipocytes (untreated adipocytes); PIT(750) = PIT at 750 μg mL^{-1} treated adipocytes; SIM(1.67) = Simvastatin at 1.67 μg mL^{-1} treated adipocytes; ND = 3T3-L1 preadipocytes (nondifferentiated cells). **(a)** The integral area of nucleic acids, glycogen, and other carbohydrates. **(b)** The ratio of the integral area of lipids (CH_2/CH_3 asymmetric stretching) and proteins (CH_2 asymmetric stretching/Amide I). Means ± SD are illustrated for three replicates. Means with the same superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

the chromatograms of PIT and standards of MRM transition at m/z 515⟶353 which explained that 3,4-CQ, 3,5-CQ, and 4,5-CQ were the main ingredients of the PIT.

The quantification of the analytes was performed. We focused on the highest peak of chromatogram that was 3,5-CQ, and the result from MRM data acquisitions showed that PIT concentration 1500 μg mL^{-1} composed of 3,5-CQ 169.93 μg mL^{-1}.

4. Discussion

Obesity and hyperlipidemia are caused by an imbalance between energy intake and energy expenditure which can cause some of the health problems such as type 2 diabetes, ischemic heart disease, stroke, and cancer [2, 31]. Obesity and hyperlipidemia can be treated with conventional medicine example Simvastatin for dyslipidemia and Orlistat for obesity,

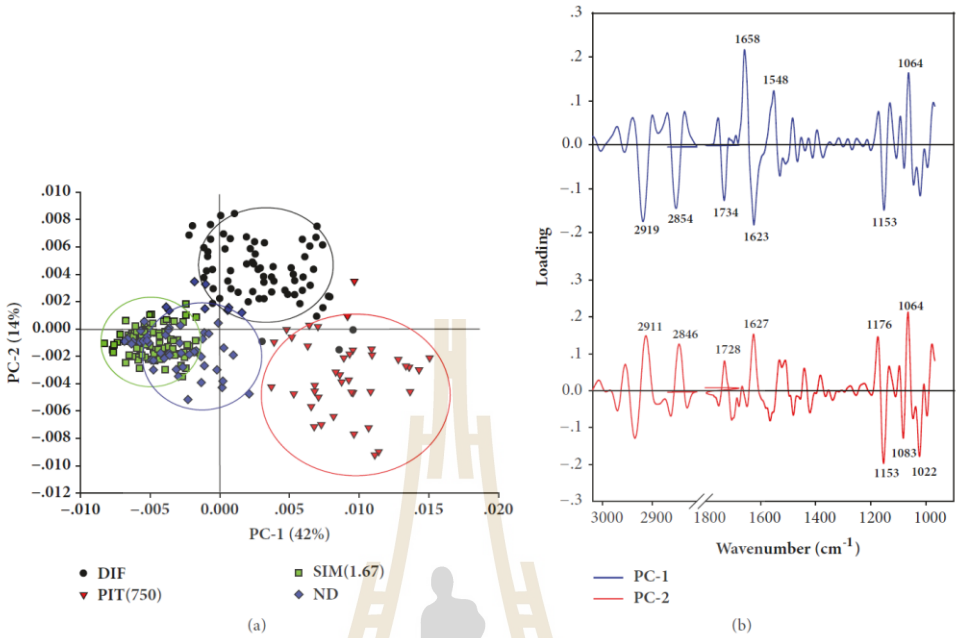

FIGURE 7: PCA analysis of FTIR spectral ranges 3000-2800 cm^{-1} and 1800-950 cm^{-1} giving PCA score plot (**a**) and PCA loading plot (**b**). The 2D PCA score plots showed the clustering separation spectra between groups. DIF = differentiate 3T3-L1 adipocytes (untreated adipocytes); PIT(750) = PIT at 750 μg mL^{-1} treated adipocytes; SIM(1.67) = Simvastatin at 1.67 μg mL^{-1} treated adipocytes; ND = 3T3-L1 preadipocytes (nondifferentiated cells) at day 10 after differentiation. The biomarker differences over a spectral range of samples are identified by PC1 and PC2 loading plots.

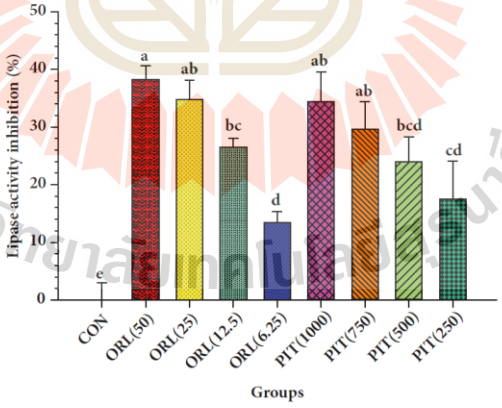

FIGURE 8: Inhibitory effects of PIT (%) at various concentrations on lipase activity. CON = Control; ORL(12.5) = Orlistat at 12.5 μg mL^{-1}; PIT(250) = PIT at 250 μg mL^{-1}. Orlistat was used as a positive control. Means ± SD are illustrated for three replicates. Means with the same superscript are not significantly different from each other (Tukey's HSD test, $p < 0.05$).

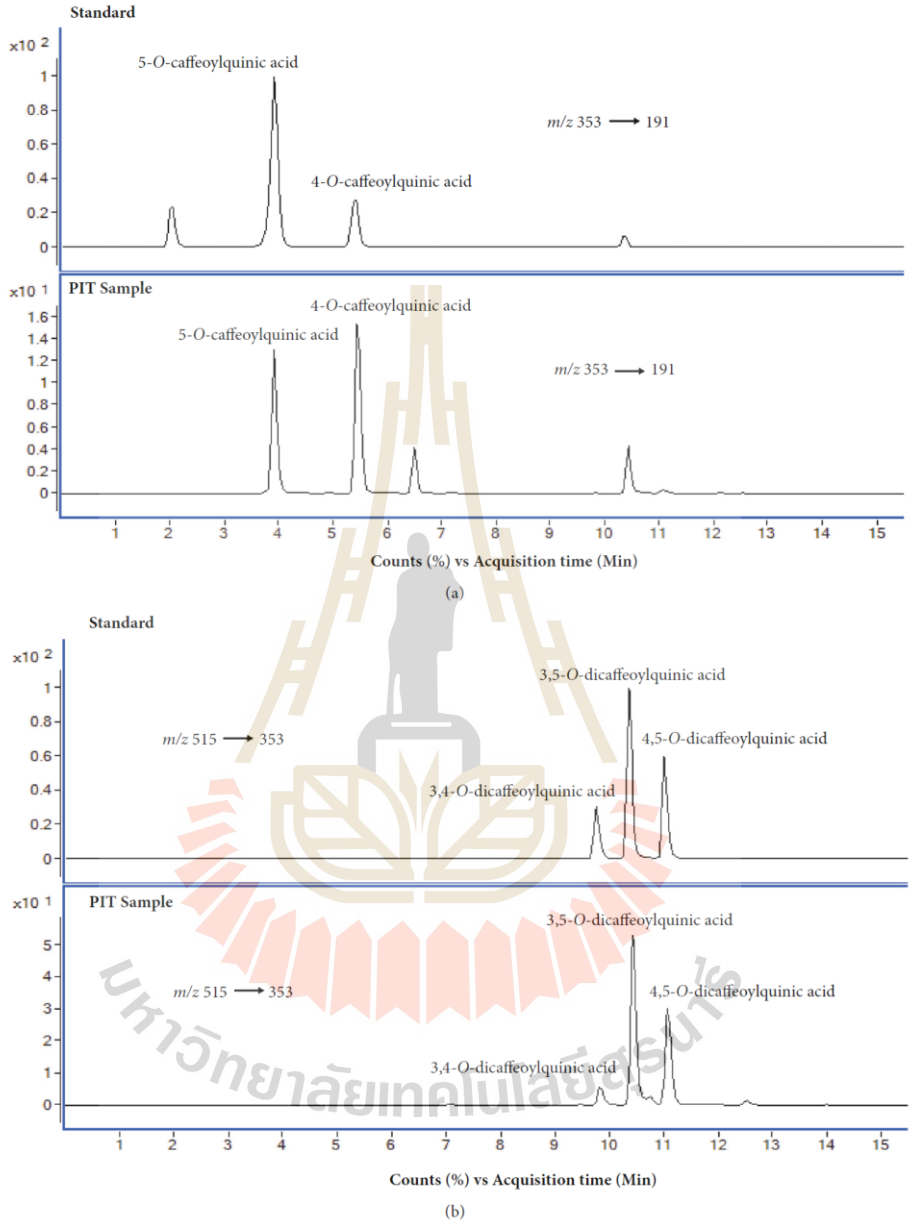

FIGURE 9: MRM chromatograms of PIT and standards (4-CQ, 5-CQ, 3,4-CQ, 3,5-CQ and 4,5-CQ) of MRM transition at m/z 353⟶191.0 **(a)** and at m/z 515⟶353 **(b)**.

but the medication has limited efficacies and has some side effects such as rhabdomyolysis [32], nausea, and vomiting [33]. Therefore, traditional medicine is an essential alternative medicine to alleviate these diseases and may have minor side effects when compared with modern medicine. Moreover, if traditional medicine can be used alone or combined with modern medicine, it will decrease the use of modern medicine or reduce the dose and side effects of modern medicine as well [34, 35].

P. indica (L.) was reported that it had many therapeutic indications such as decreasing blood sugar [19] and reducing blood pressure. In addition, folk medicine can help lower the hyperlipidemia, but this indication has not proven the efficacy by the researcher. Nevertheless, *P. indica* (L.) has the potential for prevention and treatment of metabolic syndrome because it may have effects on both lower blood glucose and blood lipid. Moreover, no work has been done on its efficacy and safety.

The inhibition of adipogenesis using 3T3-L1 cells can predict the efficacy of *P. indica* (L.) on lipid formation inhibition [23]. In addition, the use of MTT assay that has been widely used to evaluate cell viability can investigate the preliminary safety and acute toxicity of the compound. The pancreatic lipase activity investigation can suggest the efficacy of PIT on lipid absorption inhibition from the gastrointestinal tract to the blood results in blood lipid reduction [2].

The MTT assay can detect the cytotoxicity of the compound on the cells. Viable cells can generate MTT to formazan which has a purple colour. The MTT results showed that the viability of cells treated with a PIT at 250–1000 μg mL^{-1} was not significantly different compared to the controls (DIF) ($p > 0.05$). These findings provide evidence that PIT at ≤ 1000 μg mL^{-1} is safe for 3T3-L1 cells. Our results are in substantial agreement with those of Srisook et al. and Pramanik et al. that *P. indica* (L.) alone at 25 to 400 μg mL^{-1} showed no cytotoxic effect in RAW 264.7 macrophage cells [36] and at 400 mg kg^{-1} did not cause any side effects to rat [19].

The investigation of the adipogenesis of 3T3-L1 cell can be tested by using Oil Red O staining technique and illuminated by a microscope. The microscopic observation results of Oil Red O and hematoxylin-stained cells displayed that the PIT decreased Oil Red O stained droplets of mature adipocytes in a dose-dependent manner. Also, we can detect the quantity of lipid by eluting Oil Red O dye by 100% Isopropanol and measuring lipid accumulation by microplate spectrophotometer. Interestingly, the effect of the PIT at 750 and 1000 μg mL^{-1} did not show significantly different in lipid accumulation compared to 1.67 μg mL^{-1} of Simvastatin, which is a leading lipid-lowering drug.

The main active ingredients of the PIT have been investigated by using LC-MS/MS technique. The result showed that the main chemical compositions of the PIT were caffeoylquinic acid derivatives which correlated with Kongkiatpaiboon et al. [37]. Interestingly, caffeoylquinic acid has been reported for its effect on hyperlipidemia, where the enriching of caffeoylquinic acid derivatives in *Pandanus tectorius* fruit extract moderated hyperlipidemia and improved the liver lipid profile in hamsters fed high fat diet and these effects may be caused by increasing the expression of *PPARα* and its downstream genes and by upregulation of LPL and *AMPK* activities [38].

The total phenolic and total flavonoid content results are in substantial agreement with those of Susetyarini et al. [39] that tannin which is phenolic compound was found in *P. indica* (L.). Furthermore, these results are consistent with Andarwulan et al. that the active flavonoid and phenolic compounds were discovered in *P. indica* (L.) [40]. Likewise, *P. indica* (L.) appeared to possess eudesmane derivatives, terpene glycosides, benzenoids, phenylpropanoids, lignan glycosides, stigmasterol glucoside, quercetin, and chlorogenic acid [36].

Furthermore, our data are consistent with those of Hsu et al. that flavonoids and phenolic acids caused 3T3-L1 cell cycle arrest in the G1 phase that may play a role in the control adipogenesis of 3T3-L1 cell and might have the further implication of *in vivo* antiobesity effects [41]. Apart from this, the previous study reported that quercetin, which is the one of active ingredients in *P. indica* (L.) [37], could inhibit adipogenesis in 3T3-L1 adipocyte cell [42]. Moreover, quercetin was reported to have the ability to improve hypertriglyceridemia, alleviate hypercholesterolemia, and elevate HDL-cholesterol in db/db mice by decreasing the expression of peroxisome proliferator-activated receptor-α (*PPAR-α*) and sterol regulatory element-binding protein-1c (*SREBP-1c*) and reducing acetyl-CoA carboxylase (ACC) activity [43]. These results provide evidence that PIT may decrease the adipogenesis of the cells and have the potential to develop to be a herbal supplement that can prevent hyperlipidemia after efficacy and toxicity in animal and human have been investigated.

FTIR microspectroscopy has previously been used to characterise the spectral properties of biological change in various samples [11, 44]. These results showed that the integrated areas of lipids, proteins, nucleic acids, glycogen, and carbohydrates of the PIT-treated adipocytes group were significantly lower than untreated adipocytes group (DIF) ($p < 0.05$) (Figures 6(a) and 6(b)). Our results are in correspondence with those of Dunkhuntod et al. that baicalein reduces lipids, proteins, glycogen, and other carbohydrates in baicalein-treated 3T3-L1 adipocytes compared to untreated 3T3-L1adipocytes [11]. Moreover, the decreasing of integrated area of lipids of FTIR is consistent with the Oil Red O staining results. PCA analysis exhibited discrimination of four clusters of the FTIR spectra of preadipocyte (ND), untreated adipocyte (DIF), and Simvastatin- and PIT-treated adipocytes (Figures 7(a) and 7(b)).

The lipase is the enzyme responsible for the digestion of the lipid before it is absorbed into the bloodstream. The inhibition of the enzyme can reduce the absorption of lipid which can minimise the risk of obesity and hyperlipidemia disease. The results indicated that PIT could inhibit the enzyme lipase activity in a dose-dependent manner and had potency less than Orlistat for 25.04 times. These results are in correspondence with with those of Zhang et al. and Birari that polyphenolic compound like flavonoid could inhibit the enzyme lipase activity and reduce lipid absorption in the intestine [7, 45]. Moreover, Dunkhunthod et al. reported that baicalein could inhibit pancreatic lipase activity [11]. Also,

the polyphenol-rich plants extract derived from grape seed extract, fermented oats, berry, or strawberry had been reported to inhibit lipases activity, and TPC enhanced the pancreatic lipase inhibitory effect [46, 47]. Thus, the inhibitory effect of the PIT may depend on the amount of TPC and TFC, which vary with the PIT concentrations.

5. Conclusions

Our results provide evidence that the PIT can decrease lipid accumulation in 3T3-L1 adipocytes and primarily inhibit adipogenesis. The PIT also modifies the lipid, carbohydrate, protein, nucleic acid and glycogen concentrations within the cells. Furthermore, the PIT could inhibit lipase activity *in vitro*. This study also demonstrates that FTIR microspectroscopy can provide valuable information on the biochemistry of 3T3-L1 adipocytes. The safety of PIT on 3T3-L1 adipocytes suggests that PIT may be developed to hyperlipidemia or anti-obesity herbal. However, these *in vitro* results have to be still confirmed in an animal or human test to achieve blood and tissue therapeutic levels.

Data Availability

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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References

[1] World Health Organization, *Obesity: Preventing and Managing the Global Epidemic*, World Health Organization, 2000.

[2] X. Guo, J. Liu, S. Cai, O. Wang, and B. Ji, "Synergistic interactions of apigenin, naringin, quercetin and emodin on inhibition of 3T3-L1 preadipocyte differentiation and pancreas lipase activity," *Obesity Research & Clinical Practice*, vol. 10, no. 3, pp. 327–339, 2016.

[3] W. Hardeman, S. Griffin, M. Johnston, A. L. Kinmonth, and N. J. Wareham, "Interventions to prevent weight gain: a systematic review of psychological models and behaviour change methods," *International Journal of Obesity*, vol. 24, no. 2, pp. 131–143, 2000.

[4] S. Hasani-Ranjbar, N. Nayebi, B. Larijani, and M. Abdollahi, "A systematic review of the efficacy and safety of herbal medicines used in the treatment of obesity," *World Journal of Gastroenterology*, vol. 15, no. 25, pp. 3073–3085, 2009.

[5] A. E. Chapman, G. Kiroff, P. Game et al., "Laparoscopic adjustable gastric banding in the treatment of obesity: A systematic literature review," *Surgery*, vol. 135, no. 3, pp. 326–351, 2004.

[6] N. Iordache, R. Vizeteu, A. Iorgulescu, B. Zmeu, and M. Iordache, "Laparoscopic adjustable gastric-banding treatment for morbid obesity our first year experience," *Chirurgia (Bucharest, Romania : 1990)*, vol. 98, no. 2, pp. 135–142, 2003.

[7] R. B. Birari and K. K. Bhutani, "Pancreatic lipase inhibitors from natural sources: unexplored potential," *Drug Discovery Therapy*, vol. 12, no. 19-20, pp. 879–889, 2007.

[8] D. A. Moreno, N. Ilic, A. Poulev, D. L. Brasaemle, S. K. Fried, and I. Raskin, "Inhibitory effects of grape seed extract on lipases," *Nutrition Journal* , vol. 19, no. 10, pp. 876–879, 2003.

[9] G. A. Mohamed, S. R. Ibrahim, E. S. Elkhayat, and R. S. El Dine, "Natural anti-obesity agents," *Bulletin of Faculty of Pharmacy, Cairo University*, vol. 52, no. 2, pp. 269–284, 2014.

[10] F. J. Ruiz-Ojeda, A. I. Rupérez, C. Gomez-Llorente, A. Gil, and C. M. Aguilera, "Cell models and their application for studying adipogenic differentiation in relation to obesity: A review," *International Journal of Molecular Sciences*, vol. 17, no. 7, article no. 1040, 2016.

[11] B. Dunkhunthod, K. Thumanu, and G. Eumkeb, "Application of FTIR microspectroscopy for monitoring and discrimination of the anti-adipogenesis activity of baicalein in 3T3-L1 adipocytes," *Vibrational Spectroscopy*, vol. 89, pp. 92–101, 2017.

[12] B. M. Y. Cheung, T. T. Cheung, and N. R. Samaranayake, "Safety of antiobesity drugs," *Therapeutic Advances in Drug Safety*, vol. 4, no. 4, pp. 171–181, 2013.

[13] M. A. Omar and J. P. Wilson, "FDA adverse event reports on statin-associated rhabdomyolysis," *Annals of Pharmacotherapy*, vol. 36, no. 2, pp. 288–295, 2002.

[14] K. A. Amin and M. A. Nagy, "Effect of Carnitine and herbal mixture extract on obesity induced by high fat diet in rats," *Diabetology & Metabolic Syndrome*, vol. 1, article 17, 14 pages, 2009.

[15] H. P. V. Rupasinghe, L. Wang, G. M. Huber, and N. L. Pitts, "Effect of baking on dietary fibre and phenolics of muffins incorporated with apple skin powder," *Food Chemistry*, vol. 107, no. 3, pp. 1217–1224, 2008.

[16] T. Chatterjee, *Herbal Options, Books and Allied (P) Ltd*, Calcutta, India, 2000.

[17] S. Mukhopadhyay, G. A. Cordell, N. Ruangrungsi, S. Rodkird, P. Tantivatana, and P. J. Hylands, "Traditional medicinal plants of thailand. IV. 3-(2′,3′-diacetoxy-2′-methyl butyryl)-cuauhtemone from pluchea indica," *Journal of Natural Products*, vol. 46, no. 5, pp. 671–674, 1983.

[18] A. Chakravarty and S. Mukhopadhyay, "New thiophene derivatives from Pluchea indica," *Indian J Chem Sect B*, vol. 33, no. 10, pp. 978–980, 1994.

[19] K. C. Pramanik, P. Bhattacharya, R. Biswas, D. Bandyopadhyay, M. Mishra, and T. Chatterjee, "Hypoglycemic and antihyperglycemic activity of leaf extract of Pluchea indica Less," *Orient Pharm Exp Med*, vol. 6, pp. 232–236, 2006.

[20] T. Sen, A. Basu, R. N. Ray, and A. K. Nag Chaudhuri, "Hepatoprotective effects of Pluchea indica (less) extract in experimental acute liver damage in rodents," *Phytotherapy Research*, vol. 7, no. 5, pp. 352–355, 1993.

[21] T. Sen, A. K. Dhara, S. Bhattacharjee, S. Pal, and A. K. Nag Chaudhuri, "Antioxidant activity of the methanol fraction of Pluchea indica root extract," *Phytotherapy Research*, vol. 16, no. 4, pp. 331–335, 2002.

[22] F. Denizot and R. Lang, "Rapid colorimetric assay for cell growth and survival—modifications to the tetrazolium dye procedure giving improved sensitivity and reliability," *Journal of Immunological Methods*, vol. 89, no. 2, pp. 271–277, 1986.

[23] J. Naowaboot, C. H. Chung, P. Pannangpetch et al., "Mulberry leaf extract increases adiponectin in murine 3T3-L1 adipocytes," *Nutrition Research*, vol. 32, no. 1, pp. 39–44, 2012.

[24] G. Eumkeb, S. Siriwong, and K. Thumanu, "Synergistic activity of luteolin and amoxicillin combination against amoxicillin-resistant *Escherichia coli* and mode of action," *Journal of Photochemistry and Photobiology B: Biology*, vol. 117, pp. 247–253, 2012.

[25] V. L. Singleton, R. Orthofer, and R. M. Lamuela-Raventós, "Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent," *Methods in Enzymology*, vol. 299, pp. 152–178, 1999.

[26] J. Chen and X. Li, "Hypolipidemic effect of flavonoids from mulberry leaves in triton WR-1339 induced hyperlipidemic mice," *Asia Pacific Journal of Clinical Nutrition*, vol. 16, no. 1, pp. 290–294, 2007.

[27] S. Settharaksa, F. Madaka, L. Sueree et al., "Effect of solvent types on phenolic, flavonoid contents and antioxidant activities of Syzygium gratum (wight) S.N," *International Journal of Pharmacy and Pharmaceutical Sciences*, vol. 6, no. 2, pp. 114–116, 2014.

[28] C. Aksoy and F. Severcan, "Role of vibrational spectroscopy in stem cell research," *Spectroscopy (New York)*, vol. 27, no. 3, pp. 167–184, 2012.

[29] J. Cao, E. S. Ng, D. McNaughton et al., "The characterisation of pluripotent and multipotent stem cells using fourier transform infrared microspectroscopy," *International Journal of Molecular Sciences*, vol. 14, no. 9, pp. 17453–17476, 2013.

[30] S. Garip, A. C. Gozen, and F. Severcan, "Use of Fourier transform infrared spectroscopy for rapid comparative analysis of *Bacillus* and *Micrococcus* isolates," *Food Chemistry*, vol. 113, no. 4, pp. 1301–1307, 2009.

[31] S. M. Artham, C. J. Lavie, R. V. Milani, and H. O. Ventura, "The obesity paradox: impact of obesity on the prevalence and prognosis of cardiovascular diseases," *Postgraduate Medical Journal*, vol. 120, no. 2, pp. 34–41, 2008.

[32] E. P. Van Puijenbroek, P. W. G. Du Buf-Vereijken, P. F. M. J. Spooren, and J. J. Van Doormaal, "Possible increased risk of rhabdomyolysis during concomitant use of simvastatin and gemfibrozil," *Journal of Internal Medicine*, vol. 240, no. 6, pp. 403-404, 1996.

[33] T. D. Filippatos, C. S. Derdemezis, I. F. Gazi, E. S. Nakou, D. P. Mikhailidis, and M. S. Elisaf, "Orlistat-associated adverse effects and drug interactions: a critical review," *Drug Safety*, vol. 31, no. 1, pp. 53–65, 2008.

[34] J. P. Liu, M. Zhang, W. Wang, and S. Grimsgaard, "Chinese herbal medicines for type 2 diabetes mellitus," *Cochrane Database of Systematic Reviews*.

[35] C. Payne, P. J. Wiffen, and S. Martin, "Interventions for fatigue and weight loss in adults with advanced progressive illness," *Cochrane Database of Systematic Reviews*, vol. 1, Article ID CD008427, 2012.

[36] K. Srisook, D. Buapool, R. Boonbai, P. Simmasut, Y. Charoensuk, and E. Srisook, "Antioxidant and anti-inflammatory activities of hot water extract from Pluchea indica Less. herbal tea," *J Med Plant Res*, vol. 6, no. 23, pp. 4077–4408, 2012.

[37] S. Kongkiatpaiboon, S. Chewchinda, and B. Vongsak, "Optimization of extraction method and HPLC analysis of six caffeoylquinic acids in Pluchea indica leaves from different provenances in Thailand," *Revista Brasileira de Farmacognosia*, vol. 28, no. 2, pp. 145–150, 2018.

[38] X. Zhang, C. Wu, H. Wu et al., "Anti-Hyperlipidemic Effects and Potential Mechanisms of Action of the Caffeoylquinic Acid-Rich Pandanus tectorius Fruit Extract in Hamsters Fed a High Fat-Diet," *PLoS ONE*, vol. 8, no. 4, Article ID e61922, 2013.

[39] R. E. Susetyarini and M. Ariesandyb, "Fructose level of male white rats semen after the treatment of pluchea indica within various observations," *Int J Appl Environ Sci*, vol. 11, no. 6, pp. 1427–1434, 2016.

[40] N. Andarwulan, R. Batari, D. A. Sandrasari, B. Bolling, and H. Wijaya, "Flavonoid content and antioxidant activity of vegetables from Indonesia," *Food Chemistry*, vol. 121, no. 4, pp. 1231–1235, 2010.

[41] C.-L. Hsu, S.-L. Huang, and G.-C. Yen, "Inhibitory effect of phenolic acids on the proliferation of 3T3-L1 preadipocytes in relation to their antioxidant activity," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 12, pp. 4191–4197, 2006.

[42] H. J. Park, J. Y. Yang, S. Ambati et al., "Combined effects of genistein, quercetin, and resveratrol in human and 3T3-L1 adipocytes," *Journal of Medicinal Food*, vol. 11, no. 4, pp. 773–783, 2008.

[43] S.-M. Jeong, M.-J. Kang, H.-N. Choi, J.-H. Kim, and J.-I. Kim, "Quercetin ameliorates hyperglycemia and dyslipidemia and improves antioxidant status in type 2 diabetic db/db mice," *Nutrition Research and Practice*, vol. 6, no. 3, pp. 201–207, 2012.

[44] R. Manoharan, J. J. Baraga, R. P. Rava, R. R. Dasari, M. Fitzmaurice, and M. S. Feld, "Biochemical analysis and mapping of atherosclerotic human artery using FT-IR microspectroscopy," *Atherosclerosis*, vol. 103, no. 2, pp. 181–193, 1993.

[45] B. Zhang, Z. Y. Deng, D. D. Ramdath et al., "Phenolic profiles of 20 Canadian lentil cultivars and their contribution to antioxidant activity and inhibitory effects on α-glucosidase and pancreatic lipase," *Food Chemistry*, vol. 172, pp. 862–872, 2015.

[46] S. Cai, O. Wang, M. Wang et al., "In vitro inhibitory effect on pancreatic lipase activity of subfractions from ethanol extracts of fermented oats (Avena sativa l.) and synergistic effect of three phenolic acids," *Journal of Agricultural and Food Chemistry*, vol. 60, no. 29, pp. 7245–7251, 2012.

[47] G. J. McDougall, N. N. Kulkarni, and D. Stewart, "Berry polyphenols inhibit pancreatic lipase activity in vitro," *Food Chemistry*, vol. 115, no. 1, pp. 193–199, 2009.

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Eumkeb, G., Tanphonkrang, S., **Sirichaiwetchakoon, K.**, Hengpratom, T., and Naknarong, W. (2017). The synergy effect of daidzein and genistein isolated from *Butea superba* Roxb. on the reproductive system of male mice. **Natural Product Research**. 31(6): 672-675.

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