EFFECTS OF RICE EXTRACTS ON ADIPOGENESIS AND ANTI-OXIDATIVE STATUS IN 3T3-L1 ADIPOCYTES

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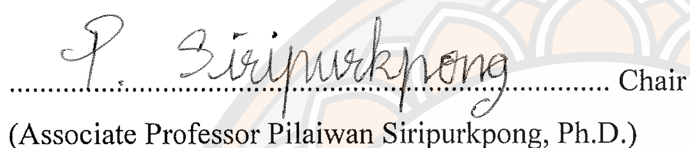

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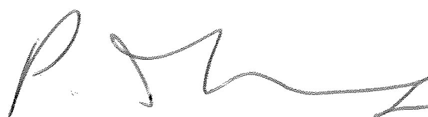

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ABSTRACT

Obesity is a serious risk factor for metabolic diseases such as atherosclerosis and type 2 diabetes mellitus. It is caused by increasing adipose tissue mass via increasing cell number (hyperplasia) and adipocytes size (hypertrophy). Obesity has been reported that excessive lipid accumulation is promoted by increasing adipogenesis, resulting in ROS production in adipocytes. The ROS production affects antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) as well as pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α) and Interleukin-6 (IL-6). Previous studies have found that natural bioactive compounds can be used to treat obesity. The objectives of this study were to identify and examine the effect of two Thai rice cultivars: *O. sativa* L. Variety phitsanulok2 (PL2) and Kaw dokmali105 (KDML105) on the adipogenesis process and the antioxidant, using 3T3-L1 adipocytes as a model. The rice extracts from these two cultivars were polished or white rice (WR), brown rice (BR), and germinated brown rice (GBR). There were two experiments in this study: the first experiment was to measure the antioxidant activity and the total phenolic content of both Thai rice extracts cultivars. The second experiment was divided into two parts: the first part was to study the effect of the rice extracts on adipogenesis in 3T3-L1 adipocytes, while the second part studied the effect of the rice extracts on oxidative status in H_2O_2-induced 3T3-L1 adipocytes. Antioxidant activity and the total phenolic content of the rice extracts were determined by using ABTS and DPPH assay, and Folin Ciocalteu assay, respectively. The results showed that the GBR, BR, and WR extracts from both KDML105 and PL2 cultivars significantly increased

antioxidant activity and total phenolic content in a dose-dependent manner. In addition, the GBR extracts showed a greater antioxidant activity and total phenolic content than BR and WR, respectively. The second experiment was separated into two parts. The first part was studied in normal 3T3-L1 adipocytes. Firstly, the cytotoxicity effect of the rice extracts was determined by MTT assay. Then, the measurement of lipid contents was determined by using Oil-Red O staining technique and TG kit assay. Furthermore, the expression of adipogenesis-related genes, including preadipocyte marker gene (*Pref-1*), adipogenic transcription factors (*PPARγ, C/EBPβ, C/EBPα, and C/EBPδ*), lipogenic (*SREBP-1c, FAS, ACC1, ACC2, aP2,* and *LPL*), lipolytic (*ATGL* and *HSL*), pro-inflammatory cytokines (*TNF-α* and *IL6*), *adiponectin*, and antioxidant (*SOD2*, *GPx4*, and *CAT*) genes by RT-PCR and qPCR techniques. The hypothesis in this part was that 1) intracellular lipid contents will decrease after the rice extract treatments; 2) GBR and BR extracts can down-regulate the expression of adipogenic and pro-inflammatory genes; 3) GBR and BR extracts up-regulate the expression of the preadipocyte marker gene, lipolytic, adiponectin, and antioxidant genes in normal 3T3-L1 adipocytes. The second part studied oxidative stress induced by H_2O_2 in 3T3-L1 adipocytes. The scope of this part was to investigate the effect of the rice extracts on ROS levels determined by DCFH-DA assay and lipid contents examined by Oil-Red O staining. The expression of genes and proteins involved in adipogenesis (*PPARγ* and *SREBP-1c*), insulin-mediated signaling (*IRS1*, *AKT2,* and *PIK3*), inflammation (*TNF-α and IL6*), *adiponectin*, and antioxidant systems (*Nrf2*, *SOD2, HO-1*, *CAT*, and *GPx4*) was measured by qPCR and western blotting, respectively. The results showed that there were no toxic effects of GBR, BR, and WR extracts at concentrations of 0.1, 0.5, and 1 mg/ml. The GBR and BR extracts of both cultivars significantly decreased (P<0.05) intracellular lipid and triglyceride contents in 3T3-L1 adipocytes in a concentration-dependent manner. These results were consistent with the positive controls, caffeine, and GABA. The underlying mechanisms were also investigated and the results were shown that the capacity of the mRNA expression of the preadipocyte marker gene (*Pref-1*) was stable in adipocytes treated with GBR extract from KDML105 cultivars throughout the differentiation. In addition, the expression of adipogenic transcription factors (*PPARγ, C/EBPβ, C/EBPα, and C/EBPδ*), lipogenic (*SREBP-1c, FAS, ACC1, ACC2, aP2,* and *LPL*), and pro-inflammatory (*TNF-α* and *IL6*) genes decreased

significantly ($P<0.05$) in adipocytes treated with BR and GBR extracts of both cultivars. Whereas mRNA expression of lipolytic (*ATGL* and *HSL*), *adiponectin*, and antioxidant (*SOD2*, *GPx4*, and *CAT*) genes increased significantly ($P<0.05$). The second part of the second experiment was examined in oxidative stress-induced 3T3-L1 adipocytes. The results showed that 100 μM H_2O_2 had no cytotoxic effect in both undifferentiated and differentiated cells. The intracellular ROS levels and intracellular lipid accumulation significantly increased ($P<0.05$) in the oxidative stress-induced 3T3-L1 cells, when compared with normal undifferentiated and differentiated adipocytes. Moreover, after being treated with GBR and BR extracts at 0.5 and 1 mg/ml from both cultivars, the intracellular ROS, and lipid contents significantly decreased ($P<0.05$) compared to untreated H_2O_2-induced 3T3-L1 adipocytes. The results examined by qPCR and western blotting showed that the expression of adipogenesis (PPARγ and SREBP-1c), insulin-mediated signaling (IRS1, AKT2, and PIK3), pro-inflammatory cytokines (TNF-α and IL6) genes and proteins significantly decreased ($P<0.05$), respectively after being treated with GBR and BR extracts at 0.5 and 1 mg/ml from both KDML105 and PL2 cultivars, when compared to control adipocytes and untreated H_2O_2-induced 3T3-L1 adipocytes. In addition, the expression of adiponectin, antioxidant-related (Nrf2, SOD2, HO-1, CAT, and GPx4) genes and proteins increased significantly ($P<0.05$) in H_2O_2-induced 3T3-L1 adipocytes treated with BR and GBR extracts of both cultivars, when compared to untreated adipocytes and H_2O_2-induced 3T3-L1 adipocytes. In conclusion, this study demonstrated that antioxidant activity and total phenolic contents comprised in GBR and BR extracts from both KDML105 and PL2 cultivars may be linked to the reduction of intracellular ROS levels in oxidative stress-induced adipocytes. Moreover, decreasing intracellular lipid accumulation may occur via inhibiting the expression of adipogenesis-related genes and proteins (PPARγ, C/EBPβ, C/EBPα, C/EBPδ, SREBP-1c, FAS, ACC1, ACC2, aP2, and LPL. In addition, the GBR and BR extracts also modulated the expression of pro-inflammatory cytokines (TNF-α and IL6), insulin signaling (IRS1, AKT2, and PIK3), adiponectin, and antioxidant (Nrf2, HO-1, SOD2, CAT, and GPx4) genes and proteins. Activation of adiponectin and antioxidant-related molecules as in adipocytes treated with GBR and BR greater than WR extract may enhance insulin sensitivity and antioxidant systems in adipocytes. The present results suggest that the consumption of GBR and BR extracts of both cultivars may have

beneficial health effects for the prevention and/or treatment of obesity and oxidative-related diseases.

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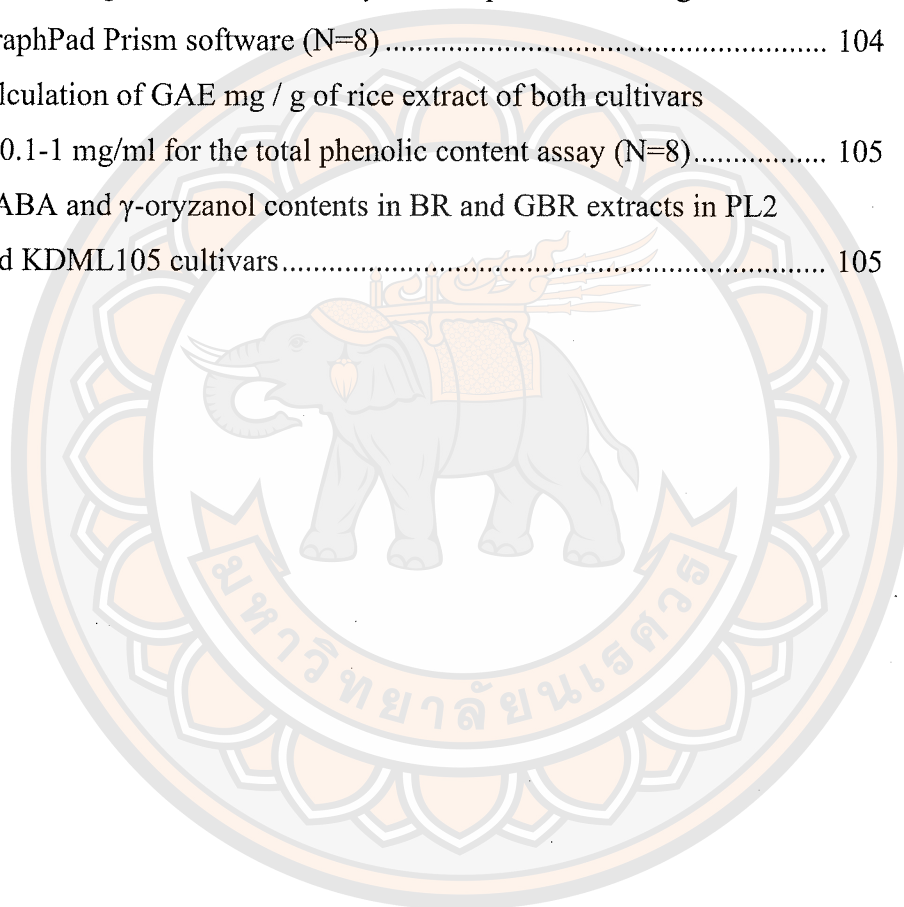

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ABBREVIATIONS

β-actin	=	Beta Actin
μg	=	Microgram
μl	=	Microliter
ACC	=	Acetyl-CoA carboxylase
AD	=	Alzheimer's disease
AGE	=	Advanced glycation end-products
Akt2	=	Threonine-protein kinases 2
ALS	=	Amyotrophic Lateral Sclerosis
AMPK	=	AMP-activated protein kinase
Ang	=	Angiotensin II
ANS	=	Autonomic nervous system
aP2	=	Adipocyte protein 2
ARE	=	Antioxidant-response element
AT	=	Adipose tissue
ATGL	=	Adipose triglyceride lipase
BAT	=	Brown Adipose Tissue
BH_4	=	Tetrahydrobiopterin
BMI	=	Body Mass Index
BTEB	=	Basic transcription element-binding protein
C/EBPs	=	CCAAT/enhancer binding proteins
CAT	=	Catalase
cDNA	=	Complementary deoxyribonucleic acid
ChREBP	=	Carbohydrate response element binding protein
CPT1	=	Carnitine/palmitoyl-transferase 1
CPT2	=	Carnitine/palmitoyl-transferase 2
CVD	=	Cardiovascular disease
Cys	=	Cysteine
DAG	=	Diacylglycerol
DCFH-DA	=	Dichloro-dihydro-fluorescein diacetate

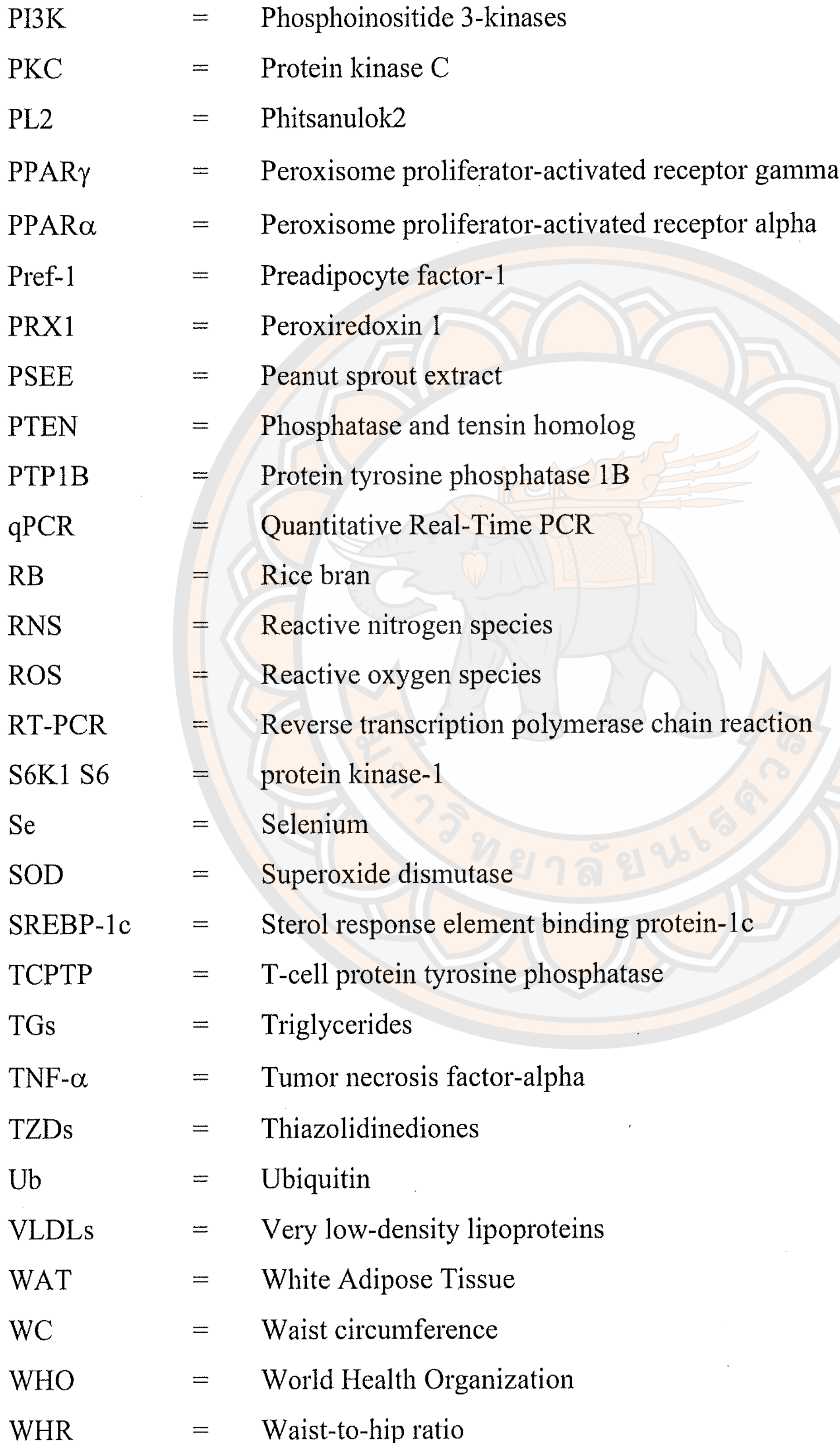
ABBREVIATIONS (CONT.)

DEPC	=	Diethylpyrocarbonate
DEX	=	Dexamethasone
dNTPs	=	Deoxyribonucleostide triphosphate (dATP, dCTP, dGTP, dTTP)
EDTA	=	Ethylenediaminetetraacetic acid
eIF4E	=	Eukaryotic translation initiation factor-4E
eNOS	=	Endothelial nitric oxide synthase
ER	=	Endoplasmic reticulum
ERK1/2	=	Extracellular signal-regulated kinase 1/2
ETC	=	Electron transport chain
FAS	=	Fatty acid synthase
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
GBR	=	Germinated brown rice
GLUT4	=	Glucose transporter 4
GPx4	=	Glutathione peroxidase 4
GR	=	Glutathione reductase
GS	=	Glycogen synthase
GSH	=	Glutathione
GT	=	Green Tea
H_2O_2	=	Hydrogen peroxidase
HFD	=	High fat diet
HGP	=	Hepatic glucose production
HO-1	=	Heme oxygenase-1
HSL	=	Hormone sensitive lipase
IBMX	=	Isobutylmethylxanthin
IC_{50}	=	Inhibitory concentration at 50%
IDLs	=	Intermediate-density lipoproteins
IL1	=	Interleukin-1
IL10	=	Interleukin-10
IL6	=	Interleukin-6

ABBREVIATIONS (CONT.)

iNOS	=	Inducible nitric oxide synthase
IR	=	Insulin resistance
IRF4	=	Interferon regulatory factor 4
IRS1	=	Insulin receptor substrate 1
KDML105	=	Kaw dokmali105
Keap1	=	Kelch-like ECH-associated protein 1
LDL	=	Low-Density Lipoprotein
LPL	=	Lipoprotein lipase
LPO	=	Lipid peroxidation
Maf	=	v-maf musculoaponeurotic fibrosarcoma oncogene homolog
MAPK	=	Mitogen-Activated Protein Kinase
MCE	=	Mitotic clonal expansion
MG	=	Monoglycerides
mRNA	=	Messenger ribonucleic acid
NADPH	=	Nicotinamide adenine dinucleotide phosphate
nm	=	Nanometer
nNOS	=	Neuronal nitric oxide synthase
NO	=	Nitric oxide
NOS	=	Nitric oxide synthase
NOX	=	NADPH oxidases
Nrf2	=	Nuclear factor erythroid 2-related factor 2
OS	=	Oxidative stress
p38 MAPK	=	Mitogen-Activated Protein Kinase p38
PBS	=	Phosphate buffered saline
PC	=	Phosphatidylcholine
PCG-1α	=	Peroxisome proliferator-activated receptor coactivator 1-alpha
PCR	=	Polymerase chain reaction
PDE3B	=	Phosphorylating phosphodiesterase 3b
PE	=	Phosphatidylethanolamine

ABBREVIATIONS (CONT.)



PI3K	=	Phosphoinositide 3-kinases
PKC	=	Protein kinase C
PL2	=	Phitsanulok2
PPARγ	=	Peroxisome proliferator-activated receptor gamma
PPARα	=	Peroxisome proliferator-activated receptor alpha
Pref-1	=	Preadipocyte factor-1
PRX1	=	Peroxiredoxin 1
PSEE	=	Peanut sprout extract
PTEN	=	Phosphatase and tensin homolog
PTP1B	=	Protein tyrosine phosphatase 1B
qPCR	=	Quantitative Real-Time PCR
RB	=	Rice bran
RNS	=	Reactive nitrogen species
ROS	=	Reactive oxygen species
RT-PCR	=	Reverse transcription polymerase chain reaction
S6K1 S6	=	protein kinase-1
Se	=	Selenium
SOD	=	Superoxide dismutase
SREBP-1c	=	Sterol response element binding protein-1c
TCPTP	=	T-cell protein tyrosine phosphatase
TGs	=	Triglycerides
TNF-α	=	Tumor necrosis factor-alpha
TZDs	=	Thiazolidinediones
Ub	=	Ubiquitin
VLDLs	=	Very low-density lipoproteins
WAT	=	White Adipose Tissue
WC	=	Waist circumference
WHO	=	World Health Organization
WHR	=	Waist-to-hip ratio

CHAPTER I

INTRODUCTION

Rationale of the study

Obesity is a chronic disease that is caused by several diverse factors including environmental factors, eating habits, and lifestyle. In addition, physiological factors such as metabolism, operation of cell function, and genetic factors, particularly maternal factors contribute to obesity. Obese patients have a high body weight due to fat deposits in the body. The World Health Organization (WHO) interprets obesity as a body mass index (BMI) > 30 and defines overweight as a BMI = 25 (Sikaris, 2004). Waist circumference is also used for screening and diagnosing overweight or obesity. (Wright and Aronne, 2012). High lipid accumulation in the fat tissue (white adipose tissue) manifests as obesity. Obesity is a prevalent health condition associated with the occurrence of hypertension (Mathew et al., 2007), hyperlipidemia (Gray, & Vidal-Puig, 2007), type 2 diabetes mellitus (Gregor, & Hotamisligil, 2007), and cardiovascular diseases (Ferrarezi et al., 2007).

An increase in the number of fat cells in an organ or tissue (hyperplasia) (Dulloo et al., 2010) has a significant impact by reducing the number of insulin receptors in the body (Deng, & Scherer, 2010). Adipocytes differentiate into mature adipocytes through the process of adipogenesis (Poulos et al., 2010) which is the process of lipid accumulation leading to obesity. The increase in the number of fat cells in an organ or tissue (hyperplasia) (Dulloo et al., 2010) has a significant impact in promoting insulin resistance (IR) (Deng, & Scherer, 2010; Bruun et al., 2003) by reducing insulin sensitivity. The expression of insulin receptor substrate 1 (IRS1) induces the activation of phosphoinositide 3-kinases (PI3K), protein kinase B (PKB or AKT), and glucose transporter 4 (GLUT4), leading to adipocyte differentiation, cell growth, and insulin sensitivity. These will enhance glucose uptake into the adipocyte cells (Khan, & Wang, 2014; Jager et al., 2007). In addition, fat tissue also plays the primary role in the accumulation of triglycerides and secretion of adipokines or adipocytokines which have a biological function similar to hormones produced from the endocrine glands. Several

adipocytokines are secreted by adipose tissue e.g., tumor necrosis factor-alpha; TNF-α, interleukin-6; IL-6, resistin, leptin, and adiponectin (Tzanavari, Giannogonas, & Karalis, 2010; Deng, & Scherer, 2010). Pro-inflammatory cytokines, TNF-α and IL-6, are found in the development of insulin resistance (Fernández-Sánchez et al., 2011), and antagonize the secretion of adiponectin (Fonsrca et al., 2007). Alternatively, adiponectin improves insulin sensitivity (Lastra et al., 2006) and increases nitric oxide (NO) which is produced in endothelial cells (Ouedraogo et al., 2006).

Adipogenesis is the process by which undifferentiated precursor cells are differentiated into fat cells (Rosen et al., 2000). Representatives of transcriptional factor genes such as peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs) such as C/EBPα, C/EBPδ, and C/EBPβ are both intricately involved in the induction of adipogenesis (Farmer, 2006). Lipogenic genes are involved in the process of lipogenesis in adipose tissue, such as the sterol response element binding protein-1c (*SREBP-1c*), lipoprotein lipase (*LPL*), fatty acid synthase (*FAS*), adipocyte protein 2 (*aP2*), and acetyl-CoA carboxylase (*ACC*) (Armani et al., 2010). In addition, lipolytic genes are known to elicit the suppression of lipid accumulation in adipocytes (Zechner et al., 2012) such as adipose triglyceride lipase (*ATGL*) and hormone sensitive lipase (*HSL*). In the present study, an increase in *PPARγ* expression was necessary for adipogenesis in 3T3-L1 adipocytes, which displayed *PPARγ* expression importance in the differentiation of adipocytes (Rios-Vazquez et al., 2006). Reactive Oxygen Species (ROS) can potentiate oxidative stress and inflammation, which can impair insulin sensitivity (Houstis et al., 2006). Glucose intolerance was observed in mice fed with a high-fat diet (HFD) that caused an increase in the size of adipocyte cells (Cani et al., 2007; Riant et al., 2009). It has also been shown that vascular ROS can promote inflammatory responses that contribute to the development of obesity (Fernández-Sánchez et al., 2011).

Adipose tissue can produce ROS via several pathways, such as the electron transport chain (ETC) in the mitochondria (Castro et al., 2016), the NADPH oxidase 4 (NOX4) enzyme (Schroder et al, 2009), and endothelial NOS (eNOS) (Le Lay et al., 2014). Therefore, an increase in the number of, and size of, adipocyte cells in adipose tissue has a positive correlation with the levels of ROS (Han, 2016). High levels of ROS can imbalance oxidative stress and antioxidant systems such as the antioxidant enzymes,

superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Amirkhizi et al., 2007). Thus, high fat accumulation can induce high levels of oxidative stress, which may cause lipid peroxidation, protein oxidation, and cellular damage (Hosogai et al., 2007). The increase in lipid accumulation associated with oxidative stress is maybe due to the possession of exorbitant adipose tissue itself because adipocytes have been identified as a source of pro-inflammatory cytokines, including TNF-α, interleukin-1 (IL-1), and interleukin-6 (IL-6). Obesity causes unbalanced antioxidant activities by decreasing enzyme activities such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Amirkhizi et al., 2007). It has been reported that increases in the size of adipocyte and glucose intolerance were observed in mice fed with a high-fat diet (HFD) (Cani et al., 2007; Riant et al., 2009). As well, vascular ROS can promote an inflammatory response that contributes to the development of obesity (Fernández-Sánchez et al., 2011).

Disposition to obesity is often based on lifestyle factors such as exercise and dietary habits (Simonyi et al., 2012). Drugs such as orlistat, sibutramine, and dinitrophenol are used to treat obesity (Filippatos et al., 2008). However, these drugs have been associated with adverse effects which include oily stools, flatulence, abdominal pain, and dyspepsia (Hanif, & Kumar, 2002; Cook, & Bloom, 2006). Therefore, it is suggested that natural medicinal products, especially the ones that exhibited antioxidant activity, might be an alternative strategy for the development of effective anti-obesity drugs and supplements. The antioxidant components of natural extracts include flavonoids, vitamins, capsaicin, and resveratrol (James et al., 2004).

Antioxidants are molecules that inhibit or quench free radicals and delay or inhibit cellular damage. They exist both in enzymatic and non-enzymatic forms in the intracellular and extracellular environments (Young, & Woodside, 2001). However, antioxidants can also assist in the prevention of diseases such as atherosclerosis, Alzheimer's disease, diabetes mellitus, and cancer (Bagchi, & Puri, 1998; Penny et al., 2004). Vitamins such as vitamins C and E (Tafazoli et al., 2005; Vojdani et al., 2000), and minerals such as selenium, carnitine (Malaguarnera et al., 2009), and coenzyme Q 10 (Crane, 2001) are also antioxidants found in foodstuffs. As well, bioactive compounds found in plants such as flavonoids, anthocyanins, and hydroxybenzoic acids (Robert, 2011) have been shown to play a vital role in scavenging ROS. Previous research has

identified the chemical composition of Thai rice cultivars and their antioxidant activities (Norhaizan et al., 2013). The bioactive compounds in these rice cultivars are mainly attributed to the phenolic compounds found in the rice: GABA, γ-oryzanol, phytic acid, and α-tocopherol (Jannoey et al., 2010; Moongngarm, & Saetung, 2010).

Rice extracts have metabolically beneficial effects. Recent studies have shown that rice extracts prevent obesity and type 2 diabetes (Sun, 2012) by improving glucose uptake (Chisayo et al., 2013). Germinated brown rice (GBR) extracts also suppressed lipid accumulation in the 3T3-L1 adipocytes and have been shown to reduce body weight, size of adipose tissue, the level of serum triglycerides, and total cholesterol in obese mice (Ho et al., 2012). In addition, GBR exhibited anti-obesity effects through the suppression of body weight gain and food intake, improvement of lipid profiles, and reduction of leptin level and white adipose tissue mass in obese rats fed with HFD (Lim et al., 2014). GBR exhibited anti-obesity effects through the suppression of body weight gain and food intake, the improvement of lipid profiles, the reduction of leptin levels, and white adipose tissue mass, in obese rats fed with a high-fat diet (Lim et al., 2016). However, the mechanisms of Thai rice extracts on the health effects of 3T3-L1 adipocytes induced by H_2O_2, have not yet been clearly examined.

Objectives of the study

The main aims of this study were as follows.

1. To study the antioxidant activity of rice extracts by 1, 1-Diphenyl-2-picryl-hydroxyl (DPPH) and 2, 2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay.

2. To study the total phenolic content of rice extracts by Folin-Ciocalteu assay.

3. To study the effect of rice extracts on cytotoxicity in 3T3-L1 adipocytes by [3-(4, 5-Dimethylthiazol-2-yl) -2, 5-Diphenyltetrazolium Bromide] (MTT) assay.

4. To study the effects of rice extracts on lipid accumulation in 3T3-L1 adipocytes by Oil-Red O staining and Triglyceride quantification kit assay.

5. To study the effects of rice extracts on ROS levels in 3T3-L1 adipocytes by Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay.

6. To study the effects of rice extracts on the expression of *preadipocyte factor1*, *adipogenic transcription factors*, *lipogenic*, *lipolytic*, *inflammations*, *insulin*

sensitivity, and *antioxidants* genes in 3T3-L1 adipocytes by reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qPCR) techniques.

7. To study the effect of rice extracts on expression of adipogenic transcription factors, insulin sensitivity, and antioxidants proteins in 3T3-L1 adipocytes by western blot technique.

The scope of the study

Experiment 1

The scope of this study was to identify the antioxidant activity of the two Thai rice extracts cultivars *O. sativa* L. Variety phitsanulok2 (PL2) and Kaw dokmali105 (KDML105), with the antioxidant activity of these rice extracts determined by using ABTS and DPPH assays and measuring the total phenolic contents by the Folin Ciocalteu assay.

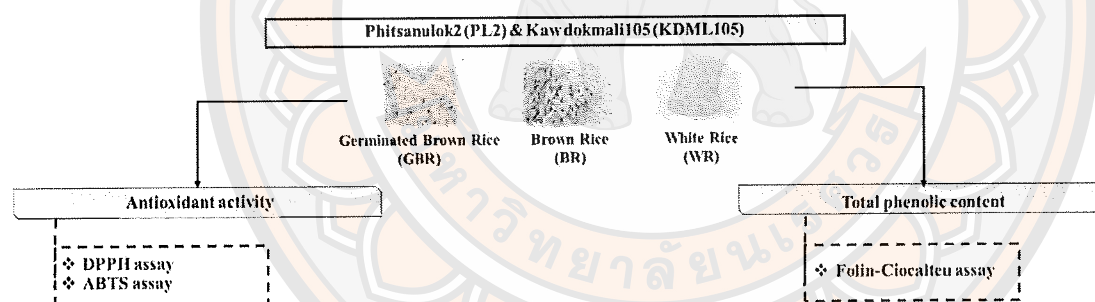


Figure 1 The scope of the study in Experiment I

Experiment I: The hypotheses are:

1. Thai rice extracts increase the antioxidant activity of rice extracts.
2. Thai rice extracts increase the total phenolic contents of rice extracts.

The scope of experiment II in part I (Normal adipocytes)

To examine the effect of the Thai rice cultivars on the adipogenesis process and the antioxidant effect of these cultivars, 3T3-L1 adipocytes were used as a model, the study was divided into sections. First, the effects of the rice extracts on cell cytotoxicity were determined by MTT assay. Oil Red O staining and a Triglyceride

quantification kit assay were used for determining lipid accumulation. Furthermore, the expression of *preadipocyte factor1*, *adipogenic transcription factors*, *lipogenic*, *lipolytic*, *inflammations*, and *antioxidants* genes in 3T3-L1 adipocytes was measured using RT-PCR and subsequently analyzed quantitatively by qPCR.

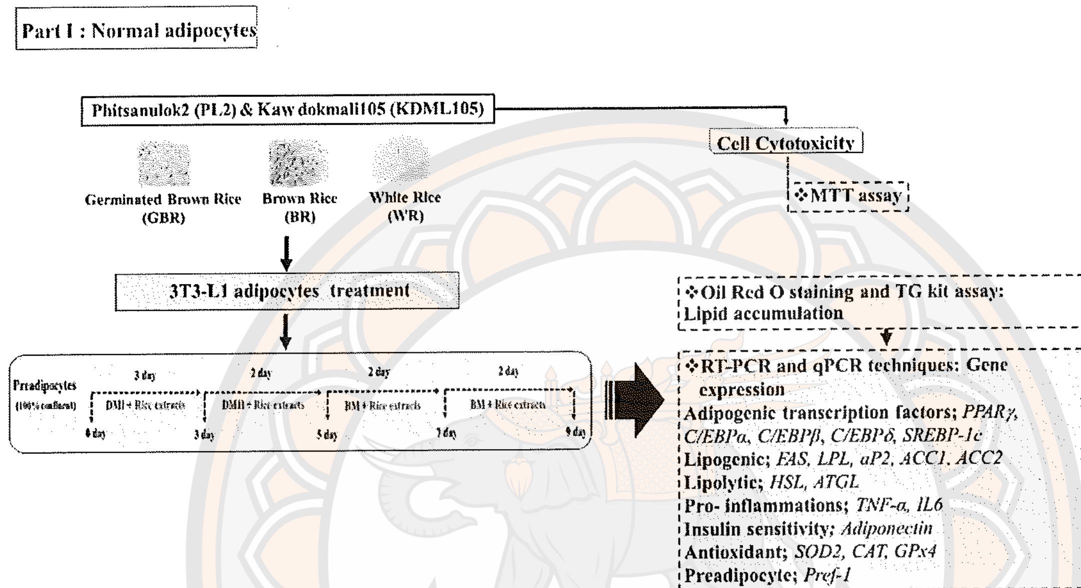


Figure 2 The scope of the study in part I.

Experiment II: The hypotheses in part I are:

1. Thai rice extracts decrease lipid contents in 3T3-L1 adipocytes.

2. Thai rice extracts down-regulate and up-regulate the expression of *adipogenic transcription factors*, *lipogenic*, *lipolytic*, *pro-inflammation*, *insulin sensitivity*, *antioxidants*, and *preadipocyte factor1* genes in 3T3-L1 adipocytes.

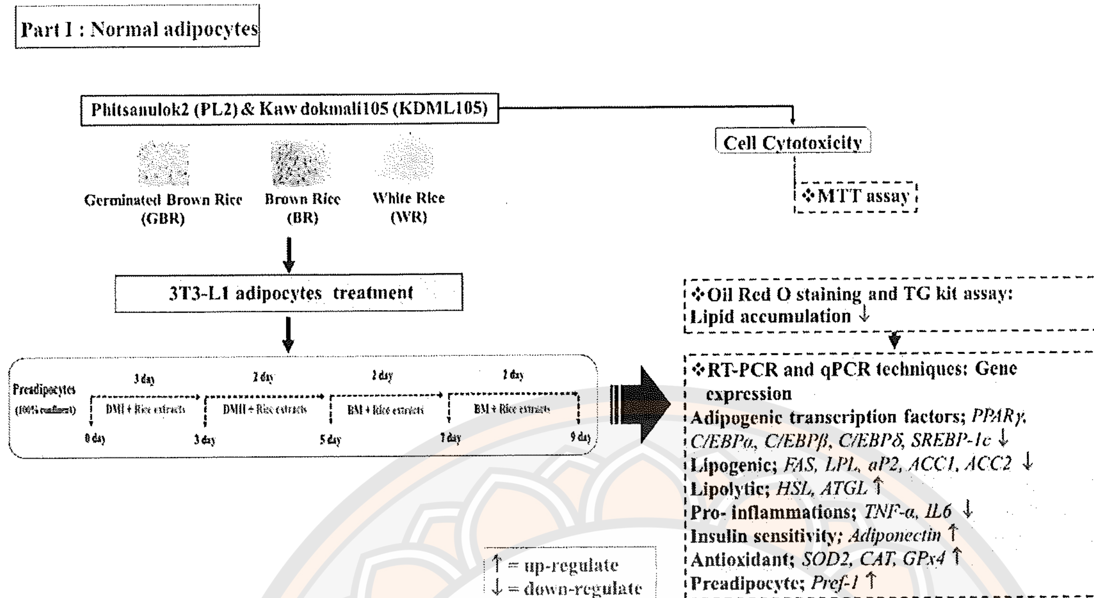


Figure 3 The hypotheses diagram of part I

The scope of experiment II in part II (H_2O_2-induced adipocytes)

The effect of rice extracts on ROS levels and lipid contents were determined by DCFH-DA assay and Oil-Red O staining, respectively. The expression of the adipogenic transcription factors, insulin-mediated signaling, pro-inflammatory cytokines, anti-inflammatory cytokines, and antioxidant-related genes and proteins in 3T3-L1 adipocytes induced by H_2O_2 was measured by qPCR and western blot, respectively.

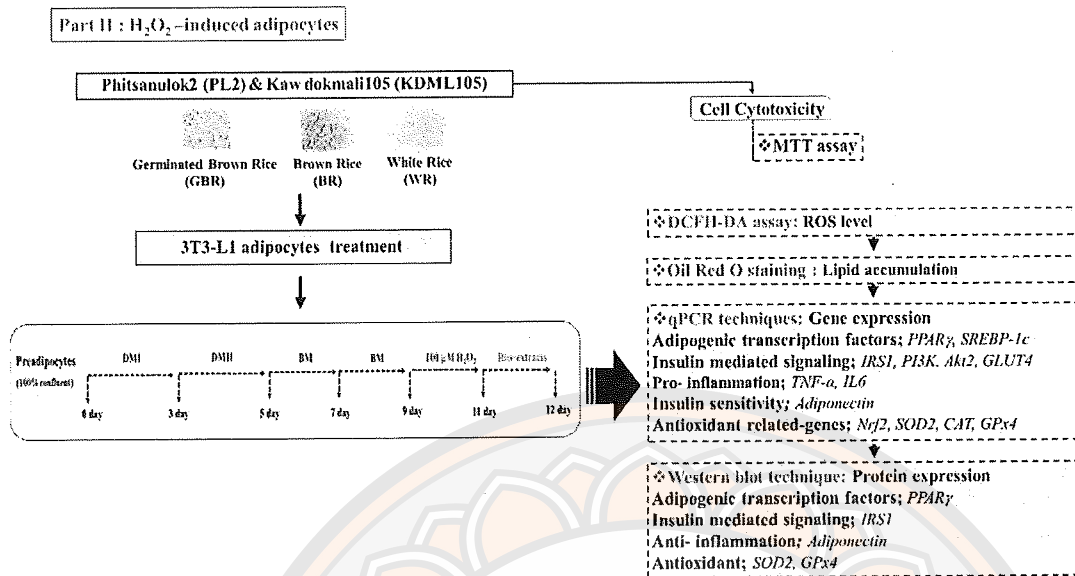


Figure 4 The scope of the study in part II

Experiment II: The hypotheses in part II are:

1. Thai rice extracts reduce lipid contents, and ROS levels in H_2O_2-induced 3T3-L1 adipocytes.

2. Thai rice extracts down-regulate the expression of adipogenic transcription factors, insulin-mediated signaling, and pro-inflammatory cytokines genes and proteins, and up-regulate the expression of anti-inflammatory cytokines, and antioxidant-related genes and proteins in H_2O_2-induced 3T3-L1 adipocytes.

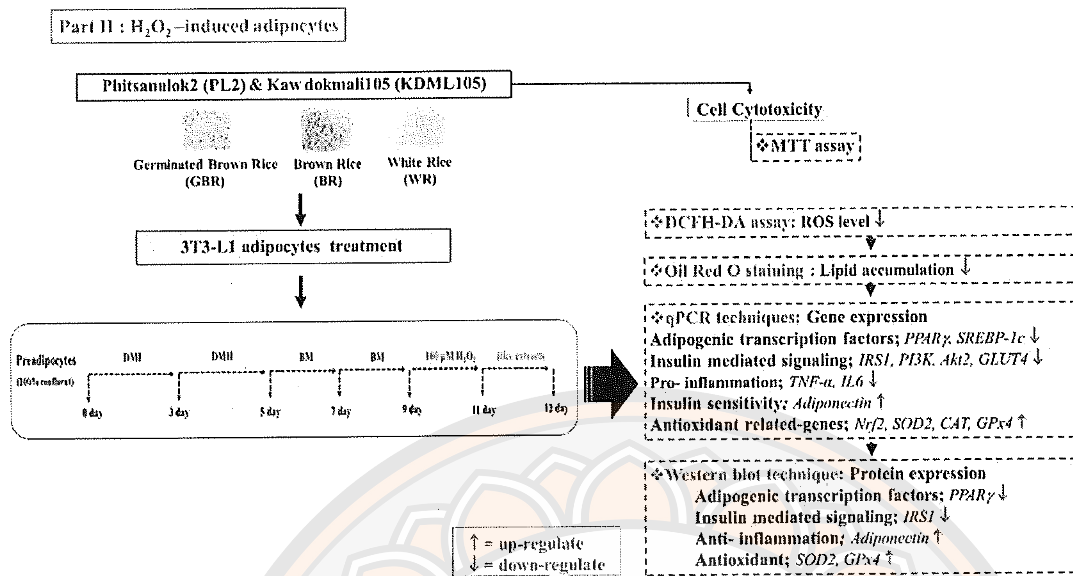


Figure 5 The hypotheses diagram of part II

Expected outcomes of the study

1. This research provides information regarding the antioxidant capacities of Thai rice extracts that can be used as a potential source of natural antioxidants.

2. This research extends the current knowledge related to the anti-obesity effect of Thai rice extracts and their molecular mechanisms.

CHAPTER II

LITERATURE REVIEW

Obesity

The World Health Organization (WHO) defines obesity as a condition of an abnormal excessive fat accumulation in adipose tissue (WHO, 2013). Obesity is a serious nutritional problem, which causes the risk of irregularity pathologies, including dyslipidemia, hypertension, type 2 diabetes, stroke, coronary heart disease, non-alcoholic fatty liver disease, osteoarthritis, sleep apnea, breast, prostate, and cancers (WHO, 2013). The WHO has defined obesity when one has body mass index (BMI) greater than 30 is obesity (Table 1). The BMI is calculated from the weight (in kilograms) divided by the square of height (in meters). The BMI valve range is based on data from America population; a BMI of ≥ 30 kg/m^2 or more is an index of obesity, while a BMI over 25 kg/m^2 is the index of overweight. For Asian, a BMI of ≥ 27.5 kg/m^2 or more is the index of obesity in the event that a BMI over 23 kg/m^2 is the index of overweight (Fernández-Sánchez et al., 2011). Waist circumference (WC) or waist-to-hip ratios (WHR) are also a useful index of visceral fat distribution: WC ≥80 cm (in women) or 94 cm (in men) and WHR above 0.90 for males and 0.85 for females are associated with obesity (Nishida et al., 2010). It is now well known that abdominal fat is a major risk for obesity-related diseases such as visceral fat accumulation contributes to pro-oxidant and pro-inflammatory states, as well as to modifications in glucose and lipid metabolisms (Lear et al., 2010).

Table 1 Body Mass Index (BMI)

Classification	Principal cut-off points (kg/m^2)	Additional cut-off points (kg/m^2)
Under Weight	< 18.50	< 18.50
Severe thinness	< 16.00	< 16.00
Moderate thinness	16.00-16.99	16.00-16.99
Mild thinness	17.00-18.49	17.00-18.49
Normal range	18.50-24.99	18.50-24.99
		23.00-24.99
Over Weigh	≥ 25.00	≥ 25.00
Pre-obese	25.00-29.99	25.00-27.49
		27.50-29.99
Obese	≥ 30.00	≥ 30.00
Obese class I	30.00-34.99	30.00-32.49
		32.50-34.99
Obese class II	35.00-39.99	35.00-37.49
		37.50-39.99
Obese class III	≥ 40.00	≥ 40.00

Source: World Health Organization (WHO), 2013

Adipose tissue

Adipose tissue can be classified into two types: white and brown adipose tissue, due to their essential functions and characteristics as shown in table 2.

Table 2 Characteristics of white adipose tissue (WAT) and brown adipose tissue (BAT)

	WAT	BAT
Function	Energy storage	Heat production
Morphology	Single lipid droplet Variable number of mitochondria (in each white adipocyte)	Multiple small vacuoles Abundant mitochondria (in each brown adipocyte)
Characteristic proteins	Leptin	UCP1
Development	From Myf5-negative progenitor cells	From Myf5-positive progenitor cells (but there are also Myf5-negative brown fat cells which are derived from other lineages)
Human data	Large amounts are associated with an increased risk of obesity-related disorders	Large amounts are associated with decreased risk of obesity-related disorders
Impact of aging	Increases with age relative to total body weight	Decreases with age

Source: Christoph et al., 2012

1. White adipose tissue (WAT)

A white adipocyte comprises a single lipid droplet, far less mitochondria, and blood vessels, thus resulting in its lighter white or yellow appearance (Wang et al., 2013). White adipocytes are sphere-shaped cells whose variant size mainly depends on the size of the single lipid droplet stored in them (Figure 6). This lipid droplet contains ≥ 90% triglycerides accounteds for the cell volume. The molecular mechanism of the predominant form of fat in the body, originates from connective tissue. Mitochondria in white adipocytes are thin, variable, and elongated in amount. The development of white adipocytes cells as adipogenesis is controlled by several signaling pathways and several genes including adipogenic transcription factors such as *PPARγ* and *C/EBPs* (Farmer,

2006) and lipogenic such as *SREBP-1c* and its downstream targets such as *LPL, FAS* (Fisher et al., 2012).

2. Brown adipose tissue (BAT)

BAT is found mainly in rodents and in newborns to play a role in cold temperature resistance. Brown adipocytes are naturally polygonal with a variable diameter. The most characterised organelles of BAT cells are the mitochondria (Cousin, 2009). BAT cells are large, spherical, packed with laminar cristae, and usually numerous (Figure 6). Because of its greater oxygen demand, brown fat contains more blood vessels than white adipocytes (Mattson, 2010). A recent has shown that research, BAT in adults also has metabolically active and that BAT may play a role in energy homeostasis (Christoph, Kathrin, and Drexel, 2012). BAT is thus of great attention as a potential target to prevent obesity and associated metabolic disorders.

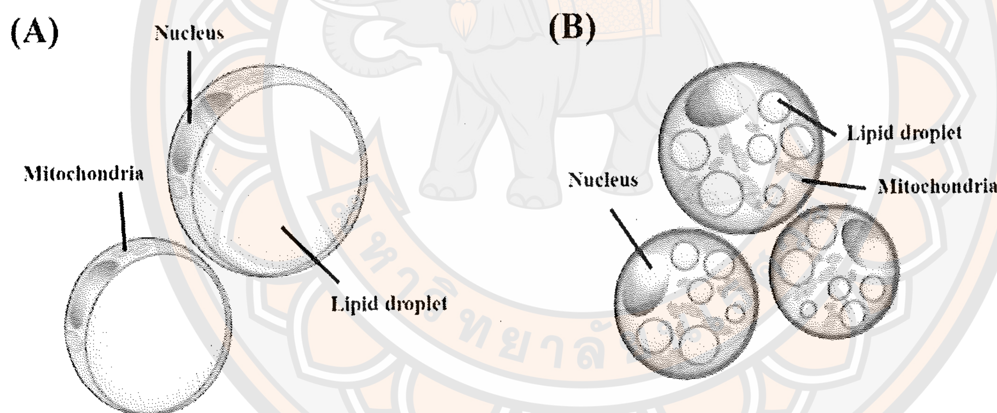


Figure 6 Morphology of (A) white adipose tissue (WAT) and (B) brown adipose tissue (BAT)

Source: https://www.bruinvet-onderzoek.nl/about

3. Lipid accumulation in liver and skeletal muscle of sedentary people is associated with impaired insulin-stimulated glucose metabolism

Lipid accumulation in the liver and skeletal muscle of sedentary people is associated with impaired insulin-stimulated glucose metabolism. A reduced capacity of oxidative tissues and organs to adjust lipid oxidation to lipid availability can lead to

tissue accumulation of lipids as triglycerides. Excess lipid accretion and/or lower triglyceride turnover can induce lipotoxicity, as reflected by the cellular accumulation of ceramides and diglycerides. These lipid species ultimately impair insulin signaling through different mechanisms, either increased serine phosphorylation of the insulin receptor and insulin receptor substrate 1 and/or reduced serine phosphorylation of PKB/Akt. Therefore, the ability to increase lipid oxidation as a function of their availability eventually reduces the formation of ceramides and diglycerides leading to improved insulin sensitivity. Fat-induced insulin resistance describing how a failure to appropriately store lipids into subcutaneous adipose tissue were led to ectopic lipid deposition into visceral fat and insulin-sensitive tissues such as liver and skeletal muscle. These tissues will progressively develop a state of lipotoxicity, altering insulin signaling and action and contributing to whole body insulin resistance and deterioration of glucose tolerance (Figure 7).

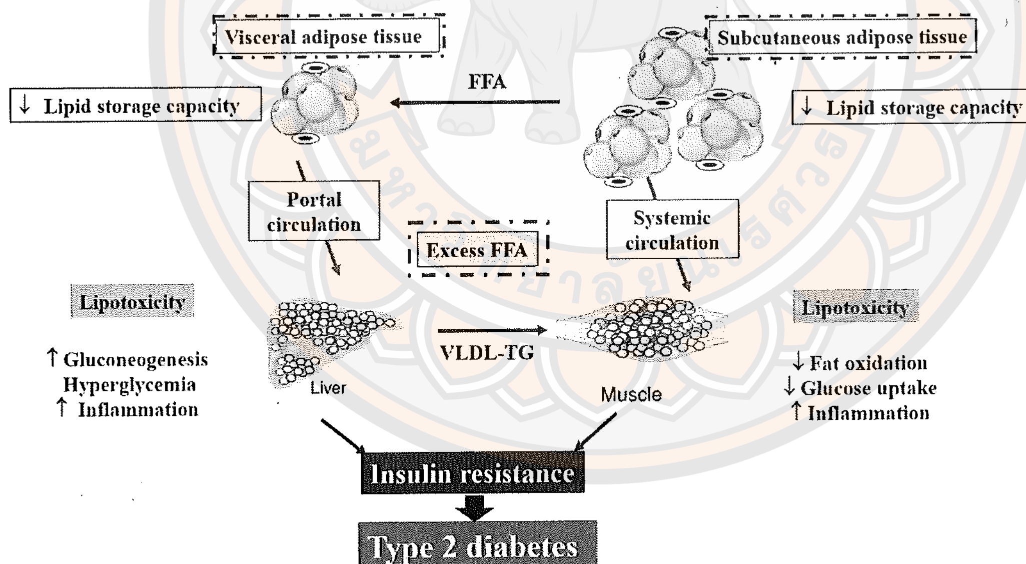


Figure 7 Model for fat-induced insulin resistance describing store lipids into subcutaneous adipose tissue

Source: Adapted from Galgani et al., 2008

Adipocytes

Adipose tissue contains adipocytes, especially white adipocytes, which store most of the energy source in humans. Adipose tissue is dispersed over multiple subcutaneous and visceral depots, typically accounting for 15–30% of total human body weight (Pi-Sunyer et al., 1998). Adipocytes are the major site for the storage of excess energy in the form of triglycerides, and they contain multiple cell types, including preadipocytes, adipocytes, endothelial cells, and immune cells. The lipid droplets of adipocytes are stored through an increase in the number of adipocytes (hyperplasia) or an expansion in the size of adipocytes (hypertrophy) (Hausman et al., 2001). In contrast to energy balance states, the energy is needed between meals or during physical exercise, triglycerides store in adipocytes can be catalyzed through lipolysis to release free fatty acids and the resulting free fatty acids are transported to other tissues to be used to the energy source (Kim et al., 2007). Obesity is associated with metabolic diseases. Metabolic dysfunction caused by hypertrophy has been shown to play an important role in the development of metabolic diseases including insulin resistance, type 2 diabetes, dyslipidemia, hypertension (Wang et al., 2010) (Figure 8).

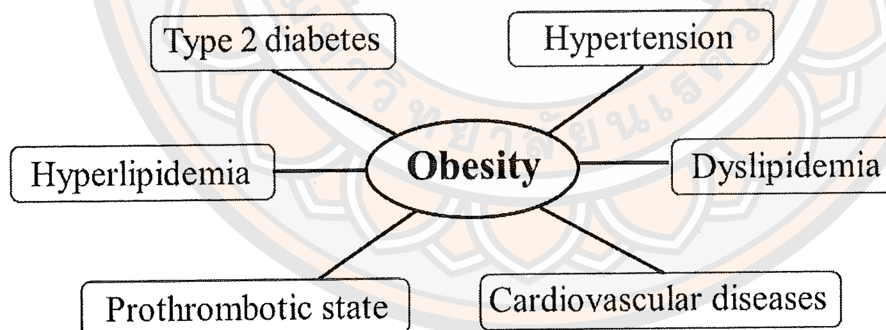


Figure 8 Obesity is associated with metabolic diseases

Source: Adapted from Jung, & Choi, 2014

Adipocyte differentiation or adipogenesis

An overview of the stages for adipocyte differentiation is shown in Figure 9. Adipocyte differentiation indicates that a pluripotent stem cell precursor gives rise to a mesenchymal precursor cell with the potential to differentiate along mesodermal

lineages of chondroblast, myoblast, osteoblast, and adipocyte. Given applicable environmental and gene expression preadipocytes undergo clonal expansion and terminal differentiation. During adipocyte differentiation, adipocyte phenotype is characterized by chronological changes in the expression of many genes such as *C/EBPs* e.g., *C/EBPα*, *C/EBPβ*, *C/EBPδ*, *PPARγ*, and lipogenic genes (e.g., *SREBP-1c*, *LPL*, etc). Overall, the adipogenesis process is regulated by the arrival of early, intermediate, and late mRNA/protein markers and triglyceride accumulation (Mandrup and Lane, 1997; Francine et al., 1995).

In humans, preadipocytes start to differentiate into adipocytes during late embryonic development, with a majority of the differentiation occur shortly after birth. This enables the newborn to survive more efficiently with interludes between nutrient intakes. Mouse preadipocytes do not begin conversion into adipocytes until after birth, but all species can differentiate preadipocytes during their life spans in response to accumulation fat storage demands the body's (Moustaid, & Sul, 1997).

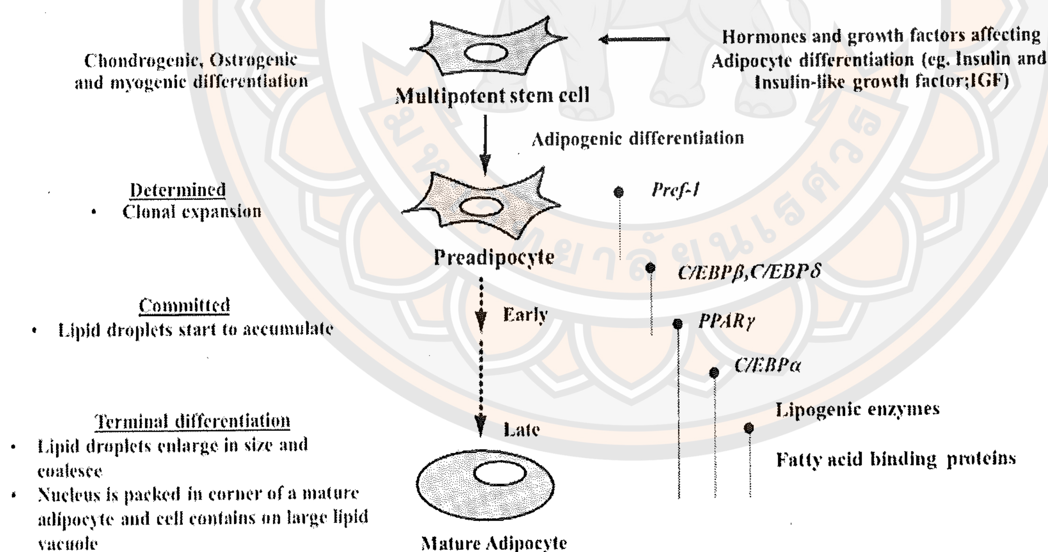


Figure 9 An overview of the stages in adipocyte differentiation

Source: Adapted from Ntambi, & Young-Cheul, 2000

Adipocyte differentiation is featured by chronological changes in the expression of various genes that lead to the syndicate of the adipocyte. These changes include the morphology conformation, intermediate gene/ protein expression, and accumulation of triglycerides (Gregoire et al., 1998). The process of adipogenesis *in vivo* occurs in four stages, including preadipocyte growth arrest, preadipocyte mitotic clonal expansion (MCE), early adipocyte differentiation, and terminal adipocyte differentiation (Farmer, 2006). Preadipocytes earn proliferation of cells before entering the growth arrest stage, at which point to express early markers of differentiation such as *C/EBPβ* and *C/EBPδ*. The cell has contacted a role in activating mechanisms that induce early differentiation markers *C/EBPα* and *PPARγ* (Tong, & Hotamisligil, 2001) as shown in Figure 10.

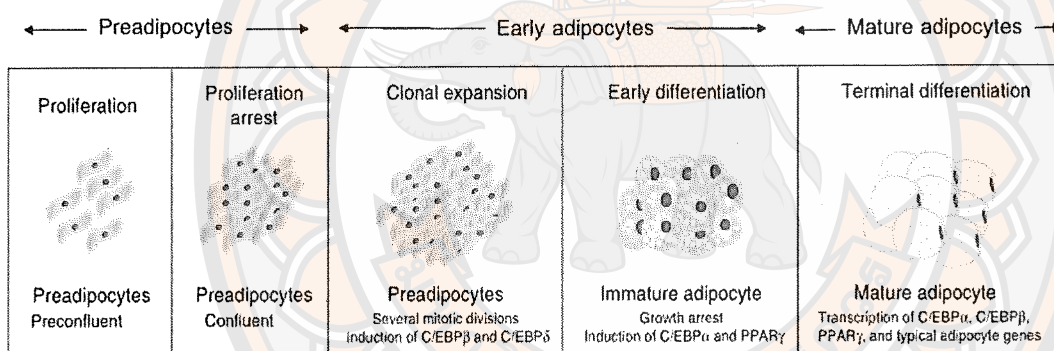


Figure 10 Stages of differentiation from preadipocytes to mature adipocytes

Source: Ntambi, & Young-Cheul, 2000

The transition from preadipocyte to adipocyte is associated with extracellular matrices (ECMs) as described in Figure 11: (a) A preadipocyte associates with a stromal ECM comprising ColI-containing fibrils (tan cross-banded strands), FN fibrils (thin blue wavy lines); and ColVI microfilaments (red beaded structures). The latter may serve to link other ECM components (e.g., ColI and FN) to each other, and cell surfaces. The proteinases MT1-MMP, CatL, and plasmin are represented by gold, green, and orange scissors, respectively. MT1-MMP and plasmin cleave both ColI and FN, whereas CatL

is thought to cleave FN, but not ColI. The fibroblastic cell secretes ColI procollagen precursors (Procol), which have small NH_2-terminal and larger COOH-terminal propeptides (yellow circles) that are cleaved off to yield the triple-helical monomers that self-associate to form ColI fibrils. Blue ovals adhered to the procollagen represent SPARC, which is thought to be involved in modulating ColI biosynthesis and ECM deposition; (b) an adipocyte containing a large internal lipid droplet is shown enveloped by a net-like ColIV framework, which forms the structural backbone of all basement membranes. In addition, ECM is now known to bind to and act as a reservoir for growth factors that affect cell behaviors, and the activity of such growth factors.

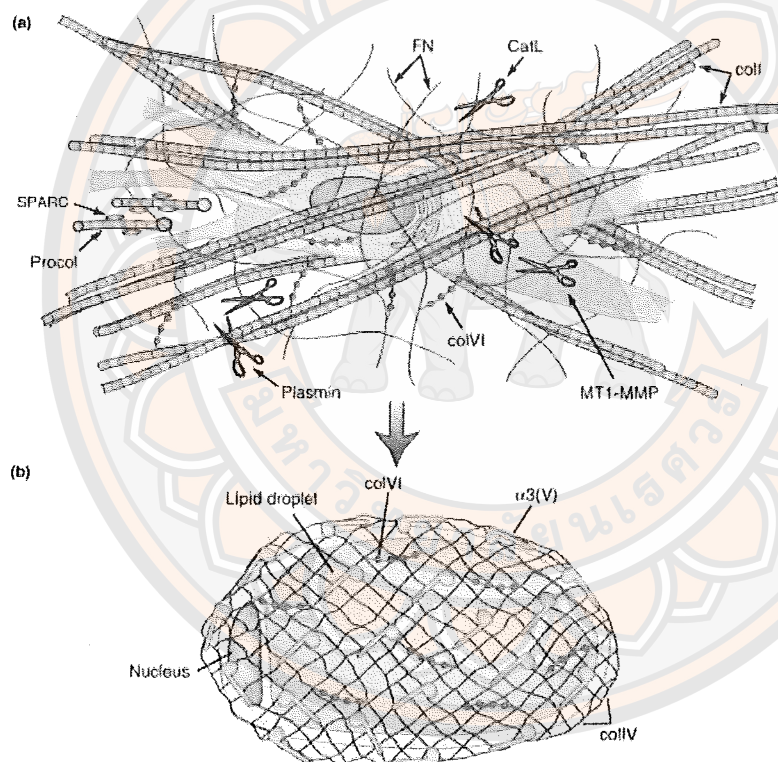


Figure 11 ECM remodelling during adipogenesis

Source: Huang, & Greenspan, 2012

The committed preadipocyte maintains the capacity for growth but has to retreat from the cell cycle before adipose conversion. *In vitro* systems have been extensively used to study adipocyte differentiation. Adipocyte differentiation

acquisition of the adipocyte phenotype is characterized by chronological changes in the expression of several genes.

Adipocyte precursor cell lines can be divided into two classes, e.g., pluripotent fibroblasts and unipotent preadipocytes. The pluripotent fibroblasts (10T1/2, Balb/c 3T3, 1246, RCJ3.1, and CHEF/18 fibroblasts) can be converted into several cell types. Multipotent fibroblast performs as a good model for understanding the events accountable for cellular determination of the separate cell fates. The second class of the unipotent preadipocytes (3T3-L1, 3T3-F422A, Ob1771, TA1, and 1246), has been widely used and can either remain as preadipocytes or undergo change to adipose tissue.

In vitro adipocyte differentiation, reasonable inducers are required for adipocytes to conduct to the mitotic clonal expansion stage and subsequent differentiation. For example, 3T3-L1 preadipocytes can differentiate into mature adipocytes upon induction using dexamethasone (DEX), Isobutylmethylxanthine (IBMX), and insulin (Farmer, 2006) (Figure 12). This cocktail activates the adipogenic program in these cells directed to the different stages of adipogenesis. In specific, DEX and IBMX have been identified as inducers of genes responsible for the expression of *C/EBPδ* and *C/EBPβ*, respectively. The role of insulin is stimulating the cells to take up glucose into cells and accumulate in the form of triacylglycerols. The early stages of differentiation found that a high expression of *C/EBPδ* and *C/EBPβ* in response to hormonal induction. Transcription factors in the late stages of the adipocytes differentiation pathway is replaced by *C/EBPα* and *PPARγ* (Yeh et al., 1995; Cao et al., 1991). *C/EBPα* and *PPARγ* function in a manner to induce adipocyte-specific genes that create the mature adipocytes and connect the adipocyte-specific gene expression such as *SREBPs*, *FAS*, *LPL,* and *aP2.*

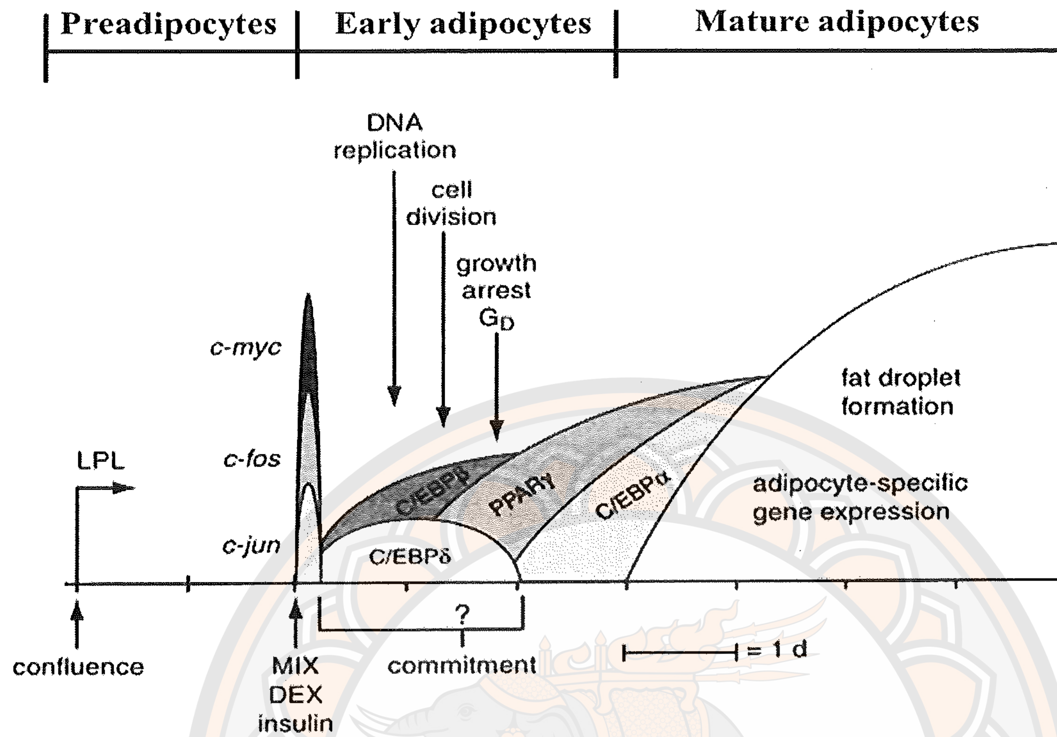


Figure 12 ***In vitro*** **adipocyte differentiation**

Source: Adapted from Ntambi, & Young-Cheul, 2000

Regulating of gene expression during adipogenesis

The growth of adipose tissue interlaces two distinct processes including hyperplasia and hypertrophy.The regulation of adipogenesis is the process of differentiation of preadipocytes into mature adipocytes and a regulation of transcription of related genes (Cristancho, & Laza, 2011).The underlying mechanism for transcriptional control of adipocyte differentiation is the study of gene expression events and activation of several keys signaling pathways (Lefterova, & Lazar, 2009; Cheguru et al., 2012). Among the transcription factors at work, peroxisome proliferator-activated receptor (*PPARγ*), CCAAT/enhancer-binding proteins (*C/EBPs*), and sterol regulatory element-binding proteins (*SREBPs*) genes play central roles in the regulation of adipocyte differentiation and adipogenesis (Christodoulides, & Vidal-Puig, 2010; Siersbaek, Nielsen, & Mandrup, 2010).

PPARγ is a master key gene in the regulation of adipocyte differentiation (Rosen et al., 2002). When this receptor is activated by an agonist ligand in fibroblast-like preadipocytes, a full program of differentiation is motivated, including morphological changes, lipid accumulation, and the expression of adipogenesis-related genes. *C/EBPs* also have a vital role in adipogenesis, with *C/EBPβ* and *C/EBPδ* driving *PPARγ* expression in the early stages and *C/EBPα* maintaining *PPARγ* expression later in the process of differentiation (Farmer, 2006). *C/EBPs* and *PPARγ* also directly activate many of the genes of terminally differentiated adipocytes such as *SREBP-1c*, *LPL*, *FAS*, *aP2,* and *ACC* to accumulation lipid droplets in mature adipocytes (Armani et al., 2010).

1. Peroxisome proliferator-activated receptors (PPARs)

Peroxisome proliferator-activated receptors PPARs are ligand-activated transcription factors of the nuclear hormone receptor superfamily including three subtypes: *PPARα* is expressed predominantly in the liver, muscle, and heart, *PPARβ/δ* is expressed in the whole body and has a role to regulate energy expenses and *PPARγ* is expressed in vascular smooth muscle cells and endothelial cells. *PPARγ* has four isoforms (Evans et al., 2004).

γ1- expressed in virtually all tissues, including the muscles, heart, colon, kidneys, and spleen.

γ2- expressed mainly in adipose tissue.

γ3- expressed in the large intestine, macrophages, and white adipose tissue.

γ4- expressed in endothelial cells

The structure of PPARs is similar to thyroid hormone or steroid receptor and is activated in response to small lipophilic ligands. Activated *PPARs* are proficient of transcriptional repression through DNA-independent protein-protein and capable interactions with other transcription factors such as transducers of transcription AP-1 and STAT-1 signaling and NFκB signal activators (Oliveira et al., 2007). *PPARs* function as a heterodimer in cooperating with the co-activator complex that binds to DNA sequence peroxisome proliferators response elements (PPREs) in the promoter of target genes which leads to transrepression and transactivation of various genes (Michalic, & Wahli, 2006). *PPARα* plays a central role in lipid and lipoprotein metabolism and thereby decreases dyslipidemia associated with metabolic syndrome.

PPAR-α ligands can be both synthetic FAs and FA-derived compounds and t decreases dyslipidemia associated with metabolic syndrome (Jay, & Ren, 2007). *PPARγ* is the thiazolidinediones (TZDs), are the most found PPARγ ligands. The mechanisms of TZDs in insulin treatment were high affinity ligands of *PPARγ* which, is a member of the nuclear receptor superfamily and a ligand-dependent transcription factor (Kubota et al., 2007). *PPARγ* regulates adipocyte differentiation, FA storage, glucose metabolism, and improves insulin resistance (Franckhauser et al., 2008). *PPARβ/δ* are promoting FA metabolism and suppresses macrophage by inflammation. *PPARδ* ligands can inhibit cardiac hypertrophy on account of their inhibitory activity on NFκB, a transcription factor that produces inflammatory cytokines (Balakumar et al., 2007). Thus, interference of PPAR can provide medication targets for a plethora of diseases such as diabetes, dyslipidemia, inflammation, obesity, and cancer.

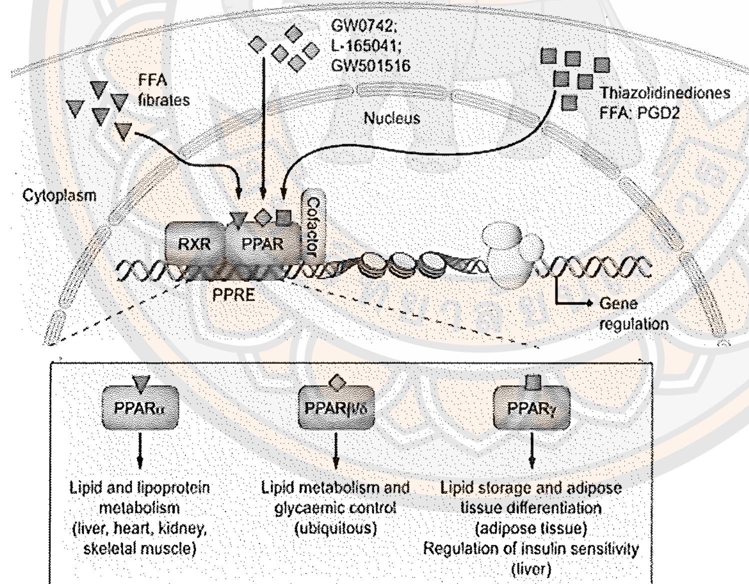


Figure 13 Localization and biological functions of peroxisome proliferator-activated receptors (PPARs)

Source: Romero et al., 2017

2. CCAAT/enhancer binding proteins (C/EBPs)

CCAAT/enhancer binding proteins (C/EBPs) are a family of transcription factors that contain a highly conserved bZIP domain at the C-terminus that is involved in dimerization and DNA binding (Williams et al., 1991). The function of the C/EBP family has been a role of the proteins in numerous cellular responses, including the control of cellular growth and immune and inflammatory processes, differentiation, and various diseases. The expression of the *C/EBPs* has been found that to change markedly during several physiological and pathophysiological conditions through the action of extracellular signals. There are six members of the *C/EBPs* family have been clone from several species, with many of them being characterized independently in different laboratories and given specific names shown in table 3. The role and the promoter regions of C/EBP isoforms α, β, δ, ε, and ζ are different in the regulation of gene expression during adipogenesis, proliferation, and inflammation.

Table 3 Nomenclature of *C/EBP* genes

Gene	Alternative name	Source
C/EBPα	*C/EBP, RcC/EBP-1*	Rat, mouse, human, chicken, bovine, *Xenopus laevis*, *Rana catesbeiana*, fish
C/EBPβ	*NF-IL6, IL-6DBP, LAP, CRP2, NF-M, AGP/EBP, ApC/EBP*	Rat, mouse, human, chicken, bovine, *Xenopus laevis*, fish
C/EBPγ	*Ig/EBP-1*	Rat, mouse, human, chicken, fish
C/EBPδ	*NF-IL6β, CRP3, CELF, RcC/EBP2*	Rat, mouse, human, *Rana catesbeiana*, fish
C/EBPε	*CRP-1*	Rat, mouse, human, ovine, fish
C/EBPζ	*CHOP-10,GADD153*	Rat, mouse, human, hamster

Source: Dipak et al., 2002

2.1 *C/EBPα*

C/EBPα gene was first characterized and shown to contain potential binding sites for C/EBP, nuclear factor (NF)-1, upstream stimulating factor (USF), Sp1, NF-Y, basic transcription element-binding protein (BTEB), and NF-κB (Wu et al., 1996; Lin et al., 1992). *C/EBPα* acts implicitly via stimulation of the DNA-binding

activity of USF, which interrelates with a site present in the proximal promoter region (Cor et al., 2000). These studies showed that the C/EBPα gene is auto-activated in a species-specific manner.

2.2 *C/EBPβ*

C/EBPβ genes have been featured and all four are subject to autoregulation. The transcription factor CREB (cAMP-response-element-binding protein) likewise controls *C/EBPβ* expression by interacting with two sites near the TATA box (Guo et al., 2008). CAMP-response element (CRE)-like sequences are also necessary for the IL-6-mediated induction of C/EBPβ transcription, during the acute-phase response (APR) through a novel pathway involving tethering of STAT-3 to a DNA-bound complex, and in joining, with a Sp1 site for the activation of gene transcription during differentiation (Niehof et al., 2001).

2.3 *C/EBPδ*

C/EBPδ is an intron less gene and the constitutive expression of *C/EBPδ* is found that in adipose, intestines, and lung, with high levels of expression. The 269-amino acid protein encodes a leucine zipper dimerization domain and DNA-binding region with *CEBPα* and *C/EBPβ* and transactivation efficiency of *C/EBPδ* is comparative to that of *C/EBPβ* and *C/EBPα*. The DNA-binding region of *C/EBPδ* differs from *C/EBPα*, which may interrupt the α-helical structure, it contains 2 proline and 4 glycines (Billiard et al., 2001; Hutt et al., 2000).

2.4 *C/EBPε*

The *C/EBPε* gene promoter is transmitted by two alternative promoters, pα and pβ, which are similar to those of several other myeloid-cell-specific genes but have several purine-rich stretches with multiple sites for the family of transcriptional regulators (Sabatakos et al., 1998).

2.5 *C/EBPζ*

C/EBPζ was first identified based on its induced expression in response to growth trap and DNA damage. *C/EBPζ* possesses a leucine zipper dimerization domain, DNA-binding region, and activator protein-1 (AP-1) which was found to be essential for this response and a role in the induction by oxidative stress. *C/EBPξ* functions as a dominant negative inhibitor of C/EBP transcriptional activation by

preventing heterodimer binding of *C/EBPα* and *C/EBPβ* to classic C/EBP enhancer sequences (Crozat et al., 1993).

3. Sterol regulatory element-binding proteins (SREBPs)

Cells need to synthesize both cholesterol and fatty acids (FA) demanded for growth. Synthesis of FA and triglycerides (TG) (Figure 14), often referred to as lipogenesis, is an energy storage system especially to lipogenic organs such as adipose tissues and the liver. Both biosynthetic pathways are controlled by a common family of transcription factors designated sterol regulatory element binding proteins (*SREBPs)* (Brown and Goldstein, 1997). *SREBPs* are the sterol regulatory element (SRE) common to LDL receptor and HMG CoA synthase genes. *SREBPs* are structurally composed of two membrane-spanning regions with four domains. *SREBPs* have been identified to three isoforms: *SREBP-1a* and *SREBP-1c* (ADD1) produced from a single gene using alternate promoters, *SREBP- 1a* and *-1c* are identical except the NH_2-terminal transactivation domains. *SREBP-1c* was cloned as a protein that binds to E-boxes and promotes adipocyte differentiation and *SREBP-2* from a separate gene. In summary, *SREBP-1a* and *SREBP-2* are likely active for the SRE-reporter gene (SRE-luciferase) except *SREBP-1c*, which is essentially inactive. In contrast, *SREBP-1a* and *SREBP-1c* are active for E-box while *SREBP-2* is inactive (Costet et al., 2000; Yoshikawa et al., 2001).

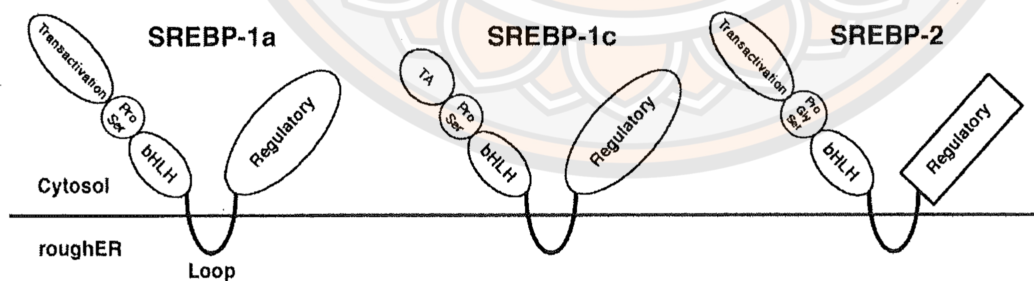


Figure 14 Domain structure of SREBP family

Source: Timothy, & Osborne, 2000

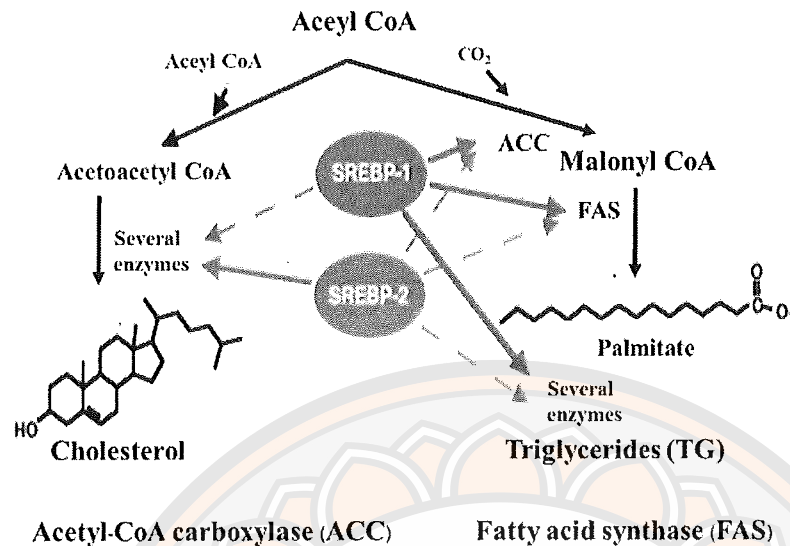


Figure 15 Pathway selective gene activation by SREBP-1 and SREBP-2

Source: Timothy, & Osborne, 2000

SREBP-1c has been suggested to be involved in adipogenesis by inducing *PPARγ*, a master gene for adipogenesis (Schultz et al., 2000). *SREBP-1c* activates promoters of *PPARγ1* and *3* and can directly induce transcription of *PPARγ* (Schultz et al., 2000). However, the role of *SREBP-1c* in adipogenesis and lipogenesis in adipose tissue seems to be very complex. Expression of *SREBP-1c* in a fact-specific manner using *aP2* promoter in transgenic mice impaired adipose tissue differentiation, causing a decreased amount of fat and hyperinsulinemia and severe insulin resistance (Korn et al., 1998).

4. Fatty Acid Synthase (FAS)

Fatty acid synthase (*FAS*) may play a role in the regulation of the development of obesity. *FAS* play an important role in energy homeostasis and providing energy when needed via β-oxidation (Roncero, 1992). *FAS* pathway has three functions:

i: storage of extra energy intake

ii: synthesis of fat from protein or carbohydrate

iii: synthesis of fat is involved in the metabolism of glucose to fatty acid

Fatty acid (FA) is synthesized from glucose to palmitate. Glucose is converted to acetyl-CoA by glycolysis and to produce citrate in the mitochondria. The citrate is transported to the cytoplasm and changed to acetyl-CoA by citrate lyase. Acetyl-CoA is carboxylated to malonyl-CoA with acetyl-CoA carboxylase. Fatty-acid synthase performs the aggregation of acetyl-CoA and malonyl-CoA change to palmitate (16-carbon saturated fatty acid), which is dependent on NADPH (Figure 16).

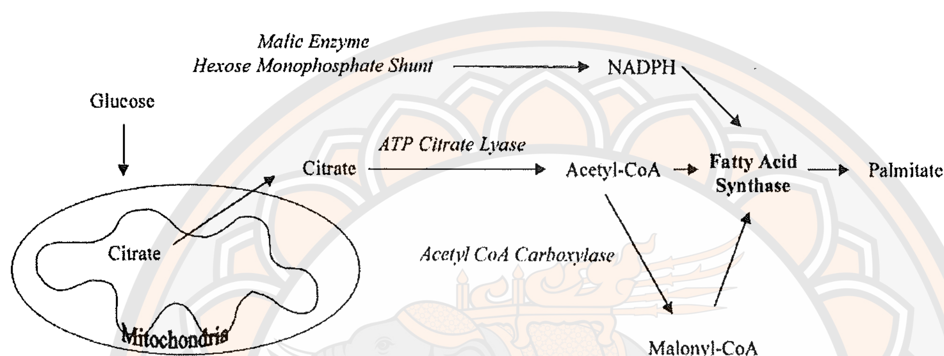


Figure 16 Fatty-acid synthesis from glucose to palmitate

Rource: Francis, & Kuhajda, 2000

5. Lipoprotein lipase (LPL)

Lipoprotein lipase (*LPL*) plays a major role in the metabolism and transport of lipids and is synthesized primarily by adipose tissue and skeletal muscle. It is the enzyme accountable for the hydrolysis of triglycerides (TGs) from lipoproteins and very low-density lipoproteins (VLDLs) in chylomicrons (Eckel, 1989) into free fatty acids (FFA) and monoglycerides (MG), producing chylomicron fragments and intermediate-density lipoproteins (IDLs). LPL has been shown to promote the change of lipids between lipoproteins, playing an important role in the kinetics of most plasma lipoproteins (Long et al., 2006) (Figure 17).

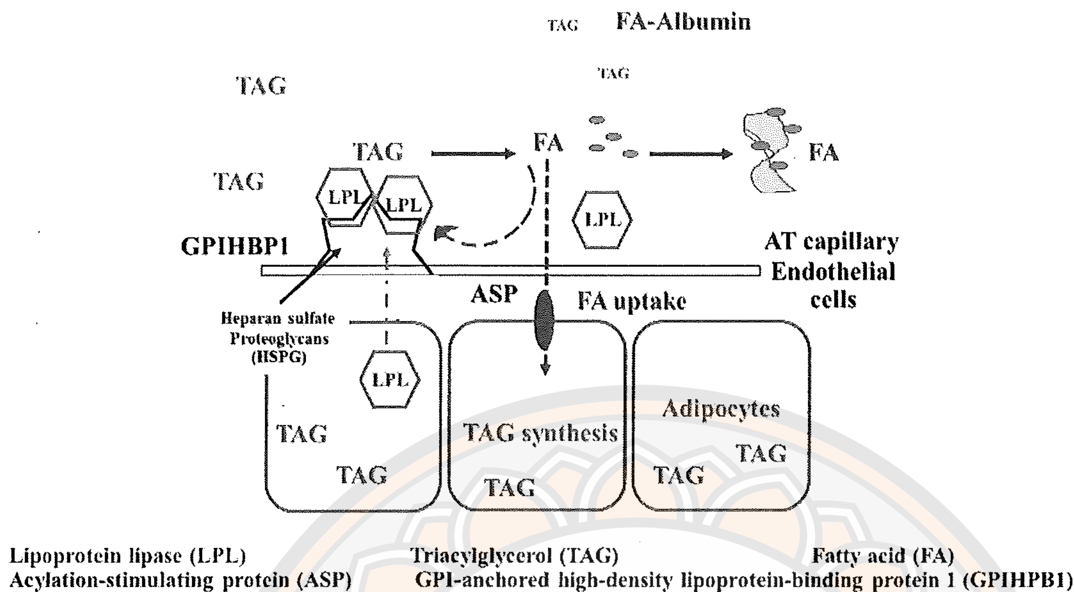


Figure 17 The major route of LPL and TAG hydrolysis for TAG deposition in human white adipose tissue

Source: Lafontan, 2008

6. Acetyl-CoA carboxylase (ACC)

The ACC carboxylates acetyl-CoA to form malonyl-CoA and regulates enzymes in the fatty acid synthesis pathway. ACC catalyzes the carboxylation of acetyl-CoA into malonyl-CoA. *ACCs* are deactivated by phosphorylation by AMP-activated protein kinase (AMPK) and regulated by many other kinases. ACCs were found in two isoforms in the human genome, ACC1 (ACCα or ACACA) which is highly in lipogenic tissues and ACC2 (ACCβ or ACACB) which occurs in oxidative tissues. Roles of *ACC1* and *ACC2* are different.Malonyl-CoA induces by *ACC*1 is a substrate for FA synthesis, while the malonyl-CoA induces by ACC2 functions while as carnitine/palmitoyl-transferase 1 (CPT1) for preventing FA degradation and the transfer of FAA through the carnitine shuttle system to the mitochondria for β-oxidation (Abu-Elheiga et al., 2000a; Abu-Elheiga et al., 2005) (Figure 18).

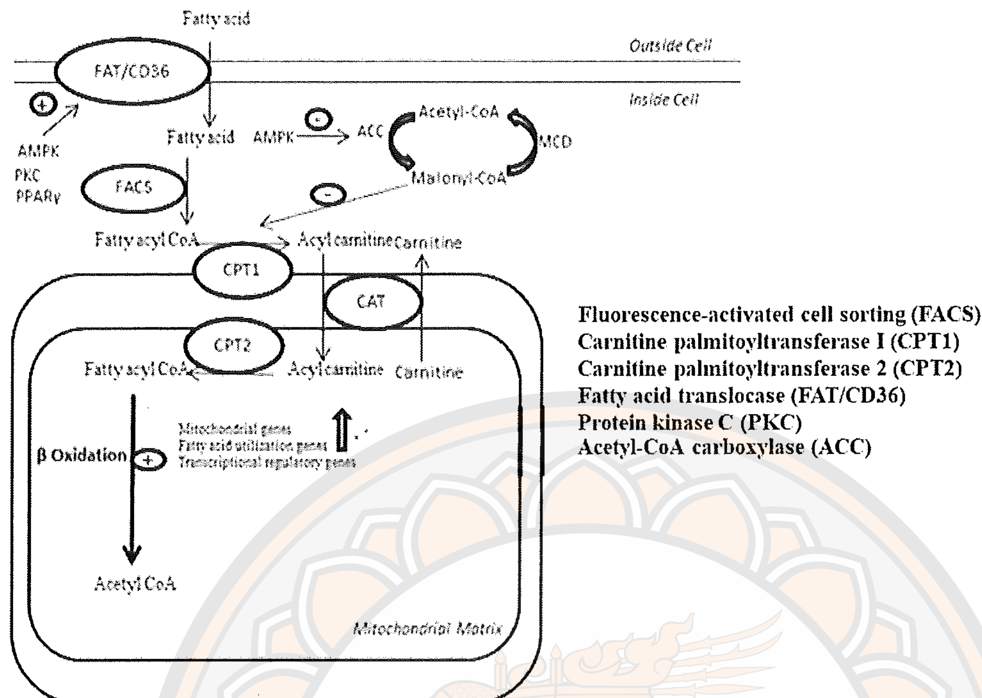


Figure 18 Major role in Acetyl-CoA carboxylase (ACC) regulation by AMPK

Source: Adapted from Fillmore et al., 2014

Regulating of gene expression during lipolytic

1. **Adipose triglyceride lipase (ATGL)**

The TG hydrolase is called adipose triglyceride lipase (*ATGL*). *ATGL* has highly expressed, and its expression increases during 3T3-L1 adipocyte differentiation and adipose tissue (Robert et al., 2004). *ATGL* expression is also observed in cardiac muscle, macrophages, skeletal muscle, liver, testis, and other cell types (Kershaw et al., 2006). *ATGL* function is the first step in TG hydrolysis into the formation of diacylglycerol (DG) and FFA. Additionally, *ATGL* was substantiating to possess transacylase and phospholipase activity. This suggested that *ATGL* accounts for the *HSL*-independent TG hydrolase activity in WAT (Jocken et al., 2008).

2. **Hormone sensitive lipase (HSL)**

HSL was shown to exhibit substrate specificity of hydrolyzing cholesterylester, retinyl ester, tri-, di-, and monoacylglycerol (TG, DG, and MG) (Holm

et al., 2000). This enzyme is considered crucial in regulating lipolysis in fat cells because of its sensitivity to neurotransmitters and hormones, specifically to catecholamines and insulin, which respectively motivate or inhibit activity (Donsmark, 2003). Activation of *HSL* occurs during thermogenesis in brown adipose tissue (BAT) and associate with the leave of free fatty acids (FFAs) from white adipose tissue (WAT) into the blood diffusion (Holm et al., 2000). Besides adipocytes, *HSL* activity has been documented in the myocardium, brain, skeletal muscles, testis, and adrenal gland macrophages. The *HSL* is present and active of pancreatic b-cells in the rat (Mulder et al., 1999) has reinforced that endogenous lipolysis participates in the regulation of insulin secretion through the generation of FFA (Prentki et al., 2002).

Perilipin is a pathway for the molecular mechanisms regulating lipolysis which controls both the ATGL and HSL activity by the ATGL activator CGI-58, and by regulating the access of HSL to the lipid droplets (LD). In the non-active state or basal state (Figure 19A) CGI-58 is bound to perilipin and *HSL* is eminently cytosolic resulting in low LD-associated *ATGL* and *HSL* activity. There are *ATGL* have on LDs at similar in non-active and activation mechanism based on translocation. In the activated state (Figure 19) phosphorylation of perilipin with PKA release of CGI-58 which then becomes available for *ATGL*. Activated *ATGL* produces DG. In parallel, *HSL* is phosphorylated by PKA and perilipin promotes the translocation of the enzyme causing ineffective DG hydrolysis.

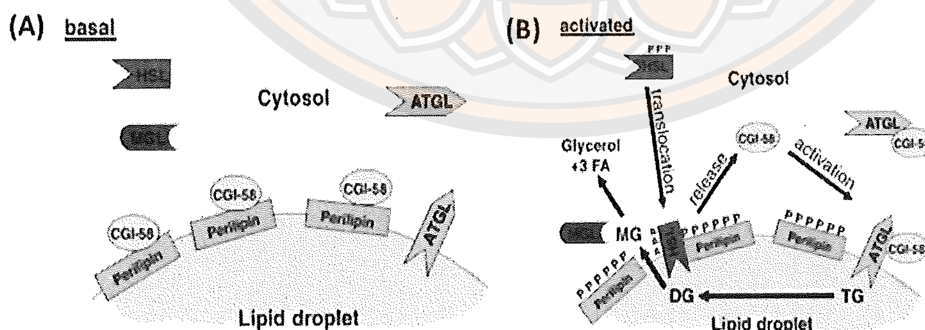


Figure 19 Regulation of ATGL and HSL activity by perilipin during basal (A) and activated (B) states

Source: Zimmermann et al., 2004

Adipocytokines or adipokines

Adipose tissue also has an endocrine function secreting multiple adipocytokines or adipokines. The adipokines are involved in inflammation and energy homeostasis, including cytokines, chemokines, and hormones (Figure 20).

1. Tumor necrosis factor-α (TNF-α)

TNF-α is a pro-inflammatory cytokine that can be contributing to obesity and insulin resistance (Skurk et al., 2007). Expression of *TNF-α* increases insulin resistance in human obesity and is positively associated with insulin resistance. However, the correlation between *TNF-α* levels and insulin resistance is relatively low (Miyazaki et al., 2003) and *TNF-α* does not improve insulin resistance in healthy overweight subjects with metabolic syndrome and insulin resistance improvements in inflammatory status (Bernstein et al., 2006). *TNF-α* is a part of complex inflammation and able of originating cytokine cascades involving inhibitory reactions and synergistic, which control the expression and synthesis of their receptors, cytokines, and hormones. For example, TNF-α in mice serum induced the IL12 levels, presenting of both cytokines act as co-stimulatory of the releasing/production of interferon-α (IFN-α), one of the essential cytokines for regulation of insulin resistance and inflammation in obesity. (Rourke et al., 2006).

2. IL6 and IL18

IL6 is a cytokine with an important role in the development of insulin resistance in obesity and hypertrophic extension of adipocytes is accompanied by increased production of IL6 by adipose tissue (Fernandez-Real and Ricart, 2003; Franckhauser et al., 2008). Expression of adipose *IL6* positively associates with insulin resistance both *in vivo* and *in vitro* (Bastard et al., 2002). Hyperglycemia can increase the IL6 levels (Pradhan et al., 2001), and treatment with IL6 induces hyperglycemia and insulin resistance in humans (Tsigos et al., 1997). IL6 seems to have different functions depending on the tissue, such as skeletal muscle and adipose tissue. The IL6 increased insulin resistance in adipocytes (Franckhauser et al., 2008), thus, its different tissue-specific functions may account for the controversial findings regarding the relationship between IL6 and insulin resistance.

IL18 is a pro-inflammatory cytokine and produced by adipose tissue (Trayhurn and Wood, 2004). IL18 levels have been shown to increase and reduce weight loss in obese (Esposito et al., 2003). Moreover, overexpression of *IL18* on insulin resistance in a rat model of metabolic syndrome (Xu et al., 2003). However, a lack of *IL8* or receptor induces hyperphagia in mice obesity and insulin resistance (Netea et al., 2009). Thus, further studies are needed to assess the role of *IL6* and *IL8* in the pathogenesis of obesity and insulin resistance.

3. Leptin

Leptin is overexpressed in adipose tissue and involved in the regulation of energy homeostasis, and it inhibits hunger and food intake and induces energy expenditure (Friedman, and Halaas, 1998). Leptin plays an important role in the regulation of glucose homeostasis, energy expenditure, or body weight, dependent on the intake of food. Concurrently, partying leptin levels and increased expression on adipose tissues in response to pro-inflammatory cytokines (*TNF, IL1*) and endotoxin (lipopolysaccharide, LPS) (Grunfeld et al., 1996). The interactions between leptin and inflammation are bidirectional: increase the synthesis of pro-inflammatory cytokines and release of leptin, which protects inflammatory in obesity (Paz-Filho et al., 2012).

4. Resistin

Resistin is an adipocyte-specific secreted adipokine and promotes inflammation and insulin resistance. Levels of circulating resistin are increased and associated with insulin resistance in obese mice (Satoh et al., 2004; Banerjee, et al., 2004). Moreover, resisting can inhibit insulin signaling in both 3T3-L1 adipocytes and murine adipose tissues (Steppan et al., 2005). But other studies have shown that the resisting levels and adipocyte expression were not related to insulin resistance in humans (Kielstein et al., 2003; Patel et al., 2003).

5. Visfatin

Visfatin is a modulator of β-cell differentiation that is expressed in tissues and cell types, including lymphocytes, bone marrow, liver, muscle, and reported to be secreted by adipose tissue and display insulin-like activities in mice. *Visfatin* was highly expressed in mice and humans in the visceral adipose tissue and treatment with visfatin improves glucose uptake in adipocytes.

6. Adiponectin

Adiponectin is an adipose-specific adipokine that produces insulin-sensitivity effects. A previous study shows that the levels of adiponectin are low in obese subjects and treatment with adiponectin increases insulin sensitivity (Berg et al., 2001).Expression of *adiponectin* on adipose tissue is decreased in obese subjects and insulin resistance than in lean subjects and is related to a higher level of insulin sensitivity and lower *TNF-α* expression in adipose tissue (Bodles et al., 2006). A shortage of adiponectin induces insulin resistance, whereas overexpression of *adiponectin* improves glucose tolerance and insulin sensitivity in mice (Maeda et al., 2002).

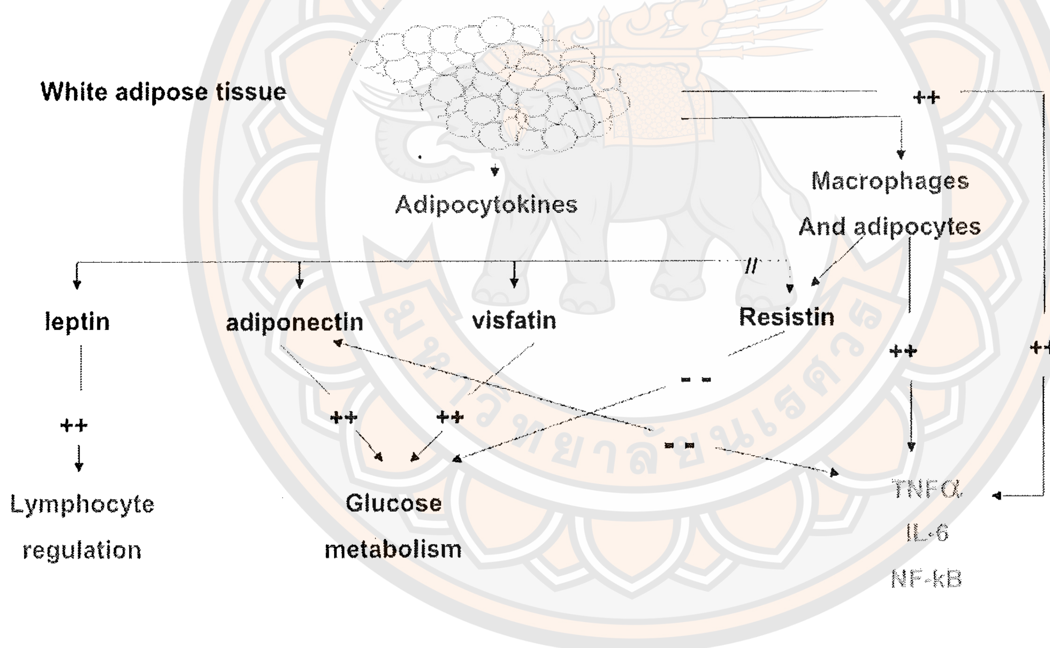


Figure 20 Secretion of adipocytokines and inflammatory cytokines by white adipose tissue

Source: Vachharajani, & Vital, 2006

Inflammatory cytokines

The adipocyte is fundamental to the development of obesity-induced inflammation by increasing various pro-inflammatory, chemokines, and cytokines. The cytokines, including TNF-α, IL1, IL6, and IL8 and promote insulin resistance (Rotter et al., 2003; Skurk et al., 2007). An increase in the adipose tissue leads to increased release of free fatty acids and dysregulated secretion of adipokines. The FFA and pro-inflammatory adipokines affect metabolic tissues, modify inflammatory responses and lipid metabolism, thereby causative to metabolic syndrome. In addition, obesity improves a phenotype in adipose tissue from anti-inflammatory (M2) to pro-inflammatory (M1) macrophages. On the other hand, the adipose tissue induces insulin-sensitizing adipokines with anti-inflammatory properties, such as decreased adiponectin in obese (Figure 21) (Jung, & Choi, 2014).

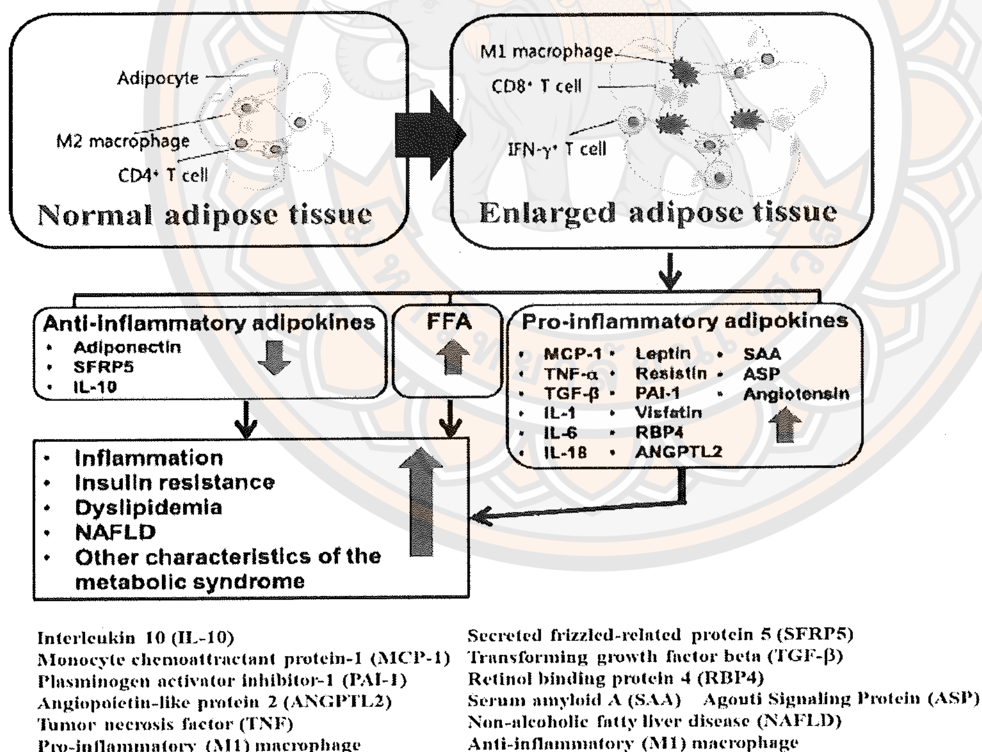


Figure 21 Secretion of inflammatory adipokines from adipose tissue in obesity state

Source: Jung, & Choi, 2014

Obesity, ROS, and insulin signaling

Adipose tissue (AT) has numerous important functions within the body. Of particular importance is its role as an endocrine organ in the control of whole-body glucose and lipid metabolism, which is achieved by the secretion of various proteins. An imbalance in the physiological levels of ROS occurs in a variety of conditions, such as obesity, and can lead to various metabolic abnormalities, including hyperglycemia and hyperlipidemia. In turn, this can contribute to insulin resistance (IR) (Khan and Wang, 2014). Hotamisligil (2006) showed that inflammation may lead to IR by inhibiting IRSs in the insulin signaling pathway, and it was well established that inflammation inhibited the action of insulin by increasing the levels of FFAs and decreasing those of adiponectin in the blood. Inflammatory cytokines and FFAs may target IRSs, leading to IR (Satoh et al., 2004). Furthermore, the High FFA levels have also been shown to downregulate PPAR-γ protein and mRNA expression, further enhancing IR (Haukeland et al., 2012). Moreover, Xu et al (2010) demonstrated a direct role for ER stress in insulin signaling in adipose cells. ER stress inducers were observed to decrease insulin signaling in 3T3-L1 adipocytes without affecting insulin-stimulated glucose uptake (Pfaffenbach et al., 2010), in a manner independent of the IRE1/JNK pathway (Xu et al., 2010).

1. ROS and the promotion of insulin sensitivity

The transient and reversible inactivation of PTPs in response to insulin is well established in cell culture systems. The protein tyrosine phosphatase 1B (PTP1B), T-cell protein tyrosine phosphatase (TCPTP), and Phosphatase and tensin homolog (PTEN) can be transiently oxidized and inhibited by H_2O_2, generated by NOXs such as NOX4, in response to insulin in various cell lines (Figure 22). Consistent with this, recent studies have confirmed the capacity of insulin-induced H_2O_2 to promote insulin signaling and sensitivity *in vivo*. The insulin-induced H_2O_2 might suppress the development of insulin resistance that is induced by an HFD by promoting PI3K-Akt signaling in muscle (Loh et al., 2009). Mice lacking functional the cytoplasmic and mitochondrial antioxidant enzyme Gpx1 (converts H_2O_2 and lipid hydroperoxides) were resistant to the development of HFD-induced. Moreover, recent studies have shown that the concomitant tyrosine phosphorylation and inactivation of PRX1 by Src family protein-tyrosine kinases in response to growth factors and mitogens facilitate the

localized accumulation of H_2O_2 around receptor complexes for the promotion of cell signaling. Notably, many studies showed that ROS were recognized as pathological mediators of insulin resistances which were characterized by hyperinsulinemia and hyperglycemia with the latter being a feature of advanced disease.

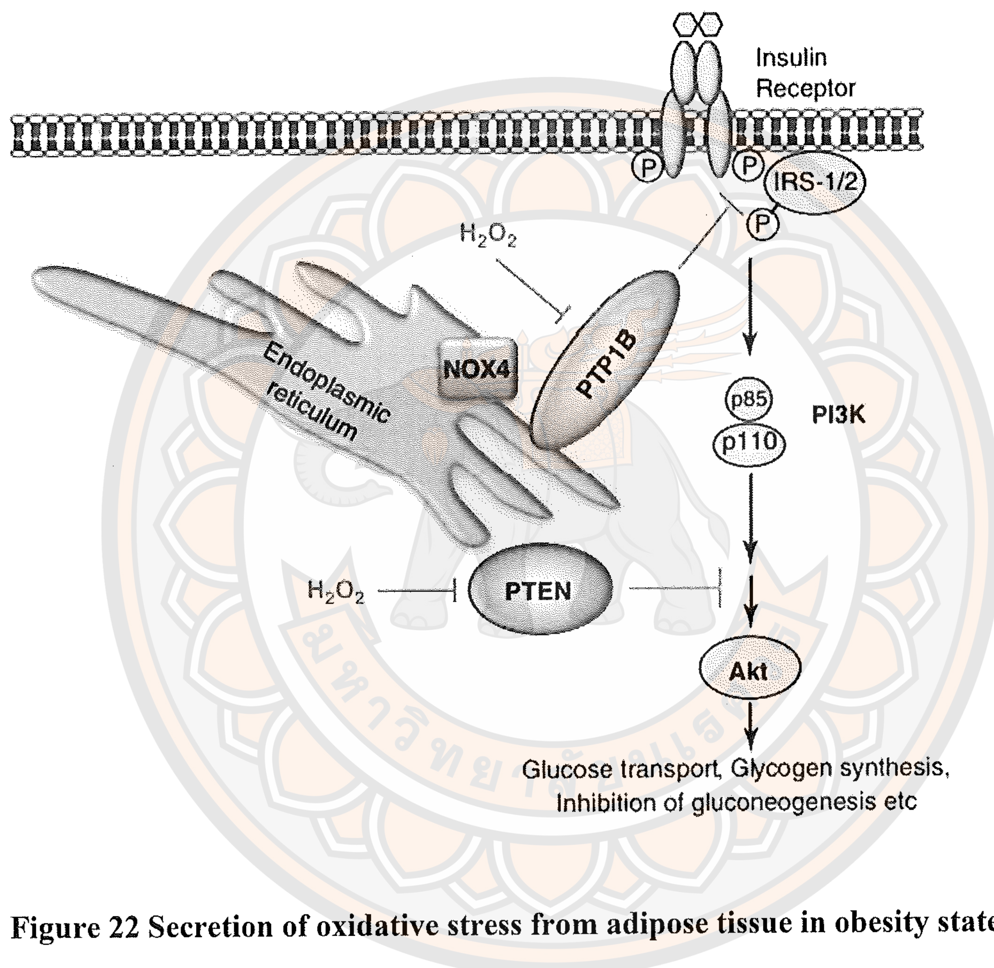


Figure 22 Secretion of oxidative stress from adipose tissue in obesity state

Source: Jung, & Choi, 2014

Insulin is a pleiotropic hormone that has diverse functions including stimulation of nutrient transport into cells, regulation of gene expression, modification of enzymatic activity, and regulation of energy homeostasis via actions in the arcuate nucleus (Cone, 2005; Luca and Olefsky, 2006). These functions of insulin are exerted across a variety of insulin target tissues through several intracellular signaling cascades. The focus of this review is the insulin action on glucose metabolism via its target tissues:

liver, adipose tissue, and skeletal muscle. In skeletal muscle, insulin promotes glucose uptake by stimulating translocation of the GLUT4 glucose transporter to the plasma membrane, and impaired skeletal muscle insulin signaling results in decreased glucose disposal. In the liver, insulin inhibits the expression of key gluconeogenic enzymes and, therefore, insulin resistance in the liver leads to elevated hepatic glucose production. Adipose tissue insulin signaling results in decreased hormone sensitive lipase activity and this anti-lipolytic effect inhibits free fatty acid (FFA) efflux out of adipocytes. Increased circulating FFA can result in decreased insulin sensitivity in skeletal muscle due to an increase in intracellular lipid products, including fatty acyl-CoA and ceramide (Pickersgill et al., 2007; Lee et al., 2006)

2. Insulin receptor substrate (IRS)

The best characterized substrates are members of the insulin receptor substrate (IRS) family of proteins, simply referred to as IRS1 through IRS-6, which act as scaffolds to organize and mediate signaling complexes (Cai et al. 2003; White 2006; Shaw 2011). At the cellular level, IRS1 is preadipocytes that show defects in differentiation in mice, whereas IRS2 is important for lipid metabolism and ERK activation (Huang et al. 2005; Bouzakri et al. 2006). IRS3 and IRS4 show a more restricted tissue distribution pattern. In rodents, IRS3 is most abundant in adipocytes, liver, and lung, which the IRS3 leads to a severe defect in adipogenesis when combined with a deletion of IRS1 (Laustsen et al. 2002). IRS4 mRNA is present in skeletal muscle, liver, heart, brain, and kidney (Fantin et al. 2000), and IRS4 show only very minimal growth retardation and glucose intolerance (Fantin et al. 2000). IRS5 (also called DOK4) and IRS6 (DOK5) has limited tissue expression (Cai et al., 2003) and is relatively poor IR substrates (Versteyhe et al. 2010). Insulin receptors and IRS proteins and enhances insulin sensitivity by promoting insulin receptor catalytic activity and by inhibiting tyrosine dephosphorylation of IRS proteins.

3. Phosphoinositide 3-kinases (PI3Ks)

Phosphoinositide 3-kinases (PI3Ks) constitute a lipid kinase family characterized by the capability to phosphorylate inositol ring 3'-OH group in inositol phospholipids (Fresno-Vara et al., 2004; Engelman et al., 2006). PI3K/AKT signaling plays a central role in cellular physiology by mediating growth factor signals during organismal growth, and critical cellular processes, such as glucose homeostasis, lipid

metabolism, protein synthesis, and cell proliferation (Abeyrathna and Su, 2015). PI3K is further divided into 3 classes: classes I, II, and III, Classes I: is the most thoroughly researched due to its various activities (Guo et al., 2015). Class II: PI3K isoforms are, PI3KC2α, PI3KC2β, and PI3KC2γ, which can be activated by RTKs, cytokine receptors, and integrins (Engelman et al., 2006; Bader et al., 2005). Class III: has been shown to function as a nutrient-regulated lipid kinase mediating signaling through mTOR (mammalian target of mTOR), indicating a potential role in regulating cell growth (Backer, 2008). Importantly, PI3K signaling pathways have been extensively studied downstream of the insulin receptor (IR). Functional insulin signaling is fundamental in obese patients, permitting the storage of their high amount of lipids in an efficient and compact form, within the adipose tissue. Insulin signaling pathways downstream IR has been thus extensively studied to elucidate novel putative therapeutic targets for treating obese patients.

4. Protein kinase B (AKT)

AKT is a serine/threonine kinase expressed as three isoforms, AKT1, AKT2, and AKT3, which are encoded by the genes PKBα, PKBβ, and PKBγ, respectively (Vivanco, & Sawyers, 2002). AKT1 expresses ubiquitously, AKT2 mainly expresses in insulin-sensitive tissues, such as skeletal muscle, adipose tissues, liver, and AKT3 expresses in the testes and brain (Abeyrathna, & Su, 2015; Zhang et al., 2015). AKT regulates lipid metabolism through sterol regulatory element-binding proteins (SREBP), which increases cholesterol and fatty acid accumulation, including SREBP-1c, SREBP-1a, and SREBP-2 (Krycer et al., 2010; Hay, 2011). AKT mediates downstream responses, including cell survival, growth, proliferation, cell migration, and angiogenesis, by phosphorylating a range of intracellular proteins (Manning, & Cantley, 2007).

5. Insulin signaling pathway in various target organs

5.1 Insulin signaling pathway in the adipose tissue

Adipose tissue has two main functions: energy storage and endocrine function, which plays an important role in maintaining body energy balance, including protecting tissues and organs from cold and hot, thermogenesis and the production of various hormones/cytokines that are collectively referred to as cytokines (Kubota et al.,

2017). Insulin-AKT signaling regulates the metabolism of adipose tissues by promoting glucose utilization, protein synthesis, and lipogenesis.

In normal physiology, feeding can activate the synthesis of SREBP-1c via insulin-dependent pathway (mTORC1) or insulin-independent pathway (carbohydrate response element binding protein (ChREBP)) (Denechaud et al., 2008). But insulin pathway is required for the expression of SREBP-1c mRNA in obesity (Haas et al., 2012). PI3K/AKT signaling pathway promotes lipid biosynthesis and inhibits lipolysis. The substrate SREBP which, regulates fatty acid synthase and cholesterol-related genes (Duvel et al., 2010). The FOXO1, which regulates lipolysis by controlling the expression of adipose triglyceride lipase (ATGL), are primary substrates for AKT-mediated lipid metabolism (Chakrabarti et al., 2009; Porstmann et al., 2005).

5.1.1 The feeding state, SREBP-1c is regulated by four pathways: activated AKT stimulates liver X receptor (LXR), which is required for SREBP-1c transcription (Chen et al., 2007); activated AKT stimulates mTORC1 to activate SREBP-1c transcription, and mTORC1 also inhibits Lipin-1, which decreases the half-life of nuclear SREBP-1c (Peterson et al., 2011; Li et al., 2010), and activates S6K1, which promotes SREBP-1c maturation (Owen et al., 2012); activated AKT suppresses insulin-induced gene 2A (INSIG2A), which strongly promotes the maturation of SREBP-1c precursors into nuclear forms (Yecies Jessica et al., 2011); and activated AKT inhibits GSK3, which extends the half-life of SREBP-1c (Bengoechea-Alonso et al., 2009).

5.1.2 In the fasting state, acute lipolysis is induced by β-adrenergic signaling, leading to cAMP accumulation and subsequent PKA-mediated phosphorylation of hormone sensitive lipase (HSL) and perilipin (Eguchi et al., 2011).

5.1.3 In the fed state, PI3K/AKT inhibits protein kinase A (PKA) and thus suppresses lipolysis. AKT-independent, PI3K-dependent pathway also regulates adipocyte lipolysis by directly regulating PKA (Choi et al., 2010), AKT regulates FoxO1 through three pathway: reducing the expression of the rate-limiting lipolytic enzyme (ATGL), which is responsible for triacylglycerol hydrolase activity (ATGL); reducing the expression of interferon regulatory factor 4 (IRF4), which promotes lipolysis, at least in part, by inducing the expression of the lipases HSL and ATGL (Eguchi et al., 2011); phosphorylating phosphodiesterase 3b (PDE3B) to reduce

intracellular cAMP levels and PKA activity, thus inhibiting lipolysis in adipocytes (Ahmad et al., 2009).

Obesity is associated with insulin resistance. Abnormal glucose metabolism caused by insulin resistance in adipose tissues impacts other tissues through its extra-adipose actions. For example, specifically knockdown of GLUT4 in adipose tissues resulted in insulin resistance in skeletal muscle and liver instead of adipose tissues (Vazirani et al., 2016). When the synthesis ability of adipose tissues decreases and fat degradation increases, the release of free fatty acids (FFAs) increases, thus reducing circulating adiponectin levels and lipid oxidation in extra-adipose tissues. However, this process triggers the accumulation of lipids and subsequently causes lipotoxicity and insulin resistance (Lionetti et al., 2009).

5.2 Insulin signaling pathway in the skeletal muscle

Insulin regulates skeletal muscle metabolism by promoting glucose transport, glycogen synthesis, and protein synthesis through PI3K/AKT signaling pathway. In skeletal muscle, insulin stimulates protein synthesis and accelerates mRNA translation by regulating the initiation steps of protein translation (Vary et al., 2001). AKT-mediated mTORC1 activation regulates several downstream effector proteins through phosphorylation, including the well-characterized effectors p70 ribosomal S6 protein kinase-1 (S6K1) and eukaryotic translation initiation factor-4E (eIF4E)-binding protein-1 (4E-BP1), to increase protein synthesis (Laplante, & Sabatini, 2012).

A previous has study found that knockout or knockdown of AKT or IRS adaptor proteins reduces insulin-induced glucose uptake, whereas overexpression of AKT increases glucose uptake (De Nyrgl, 2008). AKT directly phosphorylates AS160, inducing GLUT4 translocation, which translocates to the plasma membrane from storage vesicles and transports glucose in skeletal muscle following stimulation of insulin and AKT (Mueckler, 2010; Cesar, 2013). GagAKT, a constitutively activated form of AKT, promotes glycogen synthesis in L6 myotubes (Ueki et al., 1998). The following study found that activated AKT increases glycogen synthesis in skeletal muscle, activating glycogen synthase (GS) to redirect glucose-6-phosphate to glycogen and inhibiting GSK-3 (Wan et al., 2013). Obesity is associated with several processes in skeletal muscle metabolism, leading to insulin resistance. Insulin resistance refers to a blunted response to insulin in organs and is an important marker of T2DM

(Chen et al., 2017). Therefore, dysfunctional PI3K/Akt-mediated glucose transport and glycogen synthesis play an important role in the development of obesity and T2DM.

5.3 Insulin signaling pathway in the liver

Most of the extracellular glucose is produced in the kidneys and liver, and only the liver acutely responds to insulin by reducing glucose levels (Eileen, 2002).

5.3.1 In the fasting state, glucose is primarily utilized in the liver for gluconeogenesis and glycogenolysis, then transported to various tissues while suppressing the synthesis of new fatty acids (Titchenell et al., 2016).

5.3.2 In the fed state, the PI3K/AKT signaling pathway reduces hepatic glucose production (HGP) and glycogenolysis, increases glycogen synthesis, and the synthesis of fatty acids for storage and subsequent utilization by other tissues (Titchenell et al., 2016; Kubota et al., 2008).

In previous studies, insulin suppressed PEPCK and G6PC expression, which are known to increase hepatic gluconeogenesis by activating the PI3K/AKT pathway (Lu et al., 2012). However, insulin was recently shown to inhibit gluconeogenic gene expression (Titchenell et al., 2015). Additionally, those mice still maintained the normal postprandial state, even in the absence of canonical liver insulin signaling (Titchenell et al., 2015; Lu et al., 2012). Based on these results, AKT may not only be an indispensable intermediate for insulin action, but extrahepatic insulin may also be stimulated by other pathways to regulate glucose metabolism in the liver.

5.4 Insulin signaling pathway in the brain

Three functions of the PI3K/AKT pathway are disturbed in insulin resistance in the brain. First, PI3K generates PIP3, which regulates GHP through K (ATP) channels in the hypothalamus. Insulin signaling is blocked during insulin resistance in the brain due to increased protein kinase C (PKC) activity, ER stress, and inflammation (Yue, & Lam, 2012; Benoit et al., 2009), which blocks the ability of PI3K to generate PIP3. The second, in hypothalamic neurons, FoxO1 upregulates AgRP/NPY expression and downregulates POMC expression under normal conditions, which is inhibited by AKT. In response to insulin resistance in the brain, overexpression of FoxO1 in POMC neurons leads to obesity and hyperphagia (Iskandar et al., 2010). Third, according to studies by Ono et al. (Ono et al., 2008) and Um, S. H et al. (Um et al., 2004),

activation of S6K1, a downstream effector of mTORC1, in hypothalamic neurons leads to hepatic insulin resistance because it decreases the stimulation of IRS-1 and AKT.

5.5 Insulin signaling pathway in the pancreas

The PI3K/AKT signaling pathway regulates cell proliferation, differentiation, metabolism, and cytoskeletal reorganization, leading to apoptosis and cancer cell survival. Therefore, the pathway is associated with various diseases, such as obesity, diabetes, and cancer. In normal conditions, PI3K/AKT is activated in regulating the function of the body. When excessive energy intake occurs, the PI3K/AKT pathway is suppressed. At this point, activation of the PI3K/AKT pathway alleviates obesity and insulin resistance. However, when the regulation of PI3K is disturbed, such as overexpression and mutation, it can cause many human diseases (e.g., obesity, cancer) (Dornan and Burke, 2018).

6. The effects of insulin resistance

6.1 Lipotoxicity

One feature of metabolic syndrome is the accumulation of lipids, especially fatty acids (FA), which is believed to cause insulin resistance via multiple mechanisms. Tissue-specific increase in lipid content in nonadipose tissues provides direct evidence of lipotoxicity. Increased hydrolysis of circulating triglycerides owing to muscle-specific overexpression of lipoprotein lipase leads to skeletal muscle insulin resistance (Ferreira et al. 2001), whereas increased lipid transport in heart or liver leads to lipotoxic cardiomyopathy and nonalcoholic fatty liver disease, respectively (Chiu et al. 2005; Koonen et al. 2007). Besides the effect of increased lipid flux on insulin sensitivity, multiple lipid intermediates have been shown to promote insulin resistance.

Elevated circulating free fatty acids (FFA) are observed in obesity and induce activation of JNK, IKK, and PKC and IRS1 Ser-307 phosphorylation (Schenk et al. 2008). The fatty acid palmitate plays a particular role in promoting insulin resistance as it induces endoplasmic reticulum (ER) stress, cytokine production, and activates JNK (Ozcan et al., 2004; Shi et al., 2006). In addition, palmitate activates NF-kB signaling while inhibition of this pathway reverses lipid-induced insulin resistance (Kim et al., 2001a; Sinha et al., 2004). Interestingly, the detrimental effect of palmitate on skeletal muscle insulin resistance can be reversed by confusion with oleate, thereby changing its

conversion from phospholipids and diacylglycerol (DAG) to triglycerides (Peng et al., 2011). This indicates that FFA induces insulin resistance through multiple mechanisms, and combinations of FA can influence insulin signaling and highlight the crucial interplay of lipids for dietary interventions. The lipid metabolite DAG has also been shown to induce insulin resistance. Increased muscle DAG (intramyocellular lipid) leads to muscle insulin resistance by activating PKC-u and inducing IRS1, Ser-307 phosphorylation. Conversely, reducing DAG levels in skeletal muscle and liver protects mice against high-fat-diet-induced insulin resistance (Samuel et al., 2010). Increased plasma concentration of the sphingolipid ceramide is observed in obese and diabetic patients and is associated with severe insulin resistance (Haus et al., 2009). Ceramide has been shown to induce insulin resistance via PKC and JNK activation (Schenk et al., 2008) and, thus, inhibition of ceramide synthesis ameliorates insulin resistance (Holland et al., 2007). Ceramides also inhibit Akt activation by increasing the interaction of PP2A with Akt, and phosphorylation of Akt at Thr-34 by PKCz, resulting in reduced binding of PIP3 to Akt (Blouin et al., 2010).

In addition to effects on kinases, alteration of membrane-lipid composition affects insulin signaling. An increase in the saturated-to unsaturated FA ratio is observed in type-2 diabetic patients and is thought to reduce membrane fluidity and insulin sensitivity (Bakan et al., 2006). Moreover, an increase in the phosphatidylcholine (PC) to phosphatidylethanolamine (PE) ratio in the endoplasmic reticulum leads to the activation of ER stress and is associated with insulin resistance (Fu et al., 2011).

6.2 Endoplasmic reticulum stress

Endoplasmic reticulum (ER) stress was first found to induce chronic inflammation in obesity by activating JNK (Ozcan et al., 2004). ER is a membranous network that functions in the synthesis and processing of secretory and membrane proteins. Accumulation of unfolded or misfolded proteins is a major cause of ER stress (Schröder, & Kaufman, 2005). In obesity, ER stress may improve insulin sensitivity in the liver by activation of p38 MAP kinase (Lee et al., 2011). Inhibition of ER stress by chemical drugs is shown to improve insulin sensitivity in obese mice (Ozcan et al., 2006).

6.3 Hypoxia

Systemic hypoxia was proposed as a risk factor for insulin resistance in patients with sleep apnea. This line of evidence suggests the role of hypoxia in the pathogenesis of insulin resistance. In a recent study, acute systemic hypoxia was shown to induce insulin resistance in lean mice (Liyori et al., 2007). The study excludes the role of the autonomic nervous system (ANS) in the hypoxia-induced insulin resistance in OSA. Our study suggests that local hypoxia may contribute to the inflammatory response in adipose tissue (Ye et al., 2007).

6.4 Oxidative stress

Oxidative stress is a result of a misbalance between production and disposal of reactive oxygen species (ROS). ROS is mainly generated in mitochondria during the oxidation of fatty acids or glucose for ATP or heat production. ROS is required for normal signal transduction in cells (Bashan et al., 2009). In cells, oxidative stress is induced by fatty acids such as oleic acid (Furukawa et al., 2004), and glucose (Robertson et al., 2004). Hypoxia also induces ROS (Aragonés et al., 2009). ROS has been reported to inhibit insulin signal transduction by activation of PKC, JNKs, and NFκB (Evans et al., 2003). ROS also mediates TNF-α and glucocorticoid signals in insulin resistance (Houstis et al., 2006). In contrast, insulin sensitivity is enhanced by exercise (Hu et al., 2001). Autophagy was reported to mediate exercise signal to improve insulin sensitivity in muscle (He et al., 2012).

Obesity and oxidative stress

ROS has been shown as pathological mediators of insulin resistance characterized by oxidative stress and antioxidant system. The relative contribution of ROS to the prevention or development of insulin resistance is the use of varied animal models and HFDs. Moreover, it was found that high ROS levels within the WAT promote a disturbed redox balance and impact its function via mechanisms, such as adipogenesis and antioxidant activity, induction of insulin resistance, and adipocyte hypertrophy (Castro et al., 2016). In this view, a burst in ROS production will commit preadipocytes from proliferation to differentiation. In contrast, excessive or inadvisable redox balance affects adipocyte precursor's conversion, therefore, limiting "adipose tissue expandability" and hypertrophy (Virtue, & Vidal-Puig, 2010). Adipocytes will

indeed impair adipose tissue function by inducing inflammation, mechanical stress, and metabolism due to dysregulated adipokine secretion (Murdolo et al., 2013). Physiological levels of ROS, maintained by the capable detoxification system, can induce insulin-mimicking effects of H_2O_2 and promote preadipocyte differentiation. In contrast, high ROS or inappropriate redox balance in intracellular ROS, which will have detrimental effects, particularly by altering insulin signaling, lipid peroxidation, protein carbonylation, induce pro-inflammatory cytokines are increased and reduce adiponectin in obese adipocytes exposure to high levels of ROS (Lay et al., 2014) (Figure 23).

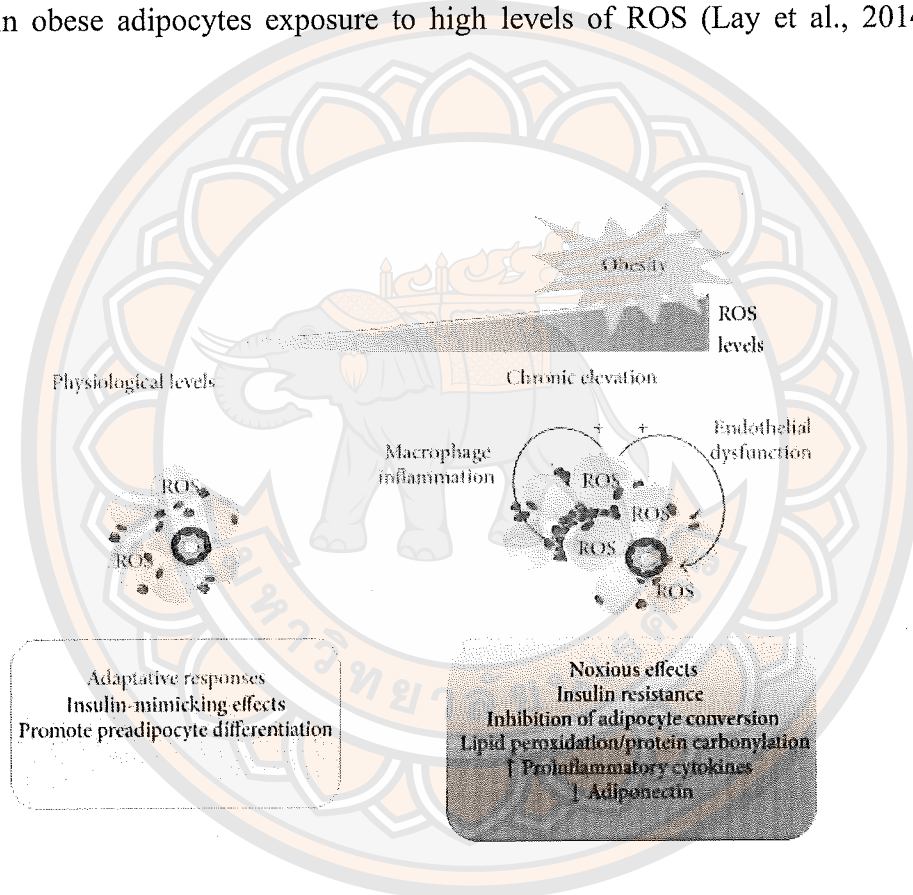


Figure 23 Adaptive or deleterious metabolic responses depending on ROS levels on adipose tissue metabolism

Source: Adapted from Lay et al., 2014

1. ROS Sources in adipocytes

Different sources of intracellular ROS detailed below might participate in ROS generation by adipocytes (Figure 24). ROS can derive 3 sources in adipocytes. 1) Mitochondria, as the core machinery for energy production through oxidative phosphorylation, is considered as a main source of O_2^- (Brownlee, 2005), predominantly produced at complexes I and III (Murphy, 2009). 2) ROS can also be produced by NADPH oxidases enzymes, the family of NADPH oxidases (NOX) is considered to be an important source of ROS generation (Mahadev et al., 2004). NOX are membrane-bound enzyme complexes that transfer electrons from NADPH to oxygen. Generated O_2^- are further converted into H_2O_2 by endogenous SOD (Bedard, & Krause, 2007). 3) Nitric oxide synthase (NOS), whose eNOS and iNOS isoforms are abundantly expressed by adipocytes, might represent another source of ROS since they can be uncoupled to produce O_2^- can react avidly with vascular $NO^{\cdot}$ to form $ONOO^-$. Under certain pathological conditions, for instance, when the availability of NOS cofactors tetrahydrobiopterin (BH_4) or substrate L-arginine is too low, the enzymatic activity of NOS can be uncoupled to produce O_2^- (Vasquez-Vivar et al., 1998; Kuzkaya et al., 2003).

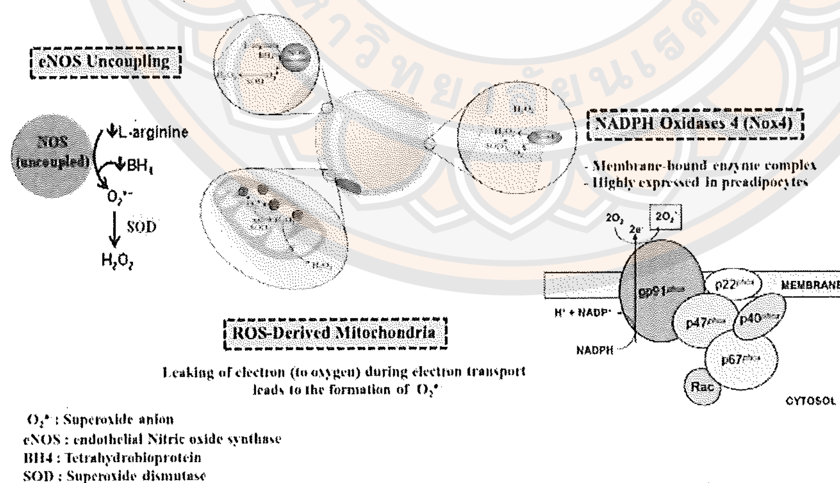


Figure 24 Different intracellular ROS sources participate in ROS generation by adipocytes

Sources: Adapted from Lay et al., 2014

2. ROS production by mitochondria

Mitochondria are necessary for fundamental functions, including respiration, oxidative energy production, and control of the fatty acid β-oxidation (Navarro, & Boveris, 2007), and is required for aerobic animals to obtain energy by oxidizing substances by transfer of electrons to electron carriers such as NAD^+, FMN, and FAD (Mitchell, & Moyle, 1965) (Figure 25).

The finding that electron-transfer is associated with the formation of ROS suggested the mitochondrial involvement in degenerative processes linked to several diseases and aging. The primary ROS generated within mitochondria by auto-oxidation of electron carriers is O_2^-, which is improved by mitochondrial SOD and CAT into H_2O_2, which can be turned into $\cdot OH^-$ via the Fenton reaction (**$H_2O_2 + Fe^{2+} \rightarrow {}^{\cdot}OH + OH^- + Fe^{3+}$**) (Quinlan et al., 2013). In the mitochondrial ROS production are localized at Complexes I and III (St-Pierre et al., 2003). Thus, both producers of Complex I and Complex III producing O_2^- into the matrix where it can damage mitochondrial DNA into the intermembrane space (St-Pierre et al., 2003).

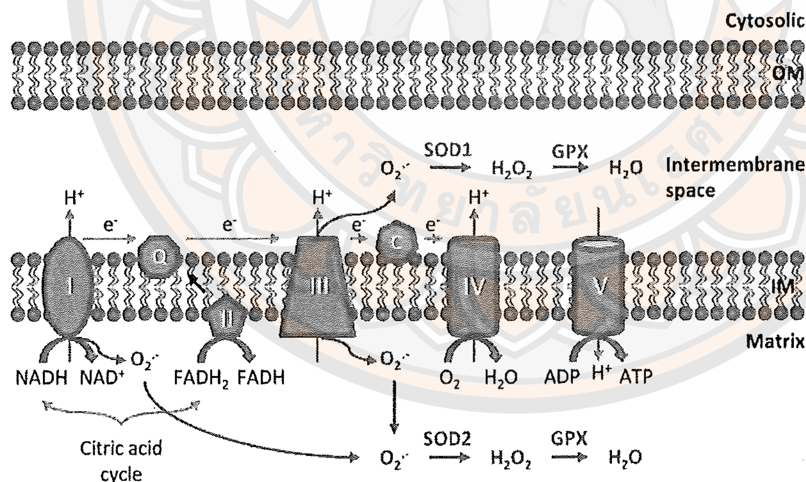


Figure 25 ROS production by mitochondria

Sources: Li et al., 2013

3. ROS production by membrane-bound enzyme NADPH oxidase

The plasma membrane is involved in cellular processes such as cell adhesion, cell signaling, and ion conductivity. ROS in tissues could be generated from dysfunctional cells. However, the source of ROS is represented by O_2^- production in the membrane-bound enzyme NADPH oxidases (Cho, Seo, & Kim, 2011). There are five isoforms of NOX (NOX1, NOX2, NOX3, NOX4, and NOX5) and two related enzymes (DUOX1 and DUOX2) were targeted to cellular membranes. The NOX proteins constitute the enzyme family with the function of producing ROS. NOX1 found that in smooth muscle cells and vascular cells, NOX2 found that in endothelial and phagocytic cells, and NOX3, found in the brain and inner ear, production O_2^-. NOX4, constitutively expressed and active in vascular smooth muscle and endothelial cells, is responsible for the generalization of H_2O_2. NOX5, found in human immature lymphatic tissues and endothelial cells, generate H_2O_2 in a Ca^{2+} conditional fashion (Katsuyama, 2010). The DUOX1 and DUOX2, originally isolated in the thyroid, produce H_2O_2 that oxidizes iodide by thyroid hormone synthesis (Kim, & Dinauer, 2006) (Figure 26).

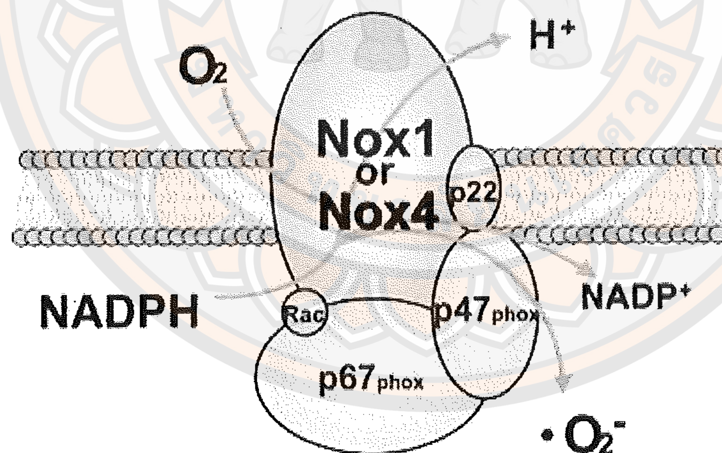


Figure 26 ROS production by NADPH oxidase

Sources: Li et al., 2013

4. ROS production by nitric oxide synthases (NOS)

The nitrogen oxide (NO^-) a product of amino acid metabolism and the enzymes catalytic in process, known as nitric oxide synthases (NOS), convert Larginine into L-citrulline and NO^- by guanidine nitrogen of L-arginine (Sanchez et al., 2006). NOS have three isoforms: two isoforms, (I) neuronal NOS (nNOS) and (III) endothelial NOS (eNOS), are regulated by the interaction of Ca^{2+} with calmodulin. The other isoform, inducible-NOS (iNOS; type II NOS), is induced in response to inflammation, trauma, or infection (Loughran et al., 2005; Parihar et al., 2008). This might represent another source of ROS since they can be uncoupled to produce O_2^-, which can react avidly with vascular NO to form ONOO−. Under certain pathological conditions, for instance, when the availability of NOS cofactor tetrahydrobiopterin (BH_4) or substrate L-arginine is too low, the enzymatic activity of NOS can be uncoupled to produce O_2^- (Vasquez-Vivar et al., 1998; Kuzkaya et al., 2003) (Figure 27).

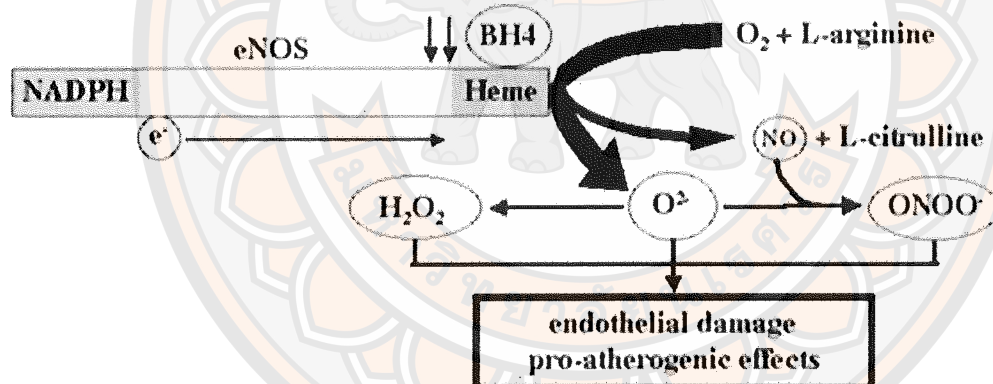


Figure 27 ROS production by nitric oxide synthases (NOS)

Sources: Li et al., 2013

Obesity-associated dysregulated metabolic parameters: contributors and amplifiers of oxidative stress

ROS occur under conditions of physiological and cause direct or indirect damage in different organs. Oxidative stress is involved in pathological processes such as obesity and diabetes. Oxidative stress is associated with a regular production of adipokines, which promotes the development of metabolic syndrome (Esposito, Ciotola,

& Giugliano, 2006). The sensitivity of biomarkers of oxidative damage is higher in personages with obesity and relates directly with BMI and the body fat, TG levels, and LDL oxidation in the body. The research study showed that a diet high in carbohydrates and fat increases oxidative stress and inflammation in people cause of obesity (Patel et al., 2004; Hartwich et al., 2007).

During WAT expansion: hypertrophia (increase size of fat cells) and hyperplasia (increase numbers of fat cells) have shown the close relationship between ROS and adipogenesis in adipocytes by a marked increase in ROS production dependent on NADPH oxidases. The silencing of NOX4 isoform of NAPDH oxidases inhibits insulin-dependent adipocytes (Schröder et al., 2009). Whereas lipid accumulation is affected following NOX4 overexpression or exogenous of H_2O_2 and increasing ROS production found in mitochondria. Thus, high levels of ROS might affect inflammation cytokine, production, metabolic dysfunctions and inhibit insulin-dependent adipocyte differentiation (Kanda et al., 2006) (Figure 28).

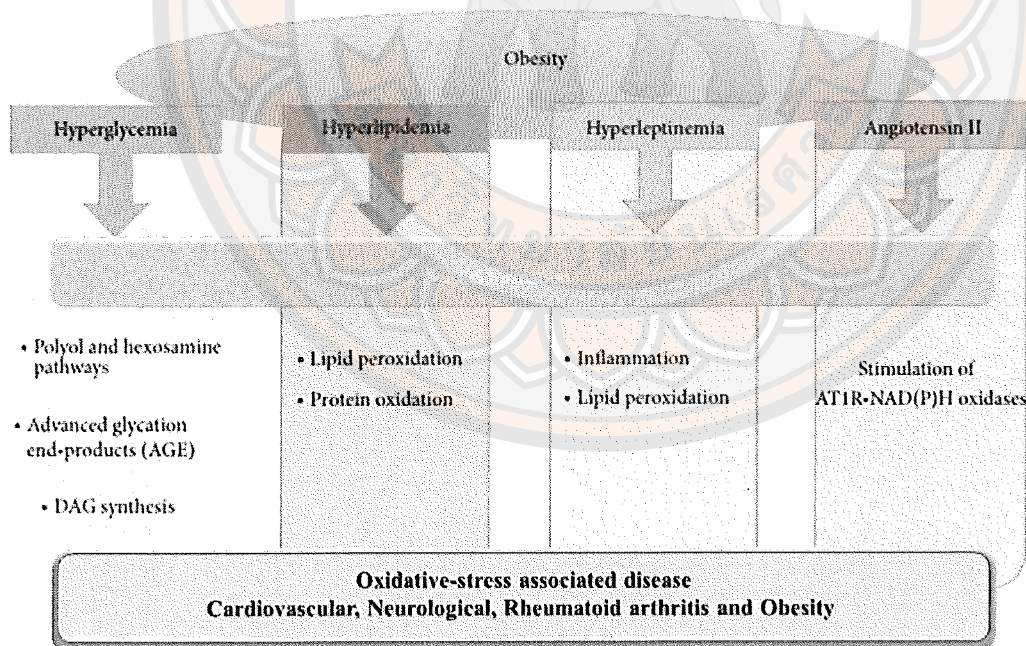


Figure 28 Systemic metabolic alterations associated with obesity contribute to the increase in oxidative stress

Sources: Lay et al., 2014

1. Hyperglycemia

Hyperglycemia induces overproduction of O_2^- by the mitochondrial ETC and reduces the activity of the key glycolytic enzyme glyceraldehyde-3 phosphate dehydrogenase (GAPDH) leading to the activation of three major pathways of hyperglycemic damage (Du et al., 2003). Increased levels of the G3P, following GAPDH inhibition, activate intracellular production of advanced glycation end-products (AGE) precursors and increased diacylglycerols (DAG) (Lee et al., 1989). In adipocytes exposed to hyperglycemic conditions result in increased ROS production and reduced insulin-stimulated glucose uptake (Tang et al., 2001), and increased inflammatory response (Lu et al., 2001).

2. Hyperlipidemaid

FFA concentrations are commonly elevated in obese associate, oxidative stress, and insulin resistance. FFAs occurring during obesity enhances oxidative stress via increased β-oxidation of acyl-CoA esters. ROS induced lipid peroxidation and increased body weight and might be a direct consequence of lipid peroxidation-associated obesity favoring the development of atherosclerosis (Paolisso et al., 1992; Gaal van et al., 1998).

3. Hyperleptinemia

Leptin, a WAT-derived hormone, has been shown to increase the generation of ROS in endothelial cells (Yamagishi et al., 2001; Mamputu et al., 2005). This effect is associated with an enhanced expression of monocyte chemoattractant protein-1 (MCP-1), which promotes atherosclerosis by favoring the migration of inflammatory cells. In addition, plasma concentrations of isoprostanes as well as lipid peroxidation products, are markedly increased following leptin infusion suggesting a proatherogenic effect of leptin on macrophages in diabetes (Maingrette, & Renier, 2003).

4. Activation of the renin-angiotensin system

The renin angiotensin system (RAS), including angiotensinogen, renin, angiotensin-converting enzyme (ACE), angiotensin II (Ang). Adipocytes are a suggested source of all components of the RAS, in which production is related to obesity-associated hypertension and Ang II-induced production of ROS is an important initiator and contributor of oxidative stress-associated obesity (Cassis et al., 2008; Kurata et al., 2006).

Oxidative-stress associated diseases

Adipocytokines and oxidative stress are a trait of adipose tissue dysfunction in obesity leading to systemic inflammation in obesity. In addition, inflammation and oxidative stress are closely interconnected, systemic oxidative stress also appears as the hallmark of metabolic syndrome. Accordingly, oxidative stress has been shown to play critical roles in the development of pathologies such as diabetes, atherosclerosis, and hypertension.

1. Cardiovascular disease

There are several risk factors associated with cardiovascular disease (CVD) including hypertension, hypercholesterolemia, smoking, and diabetes. Research data have shown that whether oxidative stress is a primary cause of many cardiovascular diseases. Further *in vivo* and *ex vivo* studies have provided supporting the role of oxidative stress in CVDs such as atherosclerosis, hypertension, cardiac hypertrophy, and congestive heart failure (Ceriello, 2006).

2. Neurological disease

Oxidative stress has been examining in neurological diseases including Parkinson's disease, Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and multiple sclerosis. A disease shown has demonstrated that oxidative damage plays an important role in the loss of neurons and the progression to dementia. The production of ß-amyloid and peptide is often found that in Alzheimer's disease, it induces oxidative stress and plays a key role in the neurodegenerative processes (Singh, Sharad, & Kapur, 2004).

3. Rheumatoid arthritis

Rheumatoid arthritis is an autoimmune disease characterized by chronic inflammation of the tissue and joints with infiltration of macrophages and activated-T cells. The pathogenesis of rheumatoid arthritis disease is induced by the generation of ROS and RNS. Oxidative damage and inflammation in rheumatic diseases were proof by increased levels of prostaglandins and isoprostanes in serum compared to controls (Walston et al., 2006; Mahajan, & Tandon, 2004).

Antioxidants and the mechanisms of action of antioxidants

Antioxidants are involved in the protection of cellular damage to the common pathway for aging, cancer, and a variation of diseases (Young, & Woodside, 2001). Specifically, enzymatic defenses include molecules that are neutralizing, capable of removing, or scavenging ROS, RNS, and their intermediates. Antioxidant defense mechanisms include the inhibition of ROS and RNS formation, the up-regulation of antioxidant activity, and the binding of metal ions needed for catalysis of ROS generation. The structure of antioxidants is strong enough to donate an electron to a rampaging free radical and reducing its capacity to damage, inhibiting cellular damage through their free radical scavenging properties (Warner et al., 2004). The molecular structure of antioxidants is stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing its capacity for damage.

1. Antioxidant defense system

An antioxidant is any substance that in low concentrations delays the oxidation of DNA, proteins, carbohydrates, and lipids. They can be distinguished into two major groups (Irshad, & Chaudhur, 2004) (Figure 29):

1. Enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx).

2. Non-enzymatic oxidants, such as glutathione (GSH), vitamin C, vitamin E, Cofactor Q 10, flavonoids, phenolic acid, and carotenoids.

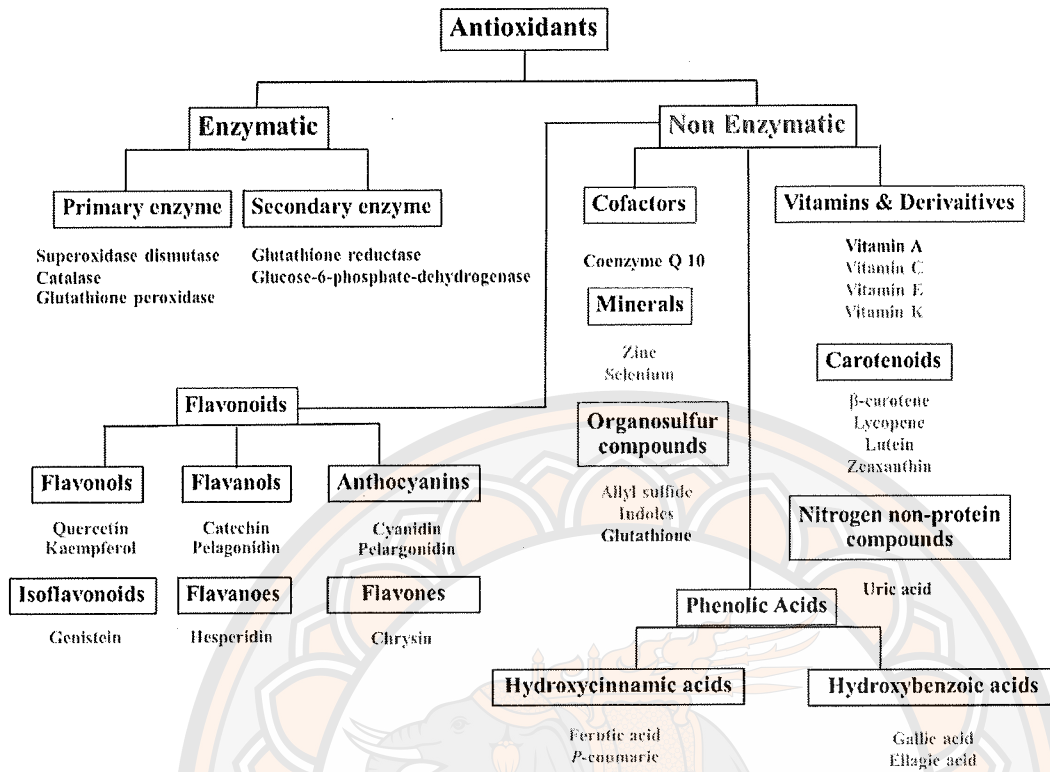


Figure 29 Natural antioxidant groups

Sources: Pietta, 2000; Ratnam et al., 2006

2. Levels of antioxidant action

2.1 Enzymatic antioxidants

2.1.1 Superoxidase dismutase (SOD)

SOD plays a critical role in endothelial and mitochondrial function by inhibiting oxidative stress. Depending on the transition metal ion found at their active site. SODs are enzymes that catalyze the dismutation of superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2) (Figure 30). Thus, they are an important antioxidant defense in nearly all cells exposed to oxygen. SODs can be categorized into three types: copper/zinc (Cu/Zn) SOD which is primarily in cytosols, manganese (Mn) SOD in the mitochondria, and iron (Fe) SOD in prokaryotes (Raha, & Robinson, 2000)

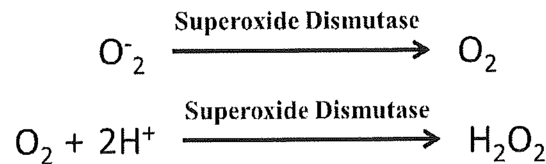


Figure 30 Function of superoxidase dismutase (SOD)

Sources: Mruk et al., 2002

2.1.2 Glutathione peroxidase (GPx)

GPx an enzyme dependent on the micronutrient selenium (Se), plays an important role in the reduction of lipid and hydrogen peroxides (Yu, & Chung, 2006). GPx has four types; GPx1 and GPx2 are found cytosol, GPx3 is found in the plasma, and GPx4 is found in mitochondria interacts with complex lipids such as lipoproteins and cholesterol damaged by free radicals (Arthur, 2000). GPx neutralizes hydrogen peroxide (H_2O_2) to molecular oxygen (O_2) and water (H_2O) (Figure 31).

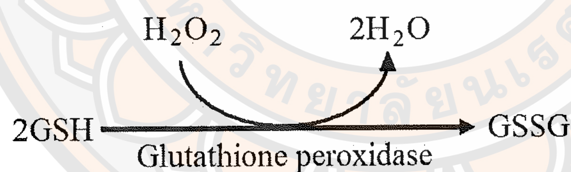


Figure 31 Function of glutathione peroxidase (GPx)

Sources: Imai, & Nakagawa, 2003

2.1.3 Catalase (CAT)

Catalase is the first enzyme to be catalases hydrogen peroxide (H_2O_2) to molecular oxygen (O_2) and water (H_2O) (Chelikani et al., 2004) (Figure 32), an enzyme that inactivates hydrogen peroxide, preventing the formation of hydroxyl radical and the hypochlorous acid. Catalase includes four protein subunits, each

containing a heme group and a molecule of NADPH. Catalase works next to SODs to protect against free radical damage to the body.

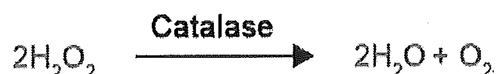


Figure 32 Function of Catalase (CAT)

Sources: Chelikani et al., 2004

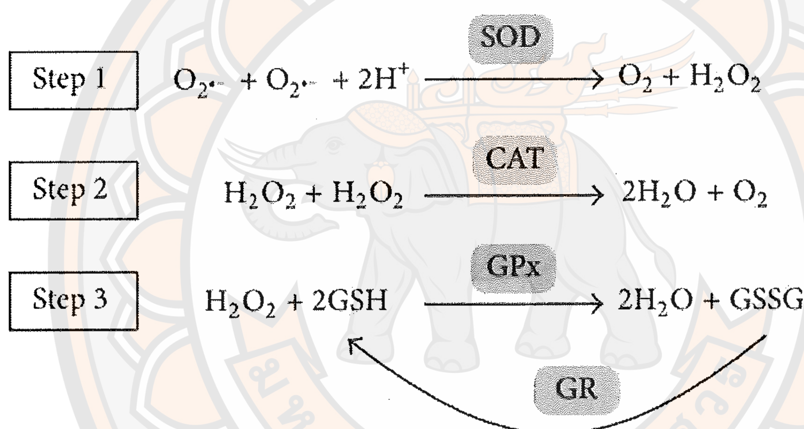


Figure 33 Mechanism of action of enzymetic antioxidants on ROS.

Sources: Hamada et al., 2014

2.2 Non-enzymatic antioxidants

2.2.1 Glutathione (GSH)

The major thiol antioxidant is the ubiquitous glutathione (GSH). GSH is a tripeptide of L-glutamate, L-cysteine, and glycine, it is a complex in cellular functions, which is considered to be a major thiol-disulfide redox is a buffer of the cell which, is a multifunctional intracellular antioxidant (Masella et al., 2005). GSH the cells protected free radicals either by donating a hydrogen atom or an electron. In reaction, GSH is oxidized to oxidized glutathione (GSSG); GSH is then remodeled from GSSG

by the enzyme GR (Dröge, 2002). GSSG is an antioxidant reaction that contains GSH and can increase accumulate of oxidative processing in cells. The proportion of GSSG/ GSH serves as a sensitive marker of oxidative stress. The functions of oxidative stress require GSH to be in a reduced form. GSSG is reduced to regenerate, GSHSG which is a reaction catalyzed by GSH reductase that reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a hydrogen donor (Figure 34).

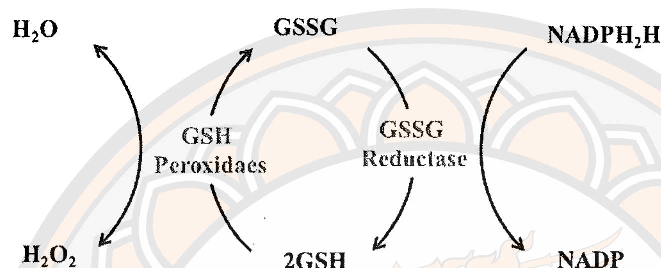


Figure 34 Function of glutathione (GSH)

Sources: Chelikani et al., 2004

2.2.2 Vitamin C and E

Vitamin C and E are generic names for ascorbic acid and tocopherols. Ascorbic acid includes two compounds with antioxidant activity: L-ascorbic acid and L-dehydroascorbic acid which are both absorbed through the gastrointestinal tract and can be interchanged enzymatically *in vivo*. Ascorbic acid is effective in scavenging the superoxide radical anion, hydrogen peroxide, hydroxyl radical, singlet oxygen, and reactive nitrogen oxide (Barros et al., 2008). Vitamin E is composed of eight isoforms, with four tocopherols (a-tocopherol, b-tocopherol, c-tocopherol, and d-tocopherol). These two vitamins also display a synergistic behavior with the regeneration of vitamin E through vitamin C from the tocopheroxyl radical to an intermediate form, therefore reinstating its antioxidant potential.

2.2.3 Coenzyme Q_{10} (Co Q_{10})

Co Q_{10} or Ubiquinone is found in all cells and membranes. Co Q_{10} plays an important role in cellular metabolism and the respiratory chain. Co Q_{10} is fat-soluble quinine that transfers electrons from complexes I and II to complex III in

the mitochondria. Co Q10 can inhibit lipid peroxidation and can protect mitochondrial inner-membrane proteins and DNA from damage, and it is used as a co-factor supplement in the treatment of mitochondrial disorders (Turunen et al., 2004) (Figure 35). Co Q_{10} is used for the treatment of Parkinson's disease, diabetes, cardiomyopathy and to prevent mitochondrial dysfunction (Littarru, & Tiano, 2005).

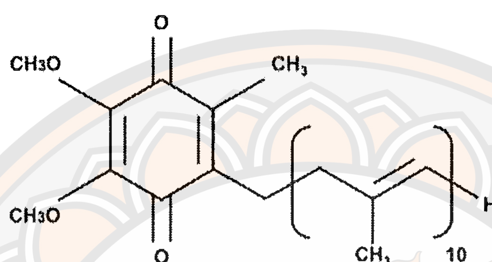


Figure 35 Structure of coenzyme Q_{10} (Co Q_{10})

Sources: Turunen et al., 2004

2.2.4 Uric acid

Uric acid is the end product of nucleotide metabolism in humans. Uric acid prevents the overproduction of oxo-hem oxidant products from the reaction of hemoglobin with peroxides (Muraoka, & Miura, 2003), these radicals seem to target predominantly lipids (LDL and membranes) rather than other cellular components. In addition, it is a potent scavenger of singlet oxygen and hydroxyl radicals and prevents lysis of erythrocytes by peroxidation (Sautin et al., 2007).

2.2.5 Flavonoids

Flavonoids are bioactive of antioxidant groups such as flavonols, anthocyanins, and isoflavonoids. Flavonoids of compounds share the same diphenyl propane ($C_6C_3C_6$) skeleton. Flavonoids are usually found in fruit and plants, some of the most important flavonoids are catechin, quercetin, and kaempferol. The antioxidant properties of flavonoids by the phenolic hydroxyl groups and attached to ring structures and they can act as reducing agents, superoxide radical, singlet oxygen quenchers, and hydrogen donator scavengers. They also activate antioxidant enzymes, reduce

α-tocopherol radicals (tocopherols), mitigate nitrosative stress, increase levels of uric acid and low molecular weight molecules and inhibit oxidases l (Rice-Evans et al., 1996; Procházková et al., 2011).

2.2.6 Phenolic acids

Phenolic acids are composed of hydroxybenzoic acids and hydroxycinnamic acids. They are ubiquitous to plant material. The antioxidant activity is free radical scavengers with a special impact over peroxyl radicals and hydroxyl radicals and superoxide anions (Terpinc et al., 2011). Phenolic acids are included in the food of human and have studied on bioactivities of phenolic acids such as antioxidant, antimicrobial, and antitumor (Fortalezas et al., 2010). Rice is the source of phenolic contents (Carocho et al, 2014), containing several phenolic acids such as gallic acid, protocatechuic acid, veratric acid, ferulic acid, and caffeic acid.

Potential for treatment obesity

Behavioral and lifestyle modifications continue to be the cornerstone of obesity treatment. Unfortunately, these modifications are often met with limited long-term success; in such cases, pharmacological therapy may be indicated. A brief review of traditional agents used for the treatment of obesity is shown in Table 4.

Table 4 Medications for the treatment of obesity

Drug	Mechanism of action	Adverse Effects
Orlistat	Lipase inhibitor	Flatulence, steatorrhea, increase stool frequency, kidney damage
Sibutramine	5-HT and NE reuptake inhibitor	Headache, insomnia, constipation
Phentermine	Stimulates NE release	Headache, insomnia, irritability, nervousness
Tesofensine	5-HT, NE, and DA inhibitor	Nausea, constipation, dry mouth
Cetilistat	GI and pancreatic lipase inhibition	Flatulence, steatorrhea, increased stool frequency

Source: Motycka et al., 2011

Anit-obesity effect of edible plants

The most effective treatment for people with metabolic syndrome is a drastic change in lifestyle, including weight loss, a healthy diet, regular physical activity, and giving up smoking. A very effective option to reduce weight is bariatric surgery, including gastroplasty, gastric banding, gastric bypass, and Biliopancreatic diversion (Buchwald et al., 2004; Rubino et al., 2009). Interestingly, gastric surgery has beneficial effects on glucose control and insulin resistance independent of weight loss within days of the surgery; the mechanisms for these effects are currently unclear (Scopinaro et al., 2005). Other than invasive surgery, medical treatment for different features of metabolic syndrome is common practice (Scott, & Grundy, 2005). A widely used drug against hyperglycemia is metformin, which lowers hepatic glucose output, partially through AMPK activation (Zhou et al., 2001). Insulin analogs and drugs increasing insulin secretion such as sulphonylureas can be used to overcome insulin resistance in the early stages of the disease. Despite certain side effects, thiazolidinediones-PPARγ agonists are also commonly prescribed in type II diabetes, as they indirectly enhance insulin sensitivity by modulating adipose tissue function (Edgerton et al., 2009).

For the treatment of obesity, most currently available drugs exert their effect on the central nervous system, for example, inhibiting noradrenergic and serotonergic reuptake in the hypothalamus (sibutramine) (Arterburn et al., 2004) or antagonizing cannabinoid receptors (Van et al., 2002). Another strategy to reduce weight pharmacological is the use of fat absorption blockers, e.g., through the inhibition of gastric and pancreatic lipases (orlistat) (Monique et al., 2005), which are most effective in combination with a reduced-calorie diet.

Anti-obesity drug administration could block approximately absorption of dietary fat (Curran et al., 2002). The drug has been reported with some adverse effects which include oily stools, diarrhea, flatulence, bloating, abdominal pain, dyspepsia, and fecal spotting (Van et al., 2005). A Research study has recently come to the fore in discovering potential anti-obesity effects, especially from natural sources with the inhibition of fat absorption and/or fat accumulation in the body by interrupting the lipase and adipocyte activity (Zheng et al., 2010). Natural products might be an excellent alternative strategy for the development of safe and effective anti-obesity drugs.

Table 5 Effects of natural extracts on anti-obesity, anti-inflammatory, and antioxidant

Plants	Bioactive compounds	Anti-obesity	Anti-inflammatory	Anti oxidant	References
Piper nigrum	Phenols, Flavonoids	√	√	√	Vadivel et al., 2016
Peanut	Genistein, Quercetin, Resveratrol	√	√	√	Kung et al., 2016
Oleuropein	Hydroxytyrosol, Tyrosol	√	√	√	Hadrich et al., 2016
Green tea	Phenol, flavonoids	√	√	√	Wolfram et al., 2006 and Choi et al., 2016
Aegle marmelos	Flavonoids, Phenolic, Saponins, Tannin content	√	√	√	Karmase et al., 2013
Cucurbita	Carotenoids, Phenolic	√	√	√	Choi et al., 2013
Red seaweed	Glutamic acid, Leucine	√	-	√	Lu et al., 2020
Taraxacum officinale	Taraxerol, chlorogenic acid	√	√	√	Rao et al., 2015
Rice	GABA, γ-orizanol, ferulic acid, phytic acid, tocotrienols	√	√	-	Ho et al., 2013

A recent study reported that green tea (GT) can decrease adipogenesis and increased lipolysis (beta-oxidation) which are beneficial in cell and animal models of obesity (Jung, & Choi, 2014). In addition, the study of the peanut sprout extract (PSEE) decreased weight gain and reduced body fat contents in adipose tissues of high-fat diets induce might. The PSEE also inhibits adipocyte differentiation by down-regulating the expressions of *PPARγ* and *adiponectin*, which are important for adipocyte differentiation (Kung et al., 2014). Moreover, it was reported that the GBR disposing of

suppressed body weight gain and lipid accumulation in the liver and decreased mRNA expression of adipogenic transcriptional factors, such as *C/EBP*, *SREBP-1c*, *PPAR*, and related genes *aP2*, and *FAS* (Ho et al., 2012). The beneficial effects of GBR on obesity seem to the synergistic effects of high minerals, dietary fibers, vitamins, and other bioactive compounds such as GABA, α-tocopherol, polyphenols, phytosterols, and γ-oryzanol (Lim et al., 2016).

As oxidative stress appears as a critical event between obesity and related chronic diseases such as type 2 diabetes, the biological effects of natural micronutrients that may increase the antioxidant capacity in the body, is of high interest. Rice extracts have metabolically beneficial effects. Recent studies have shown that rice extracts prevented obesity and type 2 diabetes (Sun et al., 2012) by improving glucose uptake (Chisayo et al., 2013). Germinated brown rice (GBR) extract also suppressed lipid accumulation in the 3T3-L1 adipocytes and reduced the body weight, size of adipose tissue, the level of serum triglycerides, and total cholesterol in obese mice (Ho et al., 2012). In addition, GBR exhibited anti-obesity effects through the suppression of body weight gain and food intake, the improvement of lipid profiles, the reduction of leptin level, and white adipose tissue mass in obese rats fed with a high-fat diet (Lim et al., 2016).

Rice

Rice (*Oryza sativa L.*) is indigenous to Asia from southern China to Southeast Asia and India. (FAO, 2012). There are hundreds of different cultivars with different grain colors, sizes, and shapes with different growing conditions and seasonality. Rice is one of the most produced grains in the world and is a staple for many millions of people but is also important in the relationship between diet and health (Melissa et al., 2013). Several antioxidant properties and bioactive compounds have been identified in rice, including its fiber content, iron, vitamins, minerals, tocopherols, tocotrienols, phenolic compounds, γ-oryzanol, and GABA (Mira et al., 2009). Furthermore, rice is rich in nutrients that may protect against various diseases including hypercholesterolemia (Eady et al., 2011), heart disease (Yu et al., 2013), and Diabetes (Meliker et al., 2007).

1. Type of rice

1.1 Polished or white rice (WR) is rice that results after polishing and milling that converts brown rice into white rice. WR has the husk, bran, and germ removed in this process resulting in a seed with a bright, white, shiny appearance (Nanri et al., 2011). WR has carbohydrates but the fiber has been dramatically reduced, the oils, most of the B vitamins, and important minerals. Therefore, the nutritional value of white rice, even when enriched, instant or parboiled, is less than both BR and GBR, having fewer vitamins and minerals than these two types of rice (Villegas et al., 2007).

1.2 Brown rice (BR) is unpolished, but the husk has been removed. BR is high in bioactive compounds such as minerals, dietary fiber, essential fatty acids, vitamins, magnesium, γ-oryzanol (Mahdieh et al., 2015), phytoestrogens, lignin, and essential fatty acids (Hsu et al., 2012). The effect of BR on some inflammatory cytokine mediators such as the tumor necrosis factor-alpha (TNF-α), Interleukin6 (IL6), and plasminogen activator inhibitor type-1 (PAI-1) in rats has been reported (Torimitsu et al., 2010) and the GABA in BR is known to be a neurotransmitter with the ability to increase insulin secretion and to regulate blood pressure (Dong et al., 2006). Also, it has been reported that brown rice is high in antioxidants that can decrease cholesterol and diabetes type 2 and have anti-cancer properties (Garrow et al., 2000).

1.3 Germinated brown rice (GBR) is also known as 'sprouted brown rice'. The process of germination enhances the bioactive compounds of nutrients as soaking in water induces a small degree of germination. BR becomes GBR by germinating BR, so in this way, it is possible to increase the levels of vitamins, minerals, fiber, phytochemicals, such as ferulic acid, GABA and α-oryzanol, (Hasmida et al., 2014). GBR has been reported to possess a high antioxidant capacity, which reduces oxidative stress (Imam et al., 2102), and several studies which have analyzed these activities are now being reviewed for their potential role in the management of oxidative stress-related chronic diseases (Zamri et al., 2014). Kayahara, & Tsukahara (2000) reported that a continuous intake of GBR is effective in preventing heart disease, headache, and cancer of the colon, relieving constipation, regulating blood sugar levels, and diabetes type 2. Okada et al. (2000) found that the intake of GABA improved sleeplessness and suppressed blood pressure and autonomic disorders observed during the menopausal or presenile period. Further, Ito, & Ishikawa (2004) concluded that

GABA might have preventive effects on Alzheimer's disease, or help lessen symptoms of the disease and other disorders, such as amnesia and dementia. In addition, GBR exhibited anti-obesity effects via the suppression of body weight gain and food intake, the improvement of lipid profiles, the reduction of leptin levels, and white adipose tissue mass in obese rats, fed with a high-fat diet (Wang et al., 2015). Rice bran (RB) has been identified bioactive compounds such as oryzanols, tocopherols, tocotrienols, and phytosterols, also suppressed oxidative stress-induced insulin resistance, by neutralizing free radicals and increasing the expression of adiponectin in the high-fat rat (Patil, & Khan, 2011).

2. Structure of rice grain

2.1 The husk or hull is the coating of the seeds, or grains, of rice. The husk covers the outside of the seed and protects it. Brown rice is usually processed by removing the paint layer of the rice husk, which unseals the rice grains and exposes the bran. White rice is usually processed by total removal of the husk, which is comprised of multiple wrapped layers, and bran. The rice bran is high in protein and high fat dietary fiber, with cellulose and hemicellulose content (Wallheimer, & Brian, 2012).

2.2 The rice germ is the reproductive part of the seed that germinates and develops into the plant; it is the embryo of the rice seed (Wittenberg, & Margaret, 2007). The germ is often a by-product of the processing of rice and are a good source of fiber and several essential nutrients including vitamin E, magnesium, zinc, thiamin, phosphorus, and folic acid. Moreover, the rice germ has essential fatty alcohols and fatty acids (Cohen, & Carson, 2013).

2.3 Bran is the hard outer layer of the grain and has a short shelf life owing to the potent enzyme lipase and a high-fat content that degrades making bran rancid and inedible. Bran is removed when the grain is polished into WR grains. Bran has dietary fiber and essential fatty acids and contains significant quantities of starch, protein, vitamins, and dietary minerals. It is also a source of phytic acid and an antinutrient (Barron, & Jon, 2010).

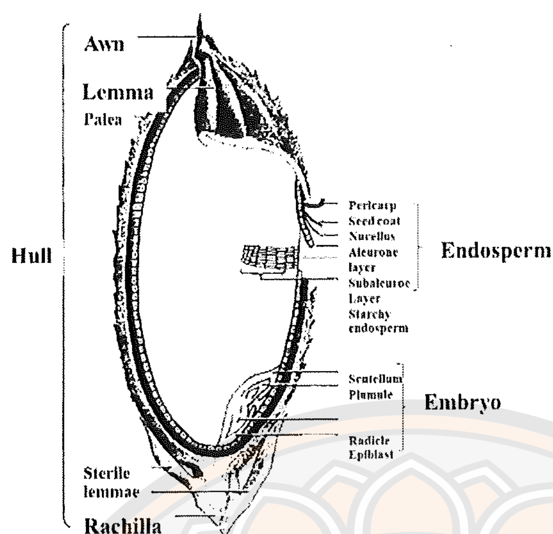


Figure 36 Structure of rice grain

Source: http://www.fao.org/docrep/t0567e/T0567E01.GIF

3. Rice seed germination process

The process of germination is a growing skill that involves helping a seed to sprout. Many, factors influence how seeds germinate (Vidal-Valverde, 2002). The germination starts with the uptake of water and terminates with the emergence of embryonic (Bewlry, & Black, 1994). The nutritional value of important nutrients of each type of rice was shown in Table 6.

Table 6 The nutritional importance of each type of rice

	White rice (WR)	Brown rice (BR)	Germinated brown rice (GBR)
Energy (kcal)	341	347	353
Protein (g)	5.8	6.7	7.1
Lipid (g)	2.9	0.8	2.0
Carbohydrate (g)	80	79.4	75.1
Fiber (g)	0.7	2.1	4.0
Vitamin			
Vitamin B1 (mg)	0.07	0.26	0.44

Table 6 (Cont.)

	White rice (WR)	Brown rice (BR)	Germinated brown rice (GBR)
Vitamin B2 (mg)	0.02	0.04	0.18
Dietary mineral			
Potassium (mg)	-	121	144
Sodium(mg)	-	79	84
Magnesium (mg)	-	27	60
Iron (mg)	-	12	1.3
Zinc (mg)	-	0.48	0.49

Source: Bewlry, & Black, 1994

4. Process of seed germination is divided into 3 steps

Step 1: The seed a rapid uptake the water and the rice seeds' weights increased rapidly during the first 20 h imbibition. Water imbibition is important for the rupture of seeds. Optimum temperatures are required. The seed morphology is not changeds significantly in step1.

Step 2: The imbibition of water of the seed coat results in germinated emergence of the radicle and the seedling rice sprouts. The temperature and the water must be required in optimum amounts. During imbibition, the water uptake restores the metabolic activity and physiological and biochemical changes.

Step 3: The final step in the germination of the seed where the cotyledons are expanded, emerging of true leaves.

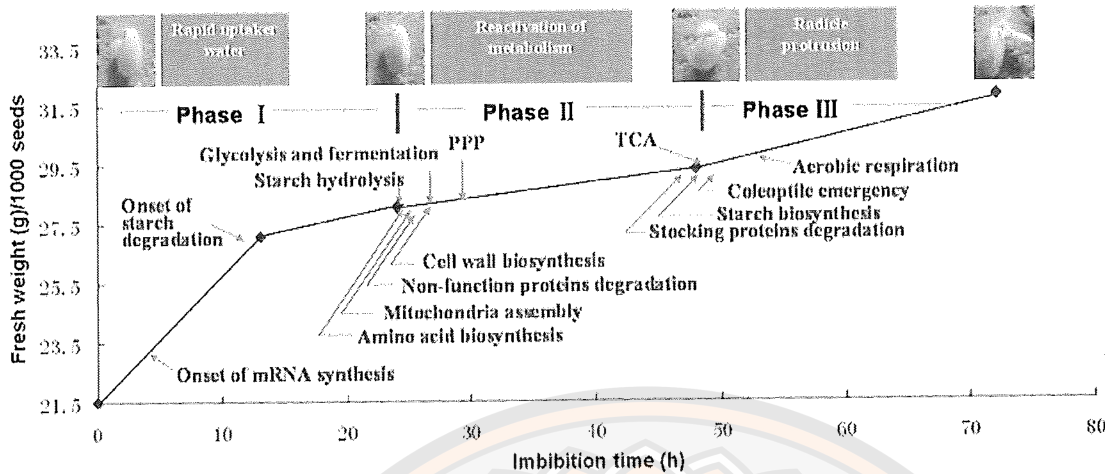


Figure 37 Sequentially processes during rice seed germination

Source: He, & Yang, 2013

5. The bioactive compounds during rice seed germination

Rice seed has a dominant endosperm for nutrient storage. The starchy endosperm is surrounded by an aleurone layer and neighbored with the embryo. Between endosperm layer embryos, there is scutellum, a metamorphosis of the cotyledon. Embryo and endosperm play different roles in rice seed germination (Ohtsubo et al., 2005). The embryo contains most of the genetic information that controls germination. Rice seed germination has a dominant endosperm for high nutrients (Ohtsubo et al., 2005). The rice seed germination of bioactive compounds, which increases gamma-aminobutyric acid (GABA), dietary fiber, ferulic acid, free amino acids, phytic acid, inositols, potassium, tocotrienols, zinc, phenolic, magnesium, and γ-oryzanol (Kayahara, & Tsukahara, 2000; Ohisa et al., 2003). The germination can also produce bioactive compounds and some antioxidants, such as tocotrienols, tocopherols, ascorbic acid, and phenolic compounds, resulting in increased antioxidant activity (Table 7) (Frias et al., 2005).

Table 7 Biological activities of nutrients in GBR

Nutrients	Biological activities in GBR
GABA	Hypotensive effect, accelerating metabolism in the brain, preventing headaches or depressions aftereffects of cerebral arteriosclerosis and cerebral apoplexy, preventing climacteric disorder
Dietary fiber	Relieving constipation, preventing cancer of the colon, regulating blood sugar levels
Inositols	Accelerating fat metabolism, preventing fatty liver, preventing atherosclerosis
Ferulic acid	Scavenging superoxides, suppressing melanogenesis
Phytic acid	Antioxidative effect, protecting cardiovascular disease, preventing platelet aggregation
Tocotrienols	Scavenging superoxides, protecting skin from ultraviolet rays
Magnesium	Preventing heart diseases
Potassium	Lowering blood pressure
Zinc	Activating reproductive function, preventing atherosclerosis
Gamma- oryzanol	Antioxidative effect, preventing skin aging, modulating cholesterol values
Prolylendopeptidase inhibitor	Possible preventing Alzheimer's disease

Source: Kayahara, & Tsukahara, 2000

CHAPTER III

RESEARCH METHODOLOGY

Materials, Equipment, and Chemicals

Cell culture

Materials and equipment

1. Culture plate and flask: T75, T25 and 24-well plate, 6-well plate, and 96- well plate (SPL, Korea)
2. Incubated (CO_2 Incubator): 5% CO_2 37 °C (SANYO, Japan)
3. Water bath: 37 °C (Julabo, Garmary)
4. Inverted microscopes (LEICA, USA)
5. Freezing box (NALGENE, USA)
6. Syringe Filter, hydrophilic 0.22 μm (Sartorius Stedim Biotech, Germany)
7. Hemocytometer (HBG, Germany)
8. Centrifuge (Biofuge, Germany)
9. Micropipette (LAMBDA, Canada)
10. Pipette aid (Trueline, France)
11. Microcentrifuge tube (SPL, Korea)
12. Vortex (IKA works, Malaysia)
13. Tip size 1000 μl, 200 μl, 10 μl (Labcon, USA)

Chemicals

1. 3T3-L1 adipocytes (#CL-173TM, ATCC, USA)

2 DMEM liquid medium with Earle' s salt, with $NaHCO_3$, with Stableglutamine (Gibco, USA) (Cat.No. 11965-092)

3. Fetal bovine serum (FBS) (Gibco, USA) (Cat.No. 10270-098)
4. Antibiotic/Antimycotic (Gibco, USA) (Cat.No. 15240062)
5. Trypsin/EDTA (Biochrom, Germany)
6. Phosphate buffered saline (PBS) (Bio Basic, Canada)

Effect of rice extracts on cytotoxicity in 3T3-L1 adipocytes by MTT assay

Materials and equipment

1. UV - visible spectrometer (Biotek, USA)
2. Shaker
3. Micropipette (LAMBDA, Canada)
4. Pipette aid (Trueline, France)
5. 96-well plate (SPL, Korea)

Chemicals

1. MTT solution: MTT (Sigma, USA) (Cat.No. M21288-500MG)
2. Dimethyl sulfoxide: DMSO (Amresco, USA) (Cat.No. 67-68-5)

Effect of rice extracts on lipid accumulation in 3T3-L1 adipocytes by Oil-red O assay and Triglyceride Quantification assay kit

Materials and equipment

1. UV - visible spectrometer (Biotek, USA)
2. Micropipette (LAMBDA, Canada)
3. Inverted microscopes (LEICA, USA)
4. Pipette aid (Trueline, France)
5. 96-well plate (SPL, Korea)
6. 24-well plate (SPL, Korea)

Chemicals

1. Oil-red O (Sigma, USA) (Cat.No. 1320-06-5)
2. Triglycerides liquicolor^{mono} (Human, USA) (Cat.No. 10725)
3. Isopropanol (ACI Labscan) (Cat. No. 67-63-0)
4. Formalin (LEICA, USA)
5. Phosphate buffered saline (PBS) (Bio Basic, Canada)

Effect of rice extracts on intracellular ROS level in 3T3- L1 by 2', 7'Dichlorofluorescin diacetate (DCFH-DA) assay kit

Materials and equipment

1. UV - visible spectrometer (Biotek, USA)
2. Micropipette (LAMBDA, Canada)
3. Pipette aid (Trueline, France)
4. 96-well plate Optical-Bottom Plate with Polymer Base (SPL, Korea)

Chemicals

1. 2', 7' Dichlorofluorescin diacetate (DCFH-DA) (Sigma, USA) (Cat.No. D6883)
2. Hydrogen peroxide (H_2O_2) (Bio Basic, Canada) (Cat.No. 7722-84-1)
3. Phosphate buffered saline (PBS) (Bio Basic, Canada)

Effect of rice extracts on Antioxidant activity and Total phenolic content by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay and Folin –Ciocalteu phenol reagent assay

Materials and equipment

1. UV - visible spectrometer (Biotek, USA)
2. Micropipette (LAMBDA, Canada)
3. Pipette aid (Trueline, France)
4. 96-well black plate (SPL, Korea)

Chemicals

1. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma, USA) (Cat.No. D9132-1G)
2. 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Sigma, USA) (Cat.No.11557-1G)
3. Folin –Ciocalteu phenol reagent (Trueline, France)
4. Methonal (Biotek, USA)
5. Trolox (Sigma, USA) (Cat.No. D9132-1G)
6. Ascorbic acid (Trueline, France)
7. Gallic acid (Biotek, USA)
8. Trolox (Sigma, USA) (Cat.No. D9132-1G)
9. Ascorbic acid (Trueline, France)

Effect of rice extracts on gene expression by Reverse Transcription Polymerase chain reaction (RT-PCR) and quantitative Real-Time PCR (qPCR) technique.

Materials and equipment

1. Heat Block (Labnet International. Inc)
2. NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA)
3. Spin down (Wealtec, Taiwan)

4. Refrigerated microcentrifuge (Heraeus, USA)
5. T100™ Thermal Cycler (Bio-Rad, USA)
6. Eso Swift™ Real-Time PCR machine (Spectrum 48 Real Time Thermal Cyclers, Singapore)
7. Eco™ Real-Time PCR System (Spectrum 48 Real Time Thermal Cyclers, USA)
8. Pipette aid (Trueline, France)

Chemicals

1. RiboZol (AMRESCO, USA)
2. Chloroform (ACI Lab scan) (Cat. No. A 3505 E)
3. Isopropanol (ACI Lab scan) (Cat. No. 67-63-0)
4. DEPC water (BIO Basic) (Cat. No. 1609-47-8)
5. Phosphate buffered saline (PBS) (Bio Basic, Canada)
6. 5x FIREPol® Master Mix Ready (Solis Biodyne, Estonia) (Cat. No. 04-12- 00125)
7. PCR Forward Primer (10 mM) (Integrated DNA Technologies, Singapore)
8. PCR Reverse Primer (10 mM) (Integrated DNA Technologies, Singapore)
9. TBE Buffer (Bioline, USA)
10. Red safe dye (Biotechnology, Korea) (Cat. No. 21141)
11. 100 bp DNA ladder (Genedrirex, Germany) (Cat. No. DM003-R500)
12. HPLC water
13. Real-Time Premi SYBR Green (2x) (RBCBioscience, Taiwan) (Cat. No. RT009)
14. Real-Time Perfe^{CTa} SYBR Green FastMix (Quantabio, Massachusetts) (Cat. No. 95074)

Effect of rice extracts on protein expression by Western blotting technique.

Materials and equipment

1. Heat Block (Labnet international Inc.)
2. Sonicate (Labnet international Inc)
3. Refrigerated microcentrifuge (Heraeus, USA)
4. 96-well black plate (SPL, Korea)

5. Pipette aid (Trueline, France)
6. SDS PAGE running
7. Polyvinylidene difluoride (PVDF) membrane (Trueline, France)
8. Heat Block (Labnet international Inc.)
9. Sonicate (Labnet international Inc.)
10. Refrigerated microcentrifuge (Heraeus, USA)
11. 96-well black plate (SPL, Korea)
12. Pipette aid (Trueline, France)

Chemicals

1. BCA Protein Assay kit (Thermo Scientific, USA)
2. RIPA buffer (Sigma, USA)
3. Protease inhibitor (Amresco, USA)
4. 1M Tris-HCl; pH 6.8 (Omnipur, USA)
5. Sodium dodecyl sulfate (SDS) (Omnipur, USA)
6. 10% glycerol
7. 14.7M β-mercaptoethanol
8. 0.5M EDTA
9. Bromophenol blue
10. 0.5M Tris-Cl; pH 8.8 (Omnipur, USA)
12. 10 % Sodium dodecyl sulfate (W/V) (10% SDS) (Omnipur, USA)
13. 30 % Acrylamide (BIO RAD, USA)
14. 10 % Ammonium Persulfate (APS) (Amresco, USA)
15. N, N, N', N'-Tetramethyl ethylenediamine (TEMED)
16. Distilled water (DW) (Omnipur, USA)
17. 0.5M Tris-Cl; pH 6.8 (Omnipur, USA)
18. 10 % Sodium dodecyl sulfate (W/V) (10% SDS)
19. 30 % Acrylamide (Bio Rad, USA)
20. Ammonium Persulfate (APS) (Amresco, USA)
21. N, N, N', N'-Tetramethyl ethylenediamine (TEMED) (Omnipur, USA)
22. 10X SDS buffer; (1.92M glycine, 0.25M Tris base, 1 % (W/V) SDS) (Omnipur, USA)

23. 10 % Sodium dodecyl sulphate (W/V) (10% SDS) (Omnipur, USA)
24. Coomassie Brilliant Blue R-250
25. Methanol (ACI Lab scan, Thailand)
26. Acetic acid (ACI Lab scan, Thailand)
27. Methanol (ACI Lab scan, Thailand)
28. Acetic acid (ACI Lab Scan, Thailand)
29. 10X transfer buffer; (1.92M glycine, 0.25M Tris base) (Omnipur, USA)
30. 0.1 % (W/V) Ponceau S (Sigma, USA)
31. Acetic acid (ACI Lab scan, Thailand)
32. Non-fat dry milk (Pacific Science, Thailand)
33. 1X TBST
34. Horseradish peroxidase (HRP) conjugates anti-rabbit (Millipore, USA)

Materials

1. Sample preparation

PL2 rice cultivar was obtained from Phitsanulok Rice Research Center and KDML105 rice cultivar was obtained from Lopburi Rice Research Center. There were 3 types of rice extracts: germinated brown rice (GBR), brown rice (BR), and white rice (WR). WR grains are polished to remove the inedible outer hull, most of the bran, the aleurone layer, and the germ of the rice, while the BR grains are only polished to remove the inedible outer hull.

The GBR rice was germinated using the method modified by Patil and Khan (2010). The dehydrated GBR, BR, and WR were powdered in a blender, then extracted using a procedure modified from Ho et al. (2012). First, 50 g of each of the GBR, BR, and WR powder were placed in 250-ml flasks containing 100 ml of 70% (v/v) methanol solution, then the mixture was shaken at 180 rpm for 20 hours at 37°C twice and 150 ml of supernatant was then collected, then filtered through a 1-micron filter paper (Whatman, USA) and evaporated using a rotary evaporator (BUCHI, Switzerland). The crude rice extracts were weighed and dissolved in dimethyl sulfoxide (DMSO) (final concentration 0.1%) as the stock solution.

2. Antioxidant activity and Total phenolic content assay

2.1 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay

Principle of DPPH assay

A DPPH assay is a measurement process based on the electron transfer that produces a violet solution in ethanol. At room temperature, a stable DPPH free radical is reduced in the presence of an antioxidant molecule, giving rise to a yellow or colorless ethanol solution (Figure 38) (Garcia et al., 2012).

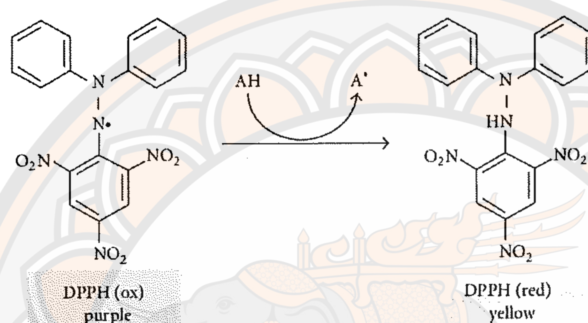


Figure 38 Principle of DPPH radical scavenging capacity assay

Source: Garcia et al., 2012

Preparation of DPPH radical solution

A stock solution (0.6 mM) of the DPPH radical was prepared by dissolving 0.0253 g DPPH in 100 ml methanol and kept in a refrigerator until required. A working solution of the radical was prepared by diluting the DPPH stock solution (0.6 mM) with ethanol to a final concentration of 0.12 mM.

Preparation of ascorbic acid (standard solution)

Standard ascorbic acid was prepared at a concentration of 1000 mg/ml with 100% methanol. It was then diluted to 0, 100, 250, 500, 750 and 1000 mg/ml. Ascorbic acid solution and 100% methanol were added into a 96-well plate, following 140 µl of the DPPH radical solution (Table 7) which was then incubated in the dark at room temperature for 30 minutes. The absorbance was then measured at 515 nm by using a microplate reader. 100% methanol was used as a blank and the DPPH radical

solution was used as the control. The percentage inhibition of DPPH radicals by the standard ascorbic acid was calculated using the equation described in the DPPH assay.

Preparation of rice extracts

Samples of rice extracts (GBR, BR, and WR) at concentrations of 0.1-10 mg/ml were prepared in a 100% methanol solution at various concentrations. All samples, together with 100 µl of 100% methanol, were then added into a 96-well plate of 140 µl of the DPPH radical solution (Table 8), and then incubated in the dark at room temperature for 30 minutes. The absorbance was measured at 515 nm by using a microplate reader. 100% methanol and the DPPH radical solution were used as a blank and control, respectively. The percentage inhibition of DPPH radicals by the rice extracts and standard ascorbic acid were calculated using the equation described in the DPPH assay. The ascorbic acid equivalent antioxidant capacity (AEAC) was then calculated. The DPPH was expressed as an inhibitory concentration (IC_{50}) using GraphPad Prism software. The data were represented as a mean ± S.D. Then a standard graph was created with the values.

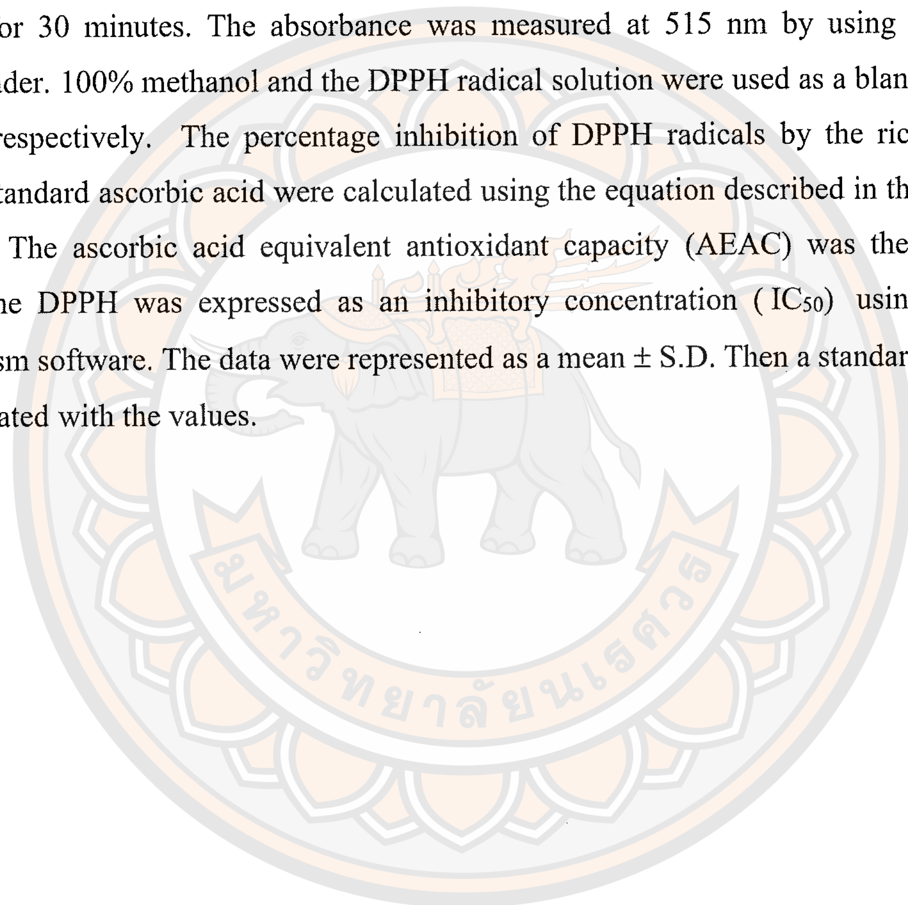


Table 8 The reaction mixture for the DPPH assay

	Blank	0	1	2	3	4	5	sample
Ascorbic acid (µl)	-	- (0 mg/ml)	10 µl (100 mg/ml)	10 µl (250 mg/ml)	10 µl (500 mg/ml)	10 µl (750 mg/ml)	10 µl (1000 mg/ml)	-
Final conc. of ascorbic acid (µl)	-	0 mg/ml	4 mg/ml	10 mg/ml	20 mg/ml	30 mg/ml	40 mg/ml	-
100% Methanol	250	100	100	100	100	100	100	-
Rice extracts (µl)	-	-	-	-	-	-	-	10
DPPH (µl) radical solution	-	140	140	140	140	140	140	140
Total (µl)	250	250	250	250	250	250	250	250

1. Calculation of the percentage inhibition of DPPH radical by standard ascorbic acid.

$$\textbf{DPPH inhibition (\%)} = \frac{Ab_C - Ab_{STD\ Trolox}}{Ab_C} \times 100$$

where, Ab_C is the absorbance value of the working solution of DPPH radical and $Ab_{STD\ Trolox}$ is the absorbance value of the standard ascorbic acid.

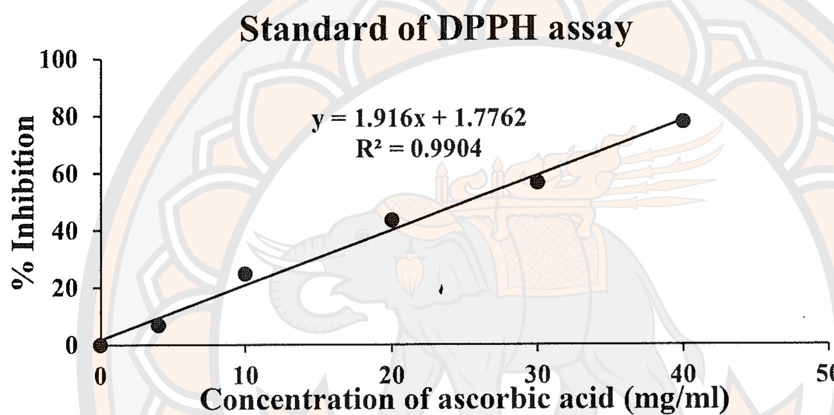


Figure 39 Standard ascorbic acid in DPPH assay

2. Linear equation plot of standard ascorbic acid

where, % inhibition (y axis) and ascorbic acid concentrations (x axis).

3. Calculation of the percentage inhibition of DPPH radical by rice extracts

$$\textbf{DPPH inhibition (\%)} = \frac{Ab_C - Ab_{Samples}}{Ab_C} \times 100$$

where, Ab_C is the absorbance value of the working solution of DPPH radicals.

$Ab_{Samples}$ is the absorbance value of the samples.

4. Calculation antioxidant capacity of rice extracts compared standard ascorbic acid (Ascorbic acid Equivalent Antioxidant Capacity, AEAC (mg/g samples)

From the Linear equation plot of standard Trolox **(2.)**

$$\mathbf{y = 1.916x + 1.7762}$$

Example: % inhibition of DPPH radical of sample A = 10.69

$$y = 1.916x + 1.7762$$

$$10.69 = 1.916x + 1.7762$$

$$\mathbf{X = 4.65\ mg/ml\ sample}$$

$$\therefore\ \mathbf{AEAC = 4.65\ mg/ml\ of\ extract}$$

Antioxidant capacity in 1 gram (g) of samples is equivalent to 4.65 mg of standard ascorbic acid.

2.2 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay

Principle

The ABTS assay is measured based on the interaction between antioxidants and the ABTS radical cation which has a characteristic color showing maxima at 645, 734, and 815 nm. An ABTS assay measures the relative ability of antioxidants to scavenge the ABTS generated in an aqueous phase, as compared with a Trolox (water soluble vitamin E analog) standard. The ABTS is generated by reacting a strong oxidizing agent (e.g., potassium permanganate or potassium persulfate) with the ABTS salt. The reduction of the blue-greenish-dyed product of ABTS by hydrogen-donating antioxidants is measured by the suppression of its characteristic long wave absorption spectrum. During this reaction, the blue ABTS radical cation is converted back to its colorless neutral form (Figure 40). The results are usually expressed as Trolox equivalent antioxidant capacity (TEAC) (Boligon et al, 2014).

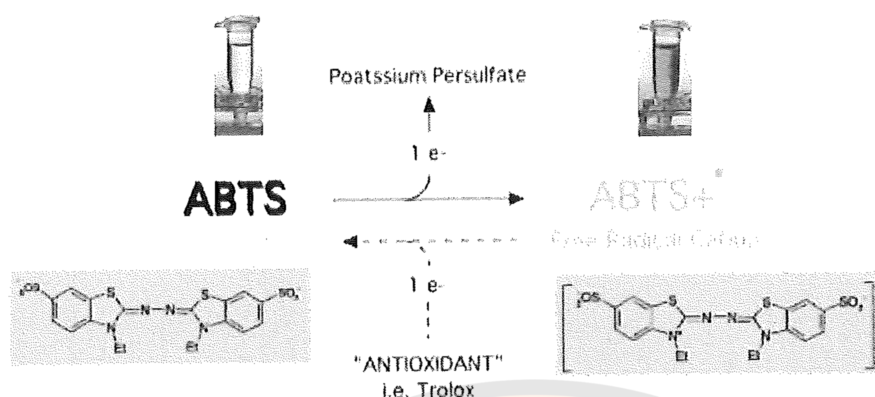


Figure 40 Principle of ABTS radical scavenging capacity assay

Source: Boligon et al., 2014

Preparation of ABTS radical solution

A stock solution (7 mM) of the ABTS radical was prepared by dissolving 0.0192 g of ABTS in 100 ml of methanol, and then 140 mM of potassium persulfate was prepared by dissolving 0.038 g of potassium persulfate in 1 ml of distilled water. A working solution of ABTS radicals was made by diluting 5 ml of ABTS radicals (7 mM) together with 88 μl of potassium persulfate (140 mM) which was then kept in the dark at room temperature, for 13-16 hours. This solution was then diluted with distilled water and the wavelength was measured at 734 nm for OD 0.70 (±0.02). The solution was then kept in a refrigerator until required.

Preparation of standard Trolox solution

A standard Trolox solution at a concentration of 2.5 mM with 100% methanol was prepared and the solution was added into a 96-well plate at various concentrations of 0.5, 1.0, 1.5, 2, and 2.5 mM, together with 240 μl of working solution of $ABTS^{\bullet+}$ radicals (Table 9) and left in the dark at room temperature for 30 minutes. Following this, the absorbance was measured at 734 nm by using a microplate reader. Distilled water was used as a blank and the ABTS radical solution was used as a control. The percentage inhibition of ABTS radicals by standard Trolox was calculated using the equation described in the ABTS assay.

Preparation of rice extracts

The rice extracts (GBR, BR, and WR) were prepared at concentrations of 0.1-10 mg/ml in 100% methanol. All the solution samples and distilled water were added to a 96-well plate, followed by a 240 μl of working solution of ABTS radical being added to the mix (Table 9), then left in the dark at room temperature, for 30 minutes, after which the absorbance was measured at 734 nm by using a microplate reader. Distilled water was used as a blank and the ABTS radical solution was used as a control. The percentage inhibition of ABTS radical by the oil samples and standard ORZ were calculated using the equation described in the ABTS assay. The Trolox equivalent antioxidant capacity (TEAC) was then calculated. The DPPH was expressed as an inhibitory concentration (IC_{50}) using GraphPad Prism software. The data were represented as a mean ± S.D. Then a standard graph was created with the values.

1. Calculation of the percentage inhibition of ABTS radical by standard Trolox.

$$\text{ABTS inhibition (\%)} = \frac{Ab_C - Ab_{STD\ Trolox}}{Ab_C} \times 100$$

where, Ab_C is the absorbance value of the working solution of ABTS radical and $Ab_{STD\ Trolox}$ is the absorbance value of the standard Trolox.

2. Linear equation plot of standard Trolox

where, % inhibition (y axis) and Trolox concentrations (x axis).

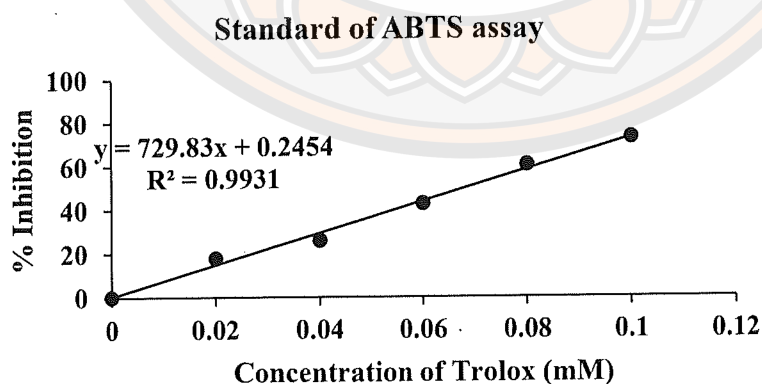


Figure 41 Standard trolox in ABTS assay

3. Calculation of the percentage inhibition of ABTS radical by oil samples and standard ORZ

$$\text{ABTS inhibition (\%)} = \frac{Ab_C - Ab_{Samples}}{Ab_C} \times 100$$

where, Ab_C is the absorbance value of the working solution of ABTS radical, and $Ab_{Samples}$ is the absorbance value of the samples.

4. Calculation antioxidant capacity of oil samples and standard ORZ compare standard Trolox (Trolox Equivalent Antioxidant Capacity, TEAC (mM/g samples))

From the Linear equation plot of standard Trolox **(2.)**

$$y = 729.83x + 0.2454$$

Example: % inhibition of ABTS radical of sample A = 5.36

$$y = 729.83x + 0.2454$$

$$5.36 = 729.83x + 0.2454$$

$$X = 0.007 \text{ mg/ml of extract}$$

$$\therefore \text{ TEAC} = 0.007 \text{ mM/mg of extract}$$

<u>Then converse TAEC value in unit mM/mg sample to mM/g sample.</u>

$$= 0.007 \text{ mM/mg of extract} \times 1000 \text{ mg}$$

$$= 7 \text{ mM/g of extract}$$

$$\therefore \textbf{ TEAC = 7 mM/g of extract}$$

Antioxidant capacity in 1 gram (g) of samples is equivalent to 7 mM of standard Trolox.

Table 9 The reaction mixture for the ABTS assay

	Blank	0	1	2	3	4	5	sample
Trolox (μl)	-	- (0 mM)	10 μl (0.5 mM)	10 μl (1 mM)	10 μl (1.5 mM)	10 μl (2 mM)	10 μl (2.5 mM)	-
Final conc. of Trolox (mM)	-	0 mM	0.02 mM	0.04 mM	0.06 mM	0.08 mM	0.1 mM	-
100% Methanol	250	10	-	-	-	-	-	-
Rice extracts (μl)	-	-	-	-	-	-	-	10
$ABTS^{\bullet +}$radical solution (μl)	240	240	240	240	240	240	240	240
Total (μl)	250	250	250	250	250	250	250	250

2.3 Total phenolic content by Folin-Ciocalteu assay

Principle

The Folin Ciocalteu assay is the most common assay used to quantify the phenolic content in both terrestrial plants and seaweeds. The Folin Ciocalteu reagent is made up of a mixture of tungsten and molybdate. The Folin Ciocalteu assay relies on the transfer of electrons from phenolic compounds to phosphomolybdic acid complexes in alkaline conditions (Figure 42). The transfer of these electrons facilitates a color change, which can then be detected at 765 nm in the visible spectrum. The blue color which occurs upon reduction of the Folin Ciocalteu reagent is thought to be due to coordinated molybdenum (V) species, although full characterization of the structure is still unknown (Ford et al., 2019).

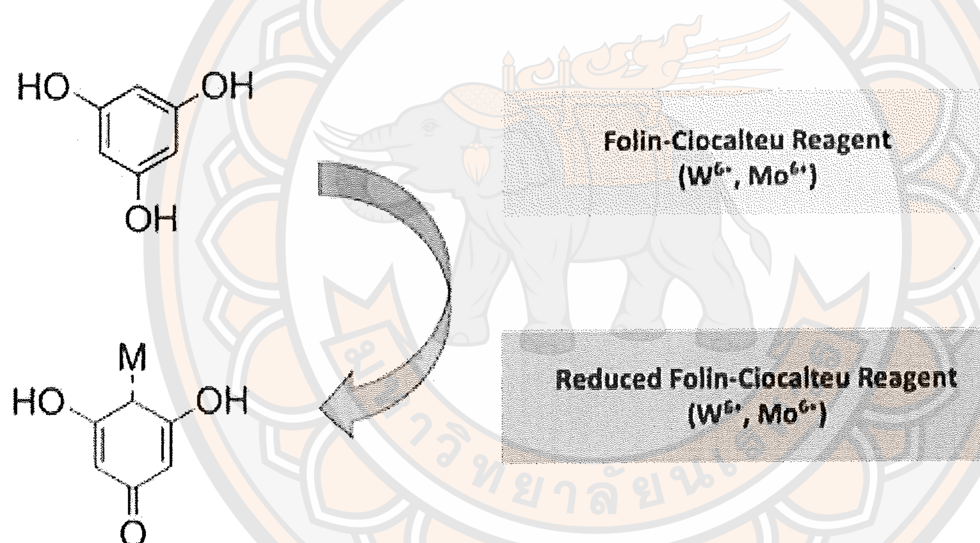


Figure 42 The reduction of the Folin Ciocalteu reagent

Source: Ford et al., 2019

Preparation of a standard of Gallic acid

Standard Gallic acid solution (2.5 mg/ml) was added to a 96-well plate together with distilled water (the final concentrations were 0.008, 0.016, 0.024, 0.032, and 0.04 mg/ml). A 25 μl volume of Folin working solution was then added to the mix

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(Table 10) and left in the dark at room temperature for 30 minutes at which time the absorbance was measured at 765 nm by using a microplate reader.

Preparation of rice extracts

Samples of rice extracts (GBR, BR, WR) at concentrations of 1-100 mg/ml in 100% methanol were prepared, and 10 µl of the solutions were added to a 96-well plate, together with distilled water, and 25 µl of Folin working solution was added to the mix (Table 10). The prepared samples were left in the dark at room temperature for 30 minutes. After that, the absorbance was measured at 765 nm by using a microplate reader. The total phenolic content was expressed as Gallic acid Equivalents (GAE). The data were represented as a mean ± S.D. Then a standard graph was created with the values.

1. Linear equation plot of standard Gallic acid

Where, OD at 765 nm (y axis) and concentrations of standard Gallic acid (x axis).

$$y = ax + b$$

$$\mathbf{y = 22.72x}$$

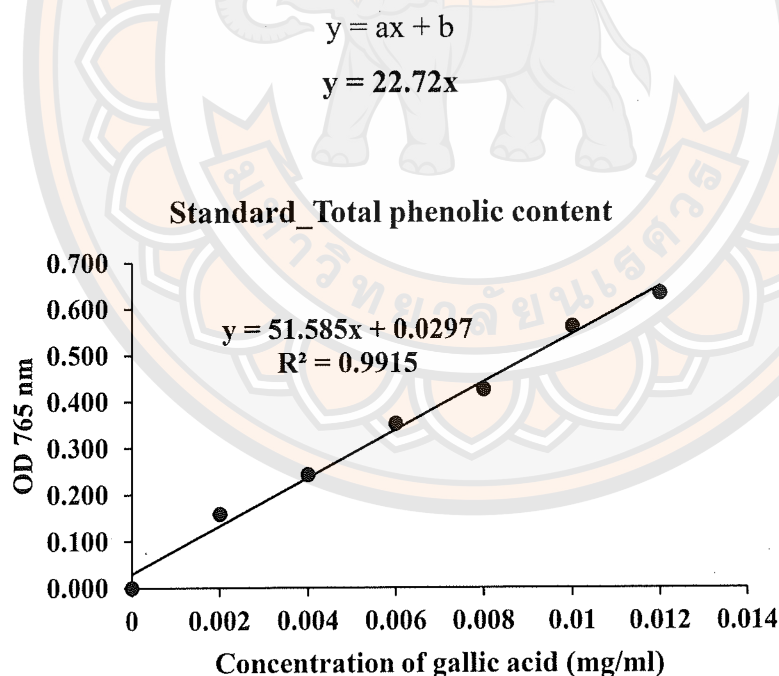


Figure 43 Standard acid in Folin Ciocalteu assay

2. Calculation of total phenolic content of oil samples and standard ORZ compare standard Gallic acid (Gallic acid Equivalents, GAE (mg/g samples)).

From the Linear equation plot of standard Gallic acid **(1.)**

$y = 51.585x+0.0296$

Example: OD of total phenolic content of extract A = 0.231

$y = 51.585x+0.0296$

$0.231 = 51.585x+0.0296$

$X = 0.004$ mg/ml of extract

$X = 0.004$ mg/ml of extract

Then converse GAE value in unit mg/mg sample to mg/g sample.

GAE = 0.004 mg/ml of extract× 1000 mg

GAE = 4 mg/ g of extract

∴ **GAE = 4 mg/ g of extract**

Total phenolic content in 1 gram (g) of samples is equivalent to 4 mg of Gallic acid.

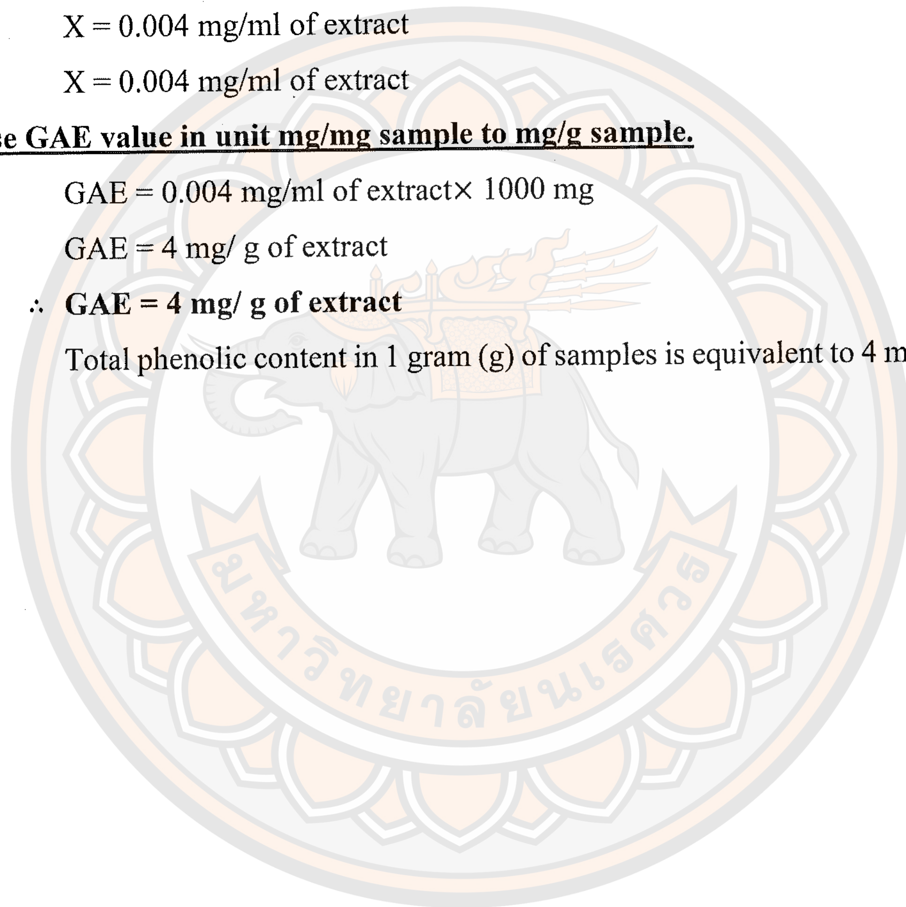


Table 10 The reaction mixture for the total phenolic content assay

	Blank	**0**	**1**	**2**	**3**	**4**	**5**	**sample**
Gallic acid (mg/ml)	-	- (0 mg/ml)	10 µl (0.2 mg/ml)	10 µl (0.4 mg/ml)	10 µl (0.6 mg/ml)	10 µl (0.8 mg/ml)	10 µl (1 mg/ml)	-
Final conc. of gallic acid (mM)	-	0 mg/ml	0.008 mg/ml	0.016 mg/ml	0.024 mg/ml	0.032 mg/ml	0.04 mg/ml	-
H_2O	250	225	215	215	215	215	215	215
Rice extracts (µl)	-	-	-	-	-	-	-	10
Folin (µl) working solution	-	25	25	25	25	25	25	25
Total (µl)	250	250	250	250	250	250	250	250

3. 3T3-L1 cell culture and treatments

3T3-L1 preadipocytes (#CL-173, ATCC) were grown in DMEM containing 10% FBS and 1% antibiotic-antifungal basal medium (BM) at 37°C in a 5% CO_2 incubator. After 2 days post-confluent, the cells ("day 0") were then differentiated for 3 days in differentiation medium 1 (DM1) with components BM, 1 mM IBMX and 2 µM DEX ("day 3") and for a further two days in differentiation medium 2 (DM2) with components BM and 1 µg/ml insulin ("day 5"). After differentiation completion, the cells were maintained in BM which was replaced with fresh BM on days 7 and 9. The cells were kept at -20°C until used.

Part 1, The mature adipocytes are treated with GBR, BR, and WR extracts to examine the effects on cell cytotoxicity, lipid accumulation, and *preadipocyte factor 1, adipogenic transcription factors*, *lipogenic*, *lipolytic, inflammatory cytokines*, and *antioxidants* genes expression (Figure 44).

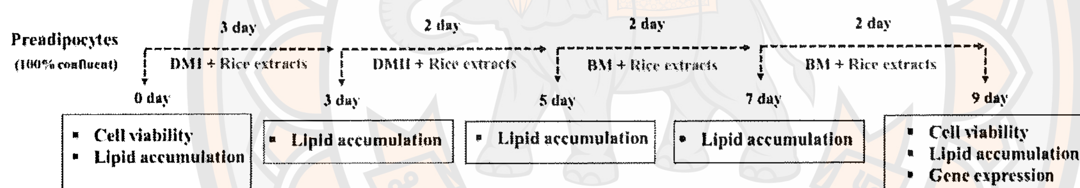


Figure 44 An experimental design for part 1

Part 2, The oxidative stress-induced cells were treated with H_2O_2 for 48 hours on day 9, after the completion of differentiation. The rice extracts (GBR, BR, and WR) at concentrations of 0.1, 0.5, and 1 mg/ml were then treated for 24 hours (Figure 45) to examine the intracellular ROS levels, lipid accumulation, as well as expression of the adipogenic, insulin sensitivity, inflammatory cytokines, and antioxidant related-genes and proteins in H_2O_2- induce adipocytes.

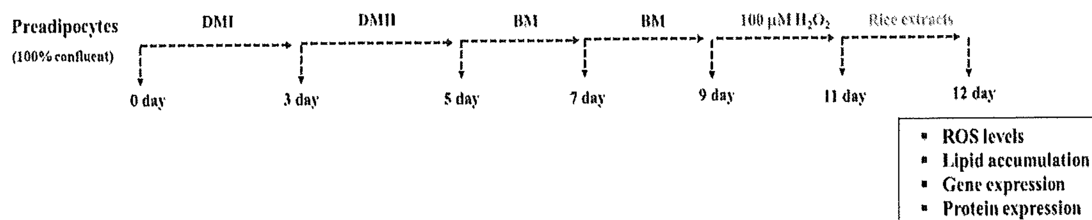


Figure 45 An experimental design for part 2

4. Cell viability by MTT assay

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) gives a yellow aqueous solution which will cause a conversion into a water insoluble violet-blue formazan after reduction by mitochondrial dehydrogenase enzyme of living cells. However, non-viable cells will not produce dehydrogenase or insoluble violet-blue formazan. (Figure 46).

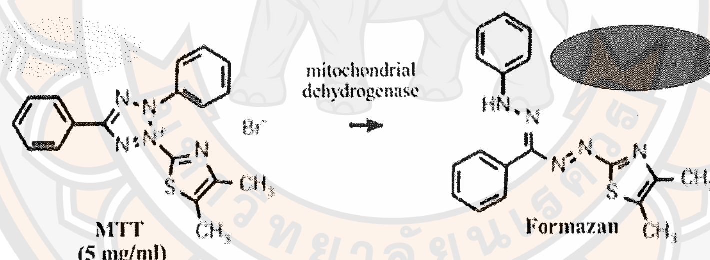


Figure 46 The MTT reaction within a viable cell

Source: www.biotek.com

To determine the effect of various concentrations of the rice extracts (0, 0.1, 0.5, 1, 2.5, 5 and 10 mg/ml) and H_2O_2 (100-700 μM) on cell viability, 3T3-L1 adipocytes (100% confluent) ("day 0") were differentiated for 3 days in differentiation medium 1 (DM1) [BM, 1 mM IBMX and 2 μM DEX] ("day 3") and for a further two days in differentiation medium 2 (DM2) [BM and 1 μg/ml insulin] ("day 5"). After the completion of differentiation, the cells were maintained in BM which was replaced on days 7 and 9. The cells were then treated with rice extracts (GBR, BR, and WR) and

H_2O_2 (100-700 μM) for 24 hours. The control cells were treated with DMSO (0.1% v/v) in the same amount. After incubation, the medium was replaced with 180 μl of serum-free medium containing 20 μl of 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (5 mg/ml) and incubated for 2 hours at 37°C. The MTT solution was discarded, and the formazan crystals in the cells were dissolved with 100 μl of DMSO which was added to each well. The absorbance was measured at 595 nm using a microplate reader (Labsystems, Finland), which was compared to the control cells.

% Cell Viability = Absorbance (OD) of Sample x 100/ Absorbance (OD) of control

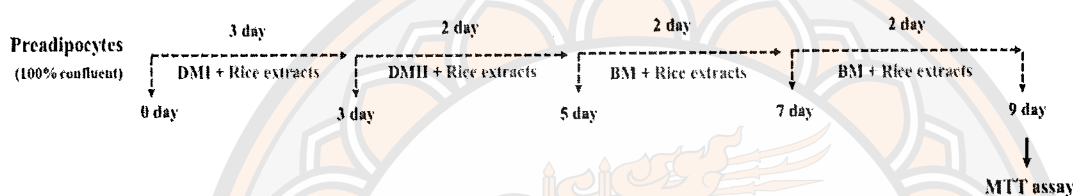


Figure 47 The measurement diagram of rice extracts on cell viability by MTT assay

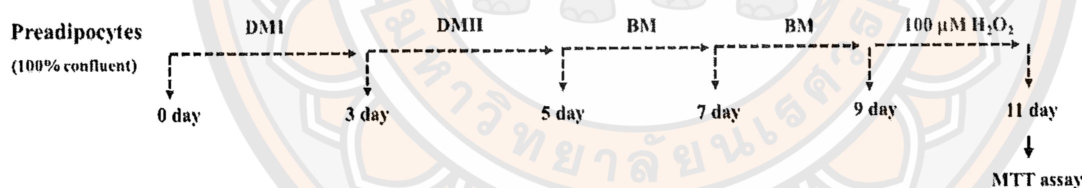


Figure 48 The measurement diagram of H_2O_2 on cell viability by MTT assay

5. Oil-Red O staining

Intracellular lipid accumulation was determined using Oil-Red O staining during adipocyte differentiation. The cells were treated with the rice extracts for 9 days from the starting of differentiation (as described above) at which time the adipocytes are washed by phosphate-buffered saline (PBS), fixed with 500 μl of 10% formaldehyde in PBS for 1 hour, and then incubated with 60% isopropanol for 5 min, followed by staining with 500 μl of 0.5% (w/v) Oil-Red O solution in 500 μl of 60% (v/v) isopropanol for 10 min at room temperature. After staining, the adipocytes were washed

twice by PBS to remove the excess stain and photographed using an inverted microscope (LEICA, USA) in combination with a digital camera at 20x magnification. The stained lipid droplets in the cells were dissolved in 500 µl of 100% isopropanol and the absorbance was quantified at 510 nm with a microplate reader.

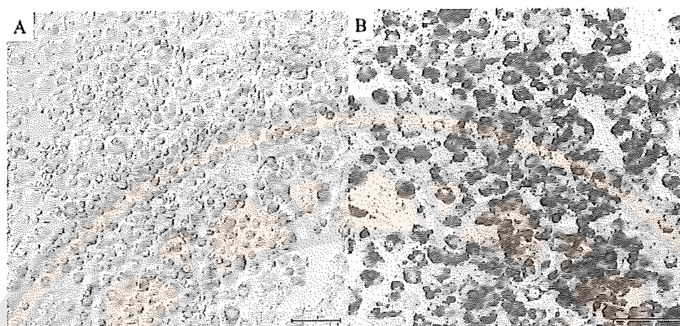


Figure 49 Unstained preadipocytes (A) and stained adipocytes (B) by Oil-Red O staining

6. Triglyceride quantification assay

The triglyceride contents in adipocytes treated with the rice extracts for 9 days were estimated using a commercial triglyceride assay kit (Triglycerides liquicolor^{mono}, Germany) and the values were compared to untreated cells. Briefly, the adipocytes were lysed by a lysis buffer (Tris-HCL 25 mM, EDTA 1 mM, pH 7.4) using a sonicator (Evan et al., 2000) and the triglycerides were then hydrolyzed with lipases. The product from the hydrolyzed triglycerides was quinoneimine which is formed from hydrogen peroxide, 4-aminoantipyrine, and 4-chlorophenol under the catalytic influence of peroxidase (Figure 50). The quinoneimine products were measured by absorbance at 500 nm. The amount of total protein was calculated with a bicinchoninic acid (BCA) protein assay kit (Thermo, USA) and normalized to the triglyceride contents. The total triglyceride levels were calculated using the following formula:

1. Triglyceride level (mg/dl) = $200 \times \left(\frac{\Delta A_{sample}}{\Delta A_{STD}}\right)$

ΔA_{sample}: the absorbance of the sample against the reagent blank (A_{sample}-A_{blank})

ΔA STD: the absorbance of the standard against the reagent blank (A_{STD}-A_{blank})

200: concentration of triglyceride standard 200 mg/dl

2. Triglyceride level (μg/μg protein) = $\frac{\textbf{Total triglyceride concentration}}{\textbf{Total protein concentration}}$

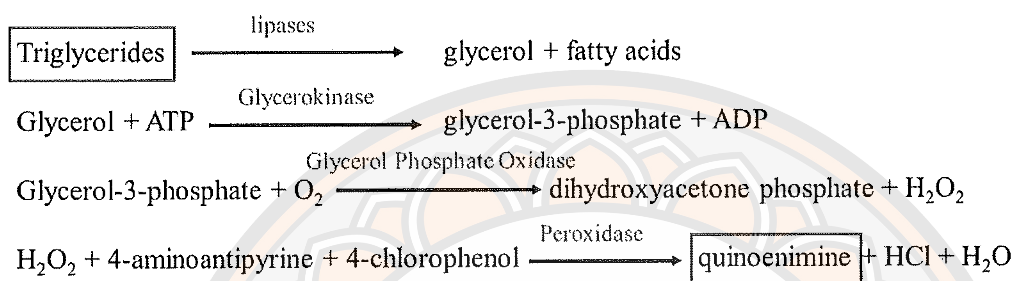


Figure 50 The reaction of Triglyceride quantification assay

7. Intracellular reactive oxygen species (ROS) determination

To determine the ROS levels within a cell, a fluorescent probe 2', 7'dichlorodihydrofluorescein diacetate (H_2DCFDA) was employed. H_2DCFDA was then hydrolyzed by esterase. After the H_2DCFDA had passed through the cell membrane into the internal membrane cell, the H_2DCFDA was deacetylated to cleave off the lipophilic groups by cellular esterases to form a non-fluorescent compound, 2', 7' dichlorodihydrofluorescein (H_2DCF), which was trapped inside the cell. Oxidation of H_2DCF by ROS/RNS converts the molecules to 2', 7'dichlorofluorescein (DCF), which is highly fluorescent (Figure 51). DCF can be detected by fluorescence absorbance with maximum excitation of 495 nm and emission spectra of 529 nm. The % of fluorescence is calculated by: **% fluorescence = (Ft_{30} -Ft_0)/Ft_0 x 100** (Ft_{30} = fluorescence at 30 min; Ft_0 = fluorescence at 0 min)

Intracellular ROS levels were quantified using a DCFH-DA fluorescent probe modified by Wang and Joseph. Briefly, 3T3-L1 cells were at 2×10^4 cells per well in 96-Well Optical-Bottom Plates with Polymer Base (Thermo Scientific™). After differentiation completion, oxidative stress in the cells was induced by 100 μM of H_2O_2 for 24 hours and then treated with the rice extracts (GBR, BR, and WR) at various

concentrations (0, 0.1, 0.5, 1 mg/ml) for 48 hours. After incubation, the cells were washed with PBS and incubated with dichlorofluorescein diacetate100 µl (DCFH-DA) (100 µM) in medium (BM) for 30 minutes at 37°C in the dark. After being washed several times with PBS, the fluorescence was measured by a microplate reader (Labsystems, Finland), using 485 nm excitation and 530 nm emission wavelengths. The % of ROS was calculated by: % fluorescence = (Ft30 -Ft0) /Ft0 x 100 (Ft30 = fluorescence at 30 min; Ft0 = fluorescence at 0 min).

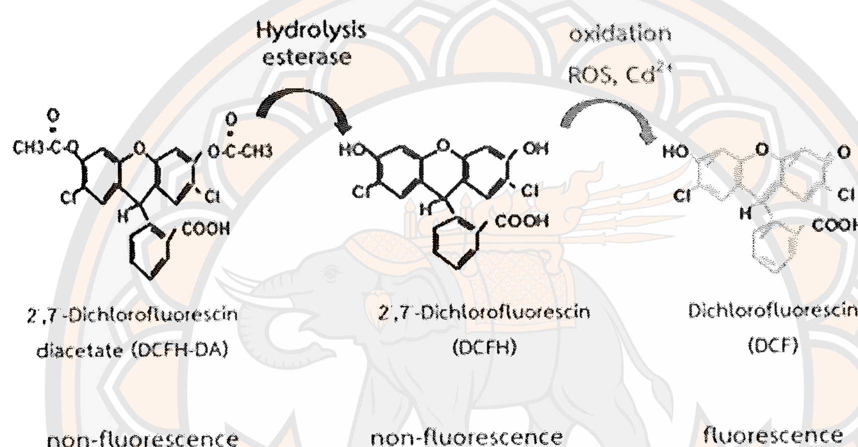


Figure 51 Formation of fluorescence compound (DCF) by ROS with the cells

Source: Adapted from Hensley et al., 2003

8. Measurement of mRNA expression by reverse transcription (RT)-PCR and quantitative real-time PCR (qPCR)

Total RNA was extracted from 3T3-L1 adipocytes treated with WR, BR, and GBR extracts using a Ribozol reagent (AMRESCO, USA), in accordance with the manufacturer's instructions. A quantity of 1 µg RNA was reverse transcribed to the complementary DNA using the random primer and MMLV reverse transcriptase (Promega, Madison, Wisconsin, USA). The mRNA expression was measured by RT-PCR (Bioline, USA) and qPCR using SYBR Green (RBC Bioscience, Taiwan). For RT-PCR, the reaction conditions were initial denaturation at 95°C for 2 min; denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 60

seconds (30 cycles) and at 72°C for 5 min (BioRad, Singapore). PCR products were electrophoresed on 2% agarose gel including fluorescence dye (RedSafe™, iNtRON Biotechnology, Korea) and visualized by UV light (Gel document, ImageQuant LAS 500).

For qPCR, the (2x) SYBR Green (RBC Bioscience, Taiwan) reaction mixtures were incubated for an initial denaturation at 95°C for 10 min, followed by 40 PCR cycles. Each cycle was performed at 95°C for 30 seconds, at 58°C for 30 seconds, and at 72°C for 30 seconds, respectively (ESCO Swift™ Real-Time PCR Thermal Cyclers, Singapore). The comparative C_T method ($2^{-\Delta\Delta CT}$) method was used to measure relative mRNA expression, and the expression was normalized to *β-actin*. The expression of each different mRNA in the untreated cells was designated as 1, and the relative levels of transcripts in treated samples were expressed as the fold change. Two independent experiments were performed, each in triplicate. The Primer sequences are shown in Table 11.

Table 11 Primer sequences used in RT-PCR and qPCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Reference
Pref-1	GCGCCAACAATGGAACTTGCGT	GAGGGGTACTCTTGTTGAGCTC	Lab design
PPARγ	CGCTGATGCACTGCCTATGA	AGAGGTCCACAGAGCTGATTCC	Ho et al., 2012
C/EBPβ	GGGGTTGTTGATGTTTTTGG	CGAAACGGAAAAGGTTCTCA	Lab design
C/EBPα	AATGGCAGTGTGCACGTCTA	CCCCAGCCGTTAGTGAAGAG	Lab design
C/EBPδ	TTCTACGAGCCAGGCAGG	TCAATGTAGGCGCTGAAGTC	Lab design
SREBP-1C	CTGCAGACCCTGGTGAGTG	GACCGGTAGCGCTTCTCAAT	Lab design
FAS	TTTGTAAACGTCCTCACCCGA	CGGAAGTTCAGAGAGGCGTAGTTAG	Ho et al., 2012
LPL	ACTTGTCATCTCATTCCTGG	TCTACTACATTCCCGTTACC	Ho et al., 2012
aP2	CATGGCCAAGCCCAACAT	CGCCCAGTTTGAAGGTC	Ho et al., 2012
ACC1	TGAGGAGGACCGCATTTATC	GAAGCTTCCTTCGTGACCAG	Lab design
ACC2	ACAGAGATTTCACCGTTGCGT	CGCAGCGATGCCATTGT	Lab design
ATGL	ATTTATCCCGGTGTAGTGTG	GGGACATGTGATGGTATTC	Ho et al., 2012
HSL	ACTCAGACCAGAAGGCACTA	TAGTTCCAGGAAGGAGTTGA	Ho et al., 2012
IRS1	CCAGCCTGGCTATTTAGCTG	CCCAACTCAACTCCACCACT	Lab design

Table 11 (Cont.)

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Reference
PI3K	AAAGACGCACTCCTGAACTG	CGAGGACATAAGCTACAC	Prabhakar et al., 2011
AKT2	GAGATTGTGTCAGCTGAG	ACAAGCCAAAGTCAGTGATCT	Boue et al., 2016
GLUT4	GACGGACACTCCATCTGTTG	GCCACGATGGAGACATAGC	Lab design
Adiponectin	CTGGCCACTTTCTCCTCATT	GTCATCTTCGGCATGACTGG	Lab design
TNF-α	CGAGATATACGCAGAGGTAAGA	CATCACGTGCAGGACACACTT	Lab design
IL-6	GATTTGCTATCTCATACCAGGAGA	AAGTCTAAGTACTTGGGCAGATTGA	Lab design
Nrf2	AGGCTTAATTACACATGTTCTCTG	TTATATCCAGTTTGGTAGCATCCAT	Lab design
HO-1	GGACATGGCCTTCTGGTATGG	ACCCAGGTAGCGGGTATATG	Lab design
SOD2	AACCCAAAGGAGAGTGCTG	CAGCAATCTGTAAGCGACCT	Lab design
GPx4	CCAATAAGAGACGTCGTGGG	GGCTTAAGTAAGCGGCTCAG	Lab design
CAT	AGCCCTGACAAAATGCTTCA	GCTGAAGCTGTTGGGGTAAT	Lab design
β-actin	TGTCCACCTTCCAGCAGATGT	AGCTCAGTAACAGTCGA	Lab design

9. Measurement of protein expression by western blot analysis

Total protein extraction of 3T3-L1 adipocytes

Different groups of cells were exposed to each of the three rice extracts for 24 hours. For each group of cells, the medium was removed, and the cells were collected using ice-cold RIPA lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail). After cell lysis, the samples were sonicated for 10 seconds three times and then centrifuged at 12,000 rpm at -4°C for 10 min. The supernatants were then transferred into new tubes and kept at -4°C until used.

Protein concentration determination

Protein concentrations were measured by BCA Protein assay using bovine serum albumin (BSA) as the standard (0-0.2 μg/ml). A 25 μl volume of each standard or sample was placed into a microplate well using a pipette, and 200 μl of BCA working reagent (50:1, Reagent A: B) were added to each well plate and mixed thoroughly on a plate shaker for 30 seconds. The plates were covered, and the cells were incubated at 37°C for 30 minutes. The absorbance was measured at 562 nm.

Table 12 Preparation of Tris-Glycine resolving gel

Conc. (μg/μl)	BSA (μl)	Distilled water (μl)	BCA (μl)	Total (μl)
0	0	25	225	250
0.04	5	20	225	250
0.08	10	15	225	250
0.12	15	10	225	250
0.16	20	5	225	250
0.20	25	0	225	250

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

A 4.2 ml volume of separating gel of gel was poured and overlaid with n-butanol and allowed to polymerize for 30 min. After that, the n-butanol was poured off and the gel was rinsed well with distilled water. Any residual water was removed, and combs were placed between the plates. Stacking gel was then made and allowed to polymerize for 30 min. 40 μg of protein sample was added to a 4x protein gel sample and loaded the SDS-PAGE gel. 3 μl of Opti-Protein Marker was loaded in each gel as a molecular weight marker. The protein was separated using a blot electrophoresis transfer unit system filled with 1x SDS-PAGE running buffer. A constant voltage of 120 V, 400 mA, then is applied for 2 hours 30 min.

Table 13 Preparation of Tris-HCl resolving gel

Separating gel (2 gel)		Stacking gel (2 gel)	
Distilled water	2.29 ml	Distilled water	3.04 ml
1.5 M Tris-HCl pH 8.8	2.5 ml	0.5 M Tris-HCl pH 6.8	1.25 ml
10% SDS	100 μl	10% SDS	50 μl
30% Acrylamide	5 ml	30% Acrylamide	0.6 ml
10% APS	100 μl	10% APS	50 μl
TEMED	10 μl	TEMED	5 μl
Total	10 ml	Total	5 ml

Western blotting

A polyvinylidene difluoride (PVDF) membrane (Amersham Protran Premium 0.45 μm) was activated in 100% methanol for 5 min and equilibrated using filter papers and a PVDF membrane in 1x transfer buffer. The protein samples were transferred to the membrane in a blot electrophoresis transfer unit, filled with 1x transfer buffer, with 50 V at 400 mA applied for 4 hours. The proteins were visualized by staining the membrane with Ponceau Red for 10-30 min and destained by repeated washing with distilled water. The membrane was incubated in 5% non-fat powdered milk (blocking) for 1 h at room temperature. The membrane was incubated overnight at 4°C in primary antibody diluted in 3% non-fat powdered milk in a ziplock bag. After overnight incubation, the membrane was washed three times with 5 ml of 1x TBST (0.1% Tween 20) for 5 min each time and incubated with horseradish peroxidase-labeled (HRP) secondary antibody diluted 1:500-1:1000 in 3% non-fat powdered milk in a ziplock bag for 1h at room temperature. The membrane was washed again for 5 min, three times, with 5 ml of 1x TBST (0.1% Tween 20) and the antigen-antigen-antibody complexes were detected with ECL^{TM} (Millipore, USA). The Excess ECL reagent mix was removed. Densitometries were carried out using a Gel document.

Table 14 Primary antibody and Secondary antibody used in Western blotting

Protein	Primary antibody	Secondary antibody	Blocking/Washing
IRS1	Rabbit mAb Antibody (1:1000) in 3% non-fat powdered milk (Cat. No: E-AB-31834) (Elabscience, USA)	Horseradish peroxidase (HRP) conjugate anti rabbit (1:1000) in 3% non-fat powdered milk (MILLIPORE, USA)	**Blocking** with 5% non-fat powdered milk **Washing** with 1xTBST
Adiponectin	Rabbit mAb Antibody (1:1000) in 3% non-fat powdered milk (Cat. No: DF7000) (Affinity, USA)		**Blocking** with 5% non-fat powdered milk **Washing** with 1xTBST

Table 14 (Cont.)

Protein	Primary antibody	Secondary antibody	Blocking/Washing
PPARγ	Rabbit mAb Antibody (1:1000) in 3% non-fat powdered milk (Cat. No: M00449) (BOSTER BIOLOGICAL TECHNOLOGY, USA)		**Blocking** with 5% non-fat powdered milk **Washing** with 1xTBST
SOD2	Rabbit mAb Antibody (1:1000) in 3% non-fat powdered milk (Cat. No: D3X8F) (Cell Signaling Technology, USA)		**Blocking** with 5% non-fat powdered milk **Washing** with 1xTBST
GPX4	Rabbit mAb Antibody (1:1000) in 3% non-fat powdered milk (Cat. No: PB9625) (BOSTER BIOLOGICAL TECHNOLOGY, USA)		**Blocking** with 5% non-fat powdered milk **Washing** with 1xTBST
β-actin	Mouse mAb (1:1000) in 3% non-fat powdered milk (Cat. No. 8H10D10) (Cell Signaling Technology, USA)		**Blocking** with 5% non-fat powdered milk **Washing** with 1xTBST

Statistical Analysis

Values were expressed as mean ± standard deviation (S.D.). The comparison between the groups was performed by one-way ANOVA analysis and multiple comparisons by Tukey's HSD. Differences were considered significant with $p < 0.05$ by SPSS 17.0 software.

CHAPTER IV

RESULTS

The objectives of this study were to identify the antioxidant activity of rice extracts was determined by using ABTS, DPPH assay, and the total phenolic contents were measured by the Folin Ciocalteu assay of the two Thai rice extracts cultivars *O. sativa* L. Variety phitsanulok2 (PL2) and Kaw dokmali105 (KDML105) and to determine the effects of the rice extracts on anti-adipogenesis and anti-oxidative stress. For the second experiments, the study was divided into the part. Firstly part, the effects of Thai rice cultivars on the adipogenesis process and their antioxidant effects were examined using 3T3-L1 adipocytes as a model.

In this study, to examine the effect of the rice extracts and H_2O_2 on cell cytotoxicity, MTT assays were done, while Oil Red O staining and TG kit assay were used for lipid accumulation. The ROS levels in the 3T3-L1 adipocytes were determined by DCFH-DA assay. *Adipogenesis, insulin singling pathway, lipolytic, pro-inflammatory cytokines,* and *antioxidant related-gene* expression were measured using RT-PCR and qPCR. Finally, the insulin singling pathway, adipogenic, and antioxidant related-protein expression, were determined by western blot.

Experiment I: Antioxidant activity and Total phenolic contents

Effects of the rice extracts on radical scavenging activity by 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay

ABTS assays were used to measure the antioxidant activity of the GBR, BR, and WR extracts from the KDML105 and PL2 cultivars at concentrations of 0.1-10 mg/ml. Both the DPPH scavenging activities of GBR, BR, and WR extracts from KDML105 and PL2 cultivars increased in a dose-dependent manner. The GBR extracts showed a greater antioxidant capacity than BR, with WR showing the least. The antioxidant activities of the GBR, BR, and WR extracts from KDML105 and PL2 cultivars were expressed as the percentage of radical inhibition (% inhibition) (Figure 52) and Trolox Equivalent Antioxidant Capacity (TEAC) (mM/g samples) values (Table

15), which shows the inhibitory concentration (IC_{50}) of the GBR, BR, and WR extracts from both the KDML105 and both the PL2 cultivars. These were expressed using the GraphPad Prism software in table 16. The GBR demonstrated greater antioxidant activity than the BR, while the BR demonstrated greater antioxidant activity than WR. As well, the KDML105 cultivars demonstrated greater antioxidant activity than the PL2 cultivars in all the GBR, BR, and WR extracts.

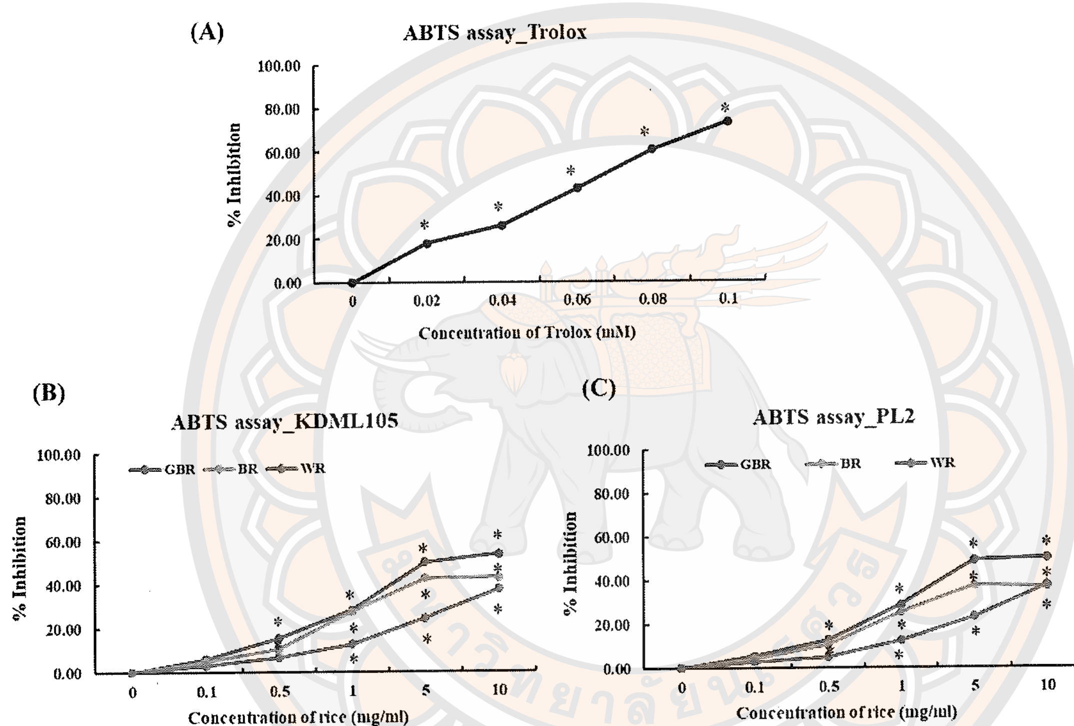


Figure 52 The percentage of radical inhibition (% inhibition) of trolox and rice extracts at concentrations of 0.1-10 mg/ml, as determined by ABTS assay (A) Trolox (B) KDML105 (C) PL2 cultivars. Data are expressed as % inhibition and are representative of 2 replicates experiments.

Table 15 Trolox Equivalent Antioxidant Capacity; TEAC) (mM/g of rice extract) of the GBR, BR, and WR extracts from KDML105 and PL2 cultivars at concentrations of 0.1-1 mg/ml and ABTS assay. (N=8)

Rice extracts (mg/ml)	TEAC (mM/g of rice extract)					
	GBR		BR		WR	
	KDML105	PL2	KDML105	PL2	KDML105	PL2
0.1	7.84±0.96	7.01±1.41	6.33±1.30	5.54±0.91	3.75±1.11	3.58±0.80
0.5	20.77±1.22	17.06±3.75	13.99±1.85	14.21±1.29	8.75±1.87	6.51±1.17
1	39.35±0.22	38.83±1.56	37.49±1.09	34.19±4.97	16.93±0.94	16.49±1.05

Table 16 The inhibitory concentration (IC_{50}) of the GBR, BR, and WR extracts from KDML105 and PL2 cultivars at concentrations of 0.1-10 mg/ml and ABTS assay as expressed using GraphPad Prism software. (N=8)

Rice extracts (mg/ml) (IC_{50})	GBR	BR	WR
PL2	1.57	2.19	6.75
KDML105	1.03	1.94	5.92

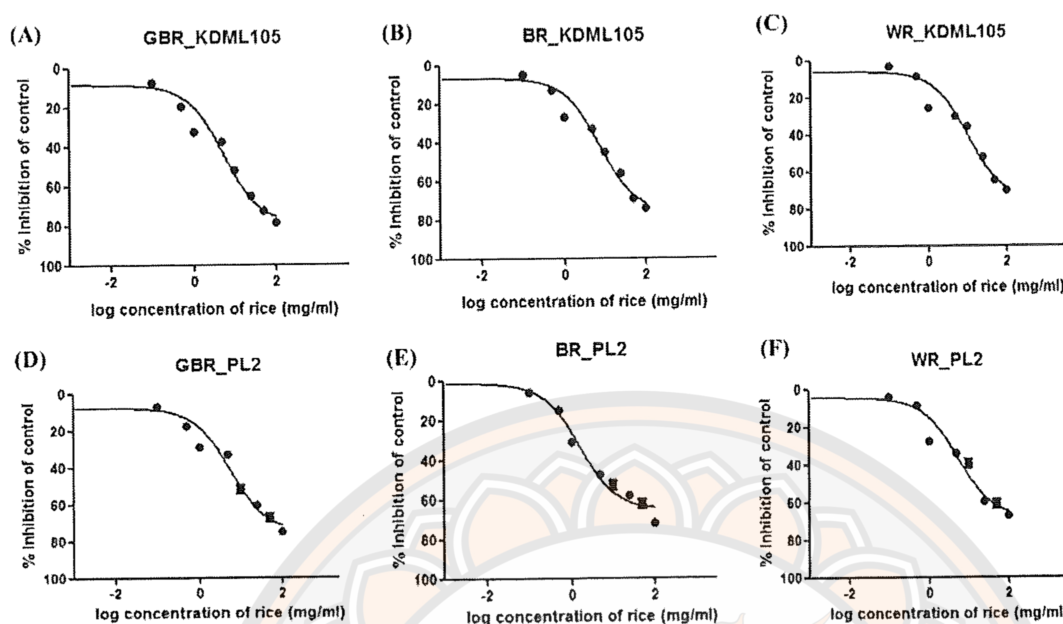


Figure 53 The graph of inhibitory concentration (IC_{50}) from KDML105 and PL2 cultivars at concentrations of 0.1-10 mg/ml by ABTS assay as expressed using GraphPad Prism software. Data are expressed as % inhibition and are representative of 2 replicated experiments

Effects of rice extracts on radical scavenging activity using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

DPPH assays were used to measure the antioxidant activity of the GBR, BR, and WR extracts from the KDML105 and PL2 cultivars at concentrations of 0.1-10 mg/ml. The DPPH scavenging activities of the GBR, BR, and WR extracts from both KDML105 and PL2 cultivars were increased in a dose-dependent manner. The GBR extracts showed the greater antioxidant capability than BR and WR showed the least. The antioxidant activities of the GBR, BR, and WR extracts from KDML105 and PL2 cultivars were expressed as the percentage of radical inhibition (% inhibition) (Figure 54) and Ascorbic acid Equivalent Antioxidant Capacity (AEAC) (mg/g samples) value (Table 17), which showed the inhibitory concentration (IC_{50}) of GBR, BR, and WR extracts from KDML105 and PL2 cultivars as expressed by the GraphPad Prism software in table 18.

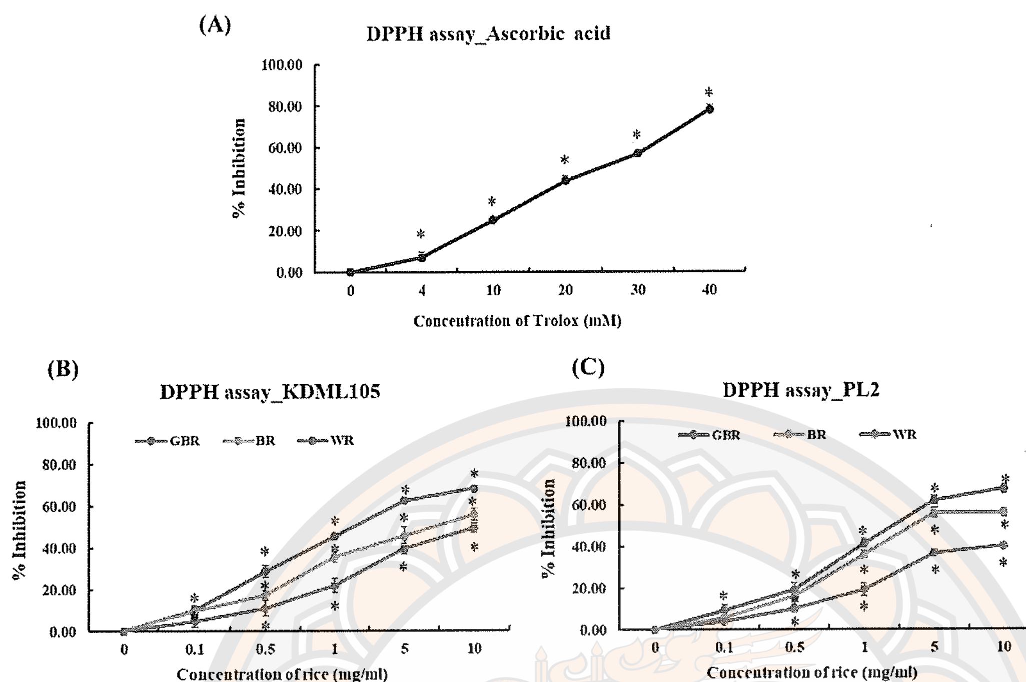


Figure 54 The percentage of radical inhibition (% inhibition) of ascorbic acid and rice extracts at concentrations of 0.1-10 mg/ml were determined by DPPH assay. (A) Ascorbic acid (B) KDML105 (C) PL2 cultivars. Data are expressed as % inhibition and are representative of 2 replicated experiments.

Table 17 Ascorbic acid equivalent antioxidant capacity; AEAC (mg/g of rice extract) of GBR, BR, and WR extracts from KDML105 and PL2 cultivars at concentrations of 0.1-1 mg/ml and DPPH assay. (N=8)

Rice extracts	AEAC (mg/g of rice extract)					
	GBR		BR		WR	
(mg/ml)	KDML105	PL2	KDML105	PL2	KDML105	PL2
0.1	4.34±0.82	3.93±1.36	4.10±1.35	2.15±0.69	1.62±1.37	1.09±1.15
0.5	14.04±4.07	9.03±1.79	7.01±1.05	6.31±0.93	4.80±1.95	4.37±1.14
1	22.81±0.84	20.67±1.06	12.37±1.35	10.54±2.60	10.50±1.68	9.12±1.01

Table 18 The inhibitory concentration (IC_{50}) of GBR, BR, and WR extracts from KDML105 and PL2 cultivars at concentrations of 0.1-10 mg/ml and DPPH assay as expressed by GraphPad Prism software. (N=8)

Rice extracts (mg/ml) (IC_{50})	GBR	BR	WR
PL2	2.65	7.18	9.25
KDML105	1.98	5.21	8.58

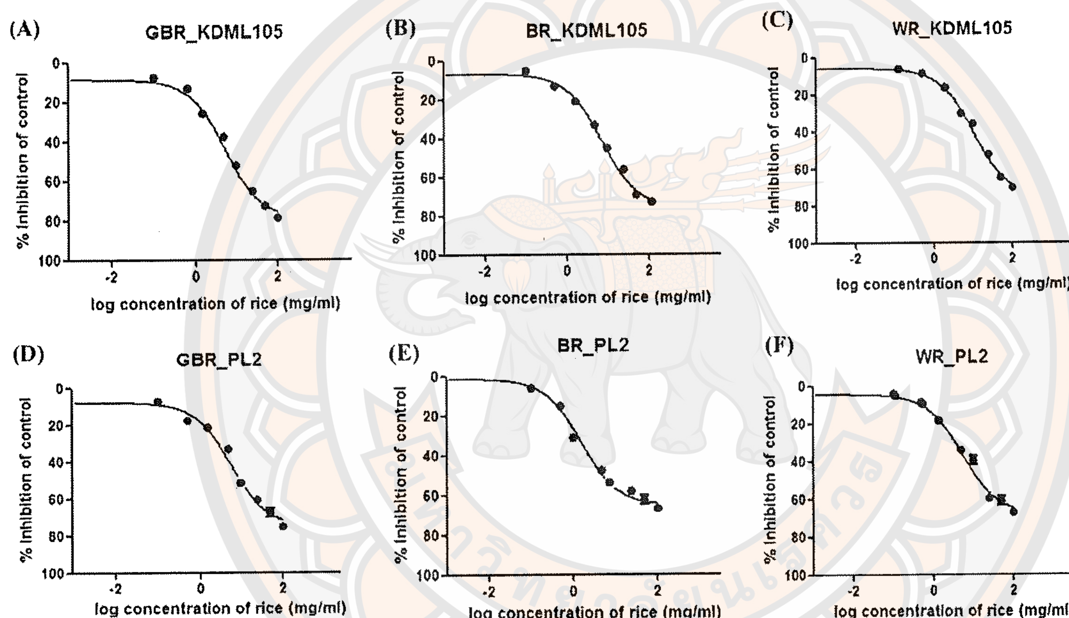


Figure 55 The graph of inhibitory concentration (IC_{50}) from KDML105 and PL2 cultivars at concentrations of 0.1-10 mg/ml by DPPH assay were expressed using GraphPad Prism software. Data are expressed as % inhibition and are representative of 2 replicated experiments.

Effects of rice extracts on total phenolic content by Folin- Ciocalteu reagent assay

Further, the results showed that the total phenolic content of the rice extracts (GBR, BR, and WR) from KDML105 and PL2 cultivars increased in a dose-dependent manner. The GBR exhibited a higher total phenolic content than BR and WR at similar

concentrations (Table 19). Total phenolic content of rice extracts (GBR, BR, and WR) from KDML105 and PL2 cultivars with different concentrations were expressed as Gallic acid Equivalent (GAE) (mg/g samples) as shown in table 19, respectively.

Table 19 The calculation of GAE (mg/g of rice extract) of both cultivars at 0.1-1 mg/ml for the total phenolic content assay. (N=8)

	GAE (mg/g of rice extract)					
Conc. of rice extract (mg/ml)	**GBR**		**BR**		**WR**	
	KDML105	**PL2**	**KDML105**	**PL2**	**KDML105**	**PL2**
0.1	4.27±0.04	3.91±0.17	1.54±0.53	1.24±0.21	1.14±0.25	1.11±0.28
0.5	5.99±0.07	6.17±0.14	3.33±0.78	3.17±0.23	2.58±0.23	2.65±0.22
1	8.07±0.13	8.13±0.05	5.06±0.17	5.22±0.37	4.56±0.10	3.78±0.06

Effects of rice extracts on GABA and γ-oryzanol contents by Gas Chromatography (GC)

In both cultivars, GBR contained more GABA than BR, while GBR contained less γ-oryzanol than BR (Table 20)

Table 20 The GABA and γ-oryzanol contents in BR and GBR extracts in PL2 and KDML105 cultivars

Rice extracts	GABA (mg/100g of rice extract)	γ-oryzanol (mg/100g of rice extract)
BR (PL2)	<1	34.88
BR (KDML105)	<1	40.97
GBR (PL2)	16.46	31.55
GBR (KDML105)	36.88	28.65

Experiment II: Cell culture and Treatments

Effects of rice extracts on Cell viability in 3T3-L1 adipocytes by [3-(4, 5-Dimethylthiazol-2-yl) -2, 5-Diphenyltetrazolium Bromide] (MTT) assay.

MTT assays were done to assess the cell viability of 3T3-L1 preadipocytes treated with various concentrations (1-10 mg/ml) of WR, BR, and GBR extracts of both cultivars. The range of the cell viabilities of the extracts at the highest concentration (10 mg/ml) were significantly lower ($P<0.05$) than the control (without treatment) (Figure 56), but the cell viability of the 3T3-L1 preadipocytes, treated with 1 mg/ml of rice extract from both cultivars, did not show significant differences ($P<0.05$) to the control. Thus, all the rice extracts (GBR, BR, and WR) from both cultivars at a concentration of 1 mg/ml were considered as non-cytotoxic to 3T3-L1 preadipocytes.

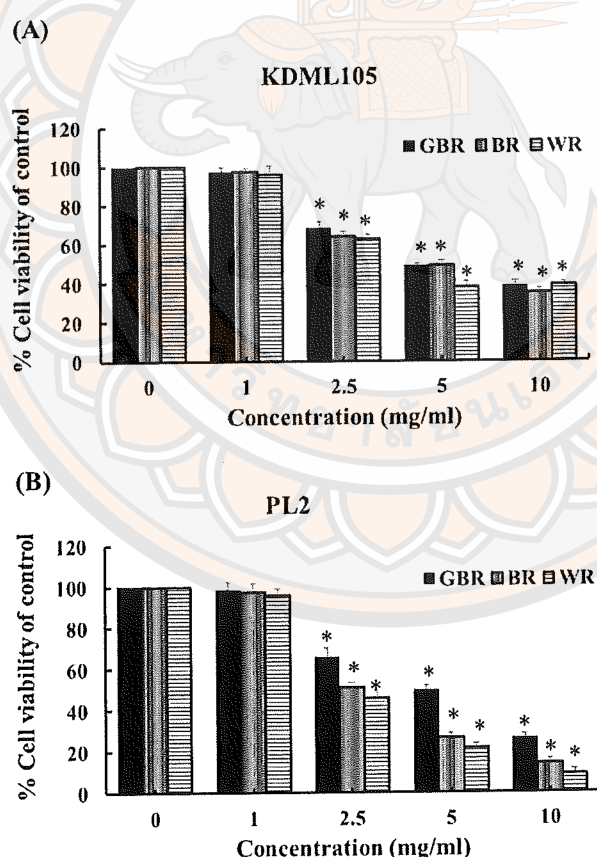


Figure 56 Effect of GBR, BR, and WR extracts on the viability of 3T3-L1 preadipocytes. Cells treated with rice extracts from KDML105 (A) and PL2 (B) cultivars (1-10 mg/ml) for 24 hours. (N=8)

After 3T3-L1 adipocytes were treated with GBR, BR, and WR extracts from both cultivars at various concentrations (0.1, 0.5, and 1 mg/ml) for 24 hours, there were no toxicity effects on the cell viability (Figure 57).

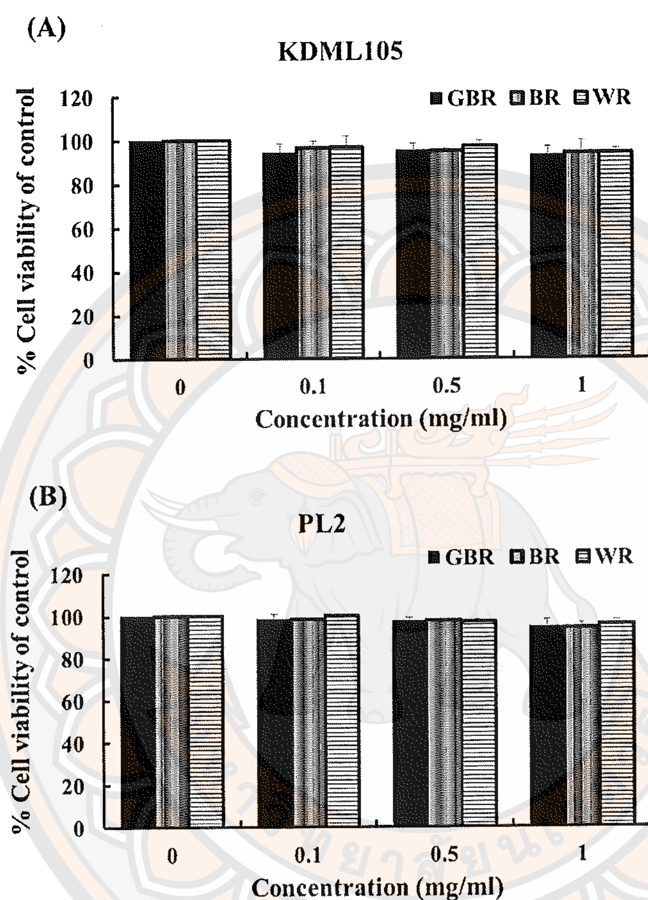


Figure 57 Effect of GBR, BR, and WR extracts on the viability of 3T3- L1 preadipocytes. Cells were treated with rice extracts from KDML105 (A) and PL2 (B) cultivars (0.1, 0.5, and 1 mg/ml) for 24 hours. (N=8)

Experiment I: Normal 3T3-L1 adipocytes treatments

Effects of rice extracts, caffeine, and GABA on lipid accumulation in 3T3-L1 adipocytes by Oil Red O staining assay.

Previous studies have shown that the rice extracts could affect the metabolic activity impairment of adipocyte cell growth. Therefore, it would be interesting to determine the effect of rice extracts on lipid accumulation. Lipid accumulation was measured at days 3, 5, 7, and 9 of incubation by Oil-Red O staining. The cells were treated with GBR and BR extracts of both cultivars. Intracellular lipid accumulation (Figure 58 and 59) was significantly decreased ($P<0.05$) in a concentration-dependent manner during adipocyte differentiation. The lipid droplets were measured at days 3, 5, 7, and 9 of incubation by Oil-Red O staining. Intracellular lipid accumulation (Figure 60) was significantly decreased ($P<0.05$) in a concentration-dependent manner during adipocyte differentiation.

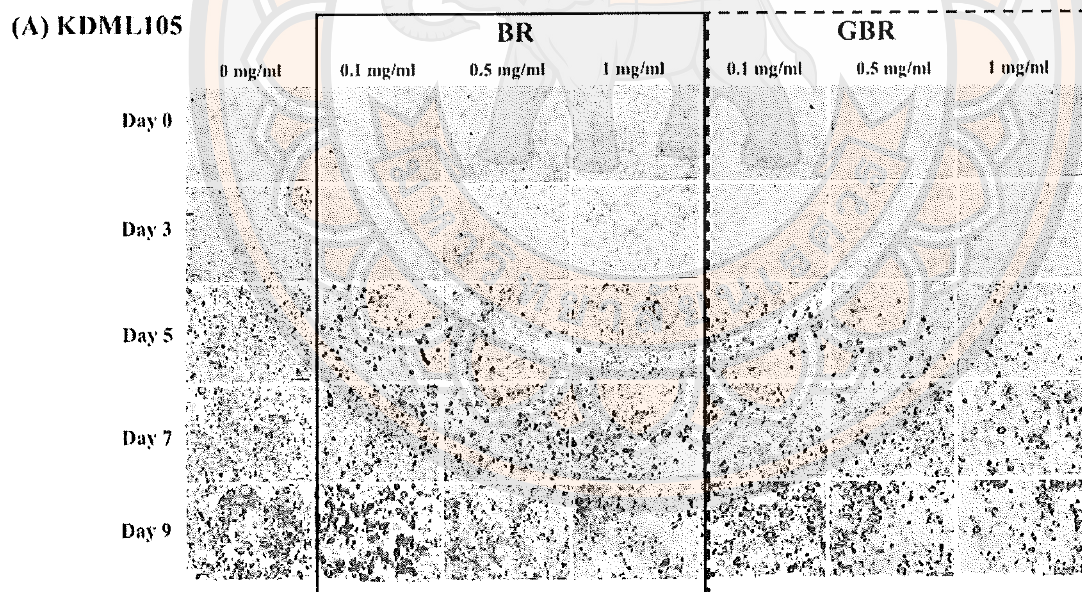


Figure 58 Effect of BR and GBR extracts on lipid droplets in 3T3-L1 adipocytes. Cells were treated with rice extracts from KDML105 (BR; __ and GBR; ---) cultivars (0.1, 0.5, and 1 mg/ml) for 9 days. (N=4)

(B) PL2

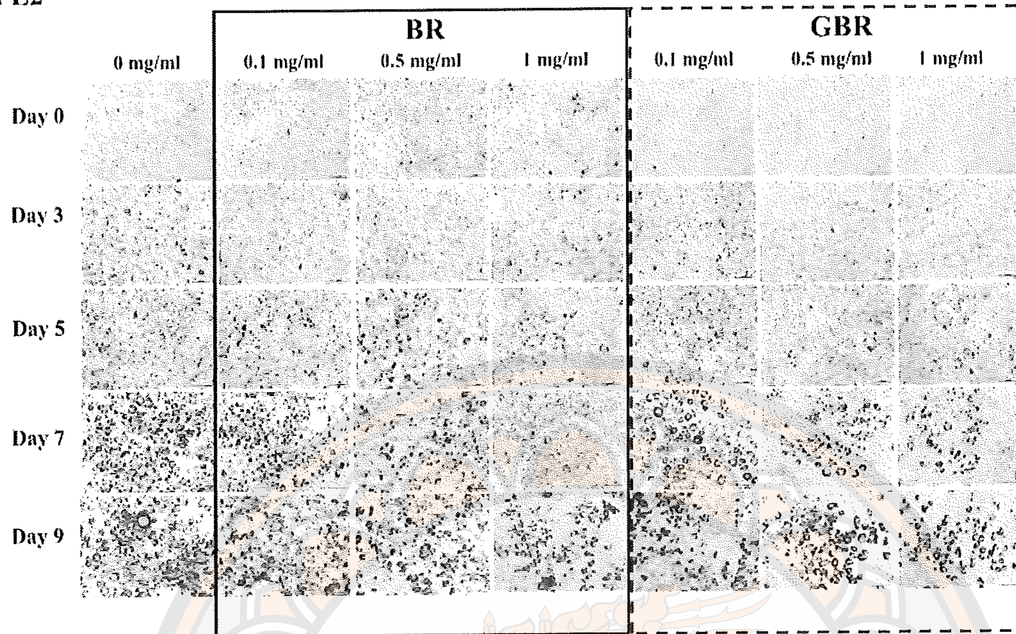


Figure 59 Effect of BR and GBR extracts on lipid droplets in 3T3-L1 adipocytes. Cells were treated with rice extracts from PL2 (BR; __ and GBR; ---) cultivars (0.1, 0.5, and 1 mg/ml) for 9 days. (N=4)

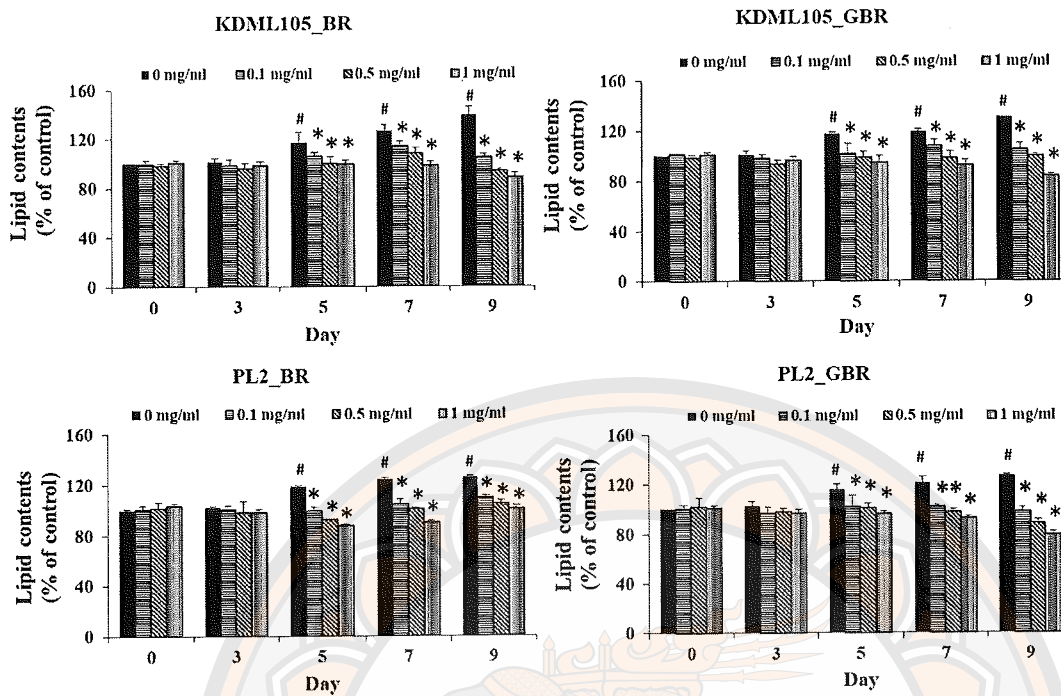


Figure 60 Effect of GBR and BR extracts on lipid droplets in 3T3-L1 adipocytes. Cells were treated with rice extracts from KDML105 (A) and PL2 (A) and cultivars (0.1, 0.5, and 1 mg/ml) for 9 days. Lipid contents were stained by Oil-Red O and quantified as absorbance at 500 nm. *P<0.05 compared to control within each day. #P<0.05 compared to control at day 0. (N=4)

3T3-L1 adipocytes treated with caffeine and GABA at concentrations of 0.1 mg/ml and 1 mg/ml also decreased intracellular lipid droplets (Figure 61).

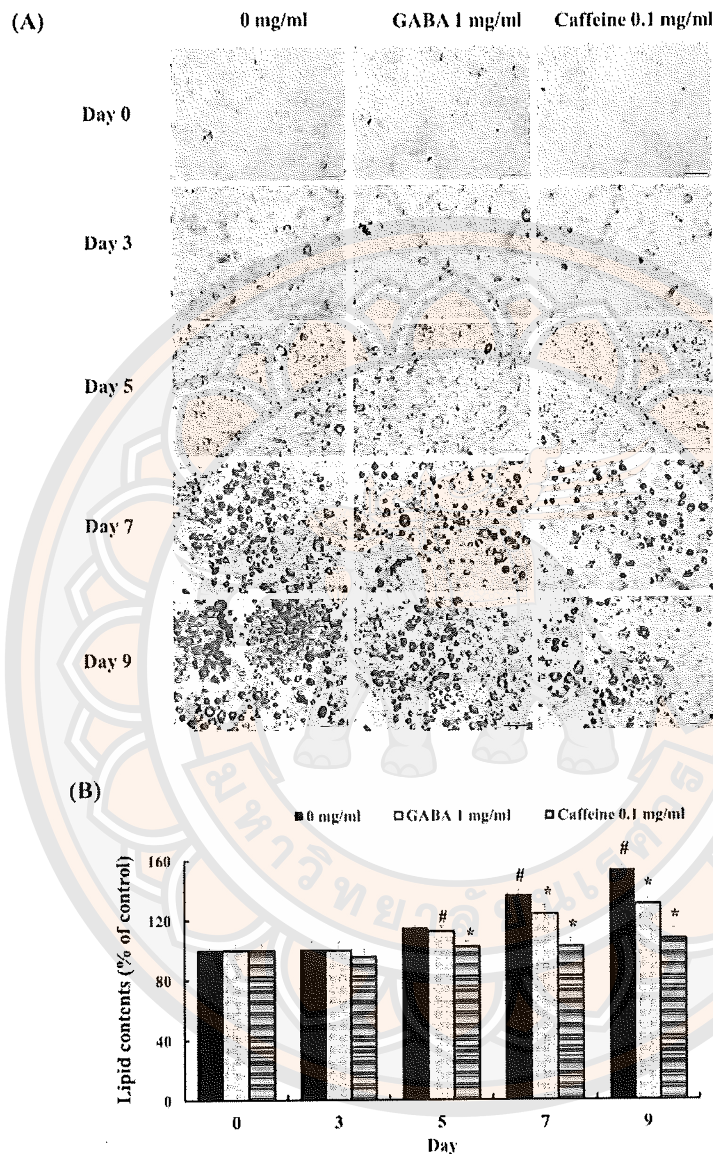


Figure 61 Effect of 1 mg/ml GABA and 0.1 mg/ml Caffeine on lipid droplets in 3T3-L1 adipocytes. Cells were treated with 1 mg/ml GABA and 0.1 mg/ml Caffeine (A) Lipid contents were stained by Oil-Red O and quantified as absorbance at 500 nm. * $P<0.05$ compared to control within each day. # $P<0.05$ compared to control at day 0. (B) for 9 days. (N=4)

Effects of rice extracts, caffeine, and GABA on lipid accumulation in 3T3-L1 adipocytes by Triglyceride quantification assay kit

Intracellular triglyceride levels were measured to confirm previous results. The triglyceride levels were measured at days 3, 5, 7, and 9 of incubation by triglyceride kit assay. Triglyceride contents (Figure 62A and B) were significantly decreased ($P<0.05$) in a concentration-dependent manner during adipocyte differentiation, and 3T3-L1 adipocytes treated with caffeine and GABA at concentrations of 0.1 mg/ml and 1 mg/ml also decreased Triglyceride contents (Figure 62C).

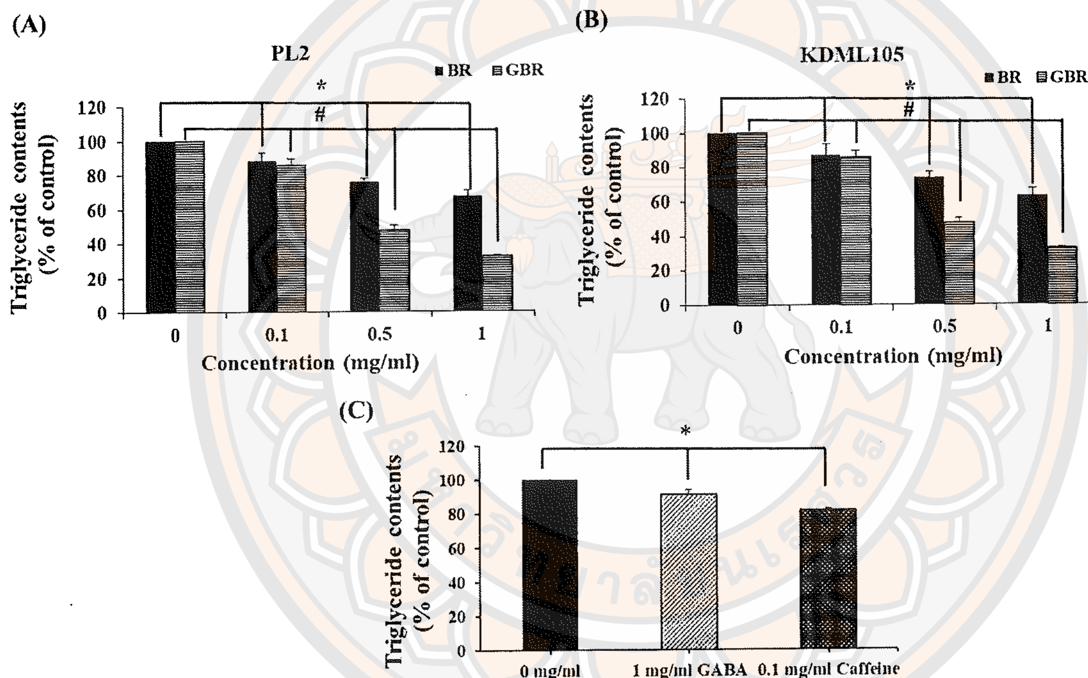


Figure 62 Effects of BR and GBR extracts on triglyceride levels in 3T3- L1 adipocytes. Cells were treated with rice extracts from PL2 (A) and KDML105 (B) cultivars (0.1, 0.5, and 1.0 mg/ml) for 9 days. The cells were then treated with caffeine and GABA at concentrations of 0.1 mg/ ml and 1 mg/ ml. Triglyceride levels were determined by Triglyceride kit assay. * P<0.05 compared to control. (N=4)

Effects of rice extracts on *adipogenic*, *lipogenic*, and *lipolytic* gene expression in 3T3-L1 adipocytes by RT-PCR

Following the studies on metabolic activity impairment of adipocyte cell growth and lipid accumulation, RT-PCR was performed to measure related-gene expression to determine the mechanisms involving in metabolic activity impairment of adipocyte cell growth and lipid accumulation. The results showed that the reduction of adipogenic and lipogenic gene expression, while expression of lipolytic genes increased (Figure 63A and B).

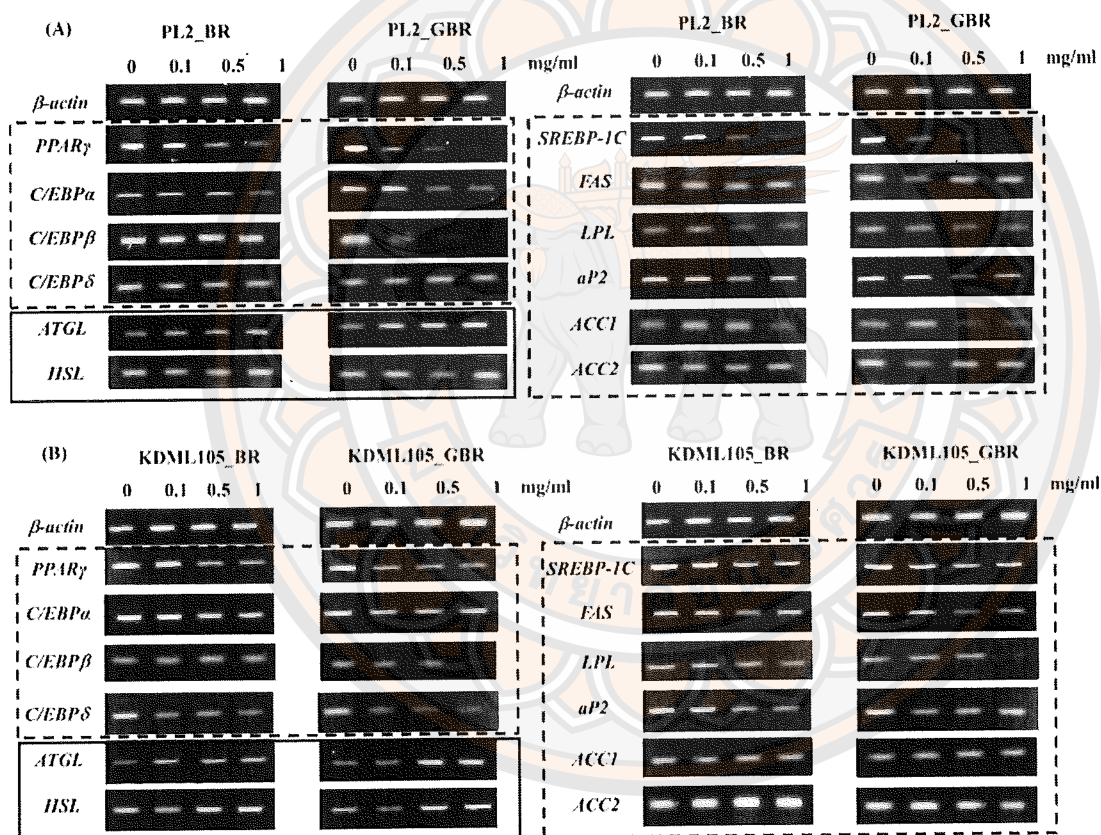


Figure 63 Effect of BR and GBR from PL2 (A) and KDML105 (B) cultivars on mRNA expression in 3T3-L1 by RT-PCR. -------, Indicates a decreasing and an increase of mRNA expression respectively. (N=3)

Effects of rice extracts (KDML105 cultivar) on *Pref-1* expression in 3T3-L1 adipocytes by RT-PCR

The expression of *Pref-1*, which is a preadipocyte marker gene, in untreated 3T3-L1 adipocytes was undetected at day 17. However, the mRNA expression of *Pref-1* was restored at day 17 in adipocytes treated with GBR from the KDML105 cultivar for 19 days from the initiation of differentiation (Figure 64).

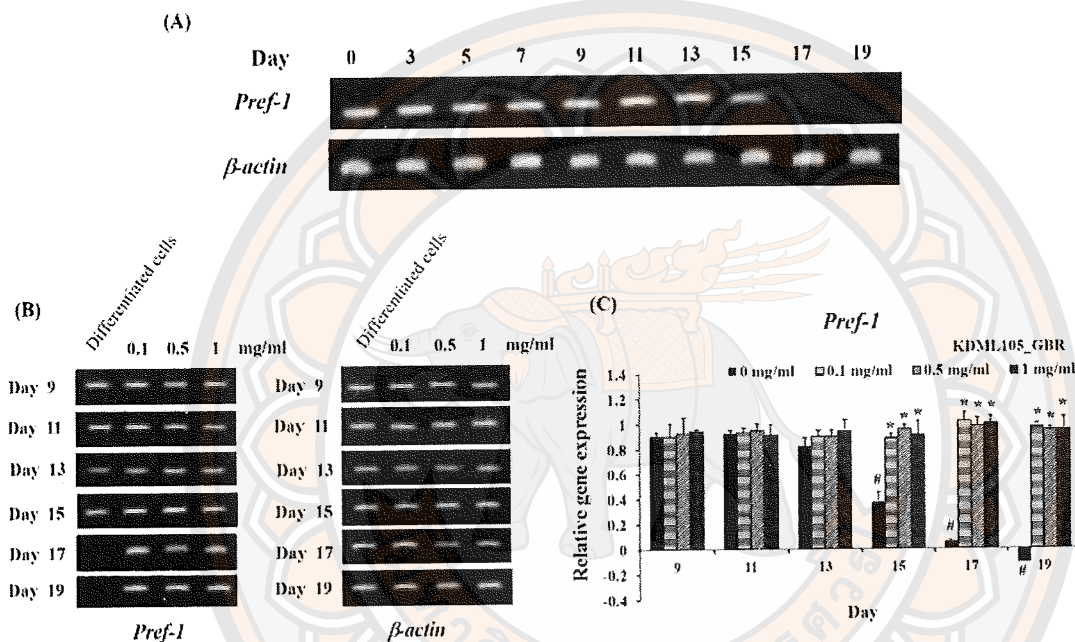


Figure 64 Effect of GBR from KDML105 cultivar on *Pref-1* gene expression. Basal expression of *Pref-1* in untreated cells (A) and cells treated with GBR (B and C) for 19 days. **P*<0.05 compared to differentiated cells within each day. #*P*<0.05 compared to differentiated cells at day 9. (N=3)

Effects of rice extracts on *adipogenic transcription factors* gene expression in 3T3-L1 adipocytes by qPCR

The study from RT-PCR shows promising results. The change of gene expression was then confirmed by qPCR. Consistent with qPCR results, it was shown that the mRNA expression of the adipogenic transcription factors *PPARγ*, *C/EBPα*, *C/EBPβ*, and *C/EBPδ* in 3T3-L1 cells treated with BR and GBR from both cultivars significantly decreased compared with the control (*P*< 0.05) (Figure 65 and 66).

(A)

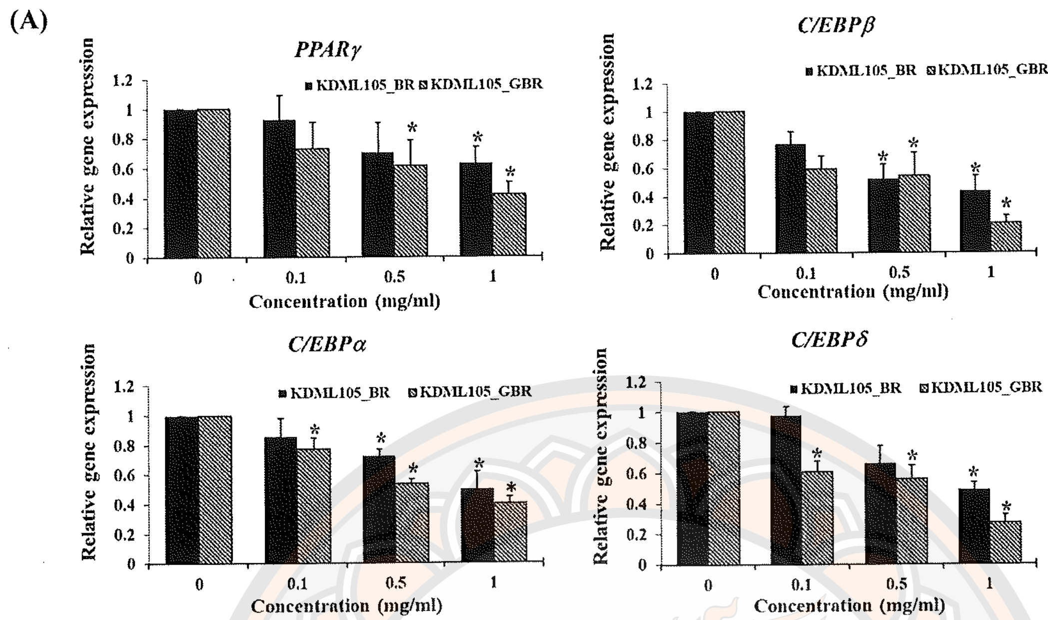


Figure 65 Effect of BR and GBR from KDML105 cultivars on *adipogenic transcription factor* genes expression by qPCR. * *P*<0.05 compared to control. (N=6)

(B)

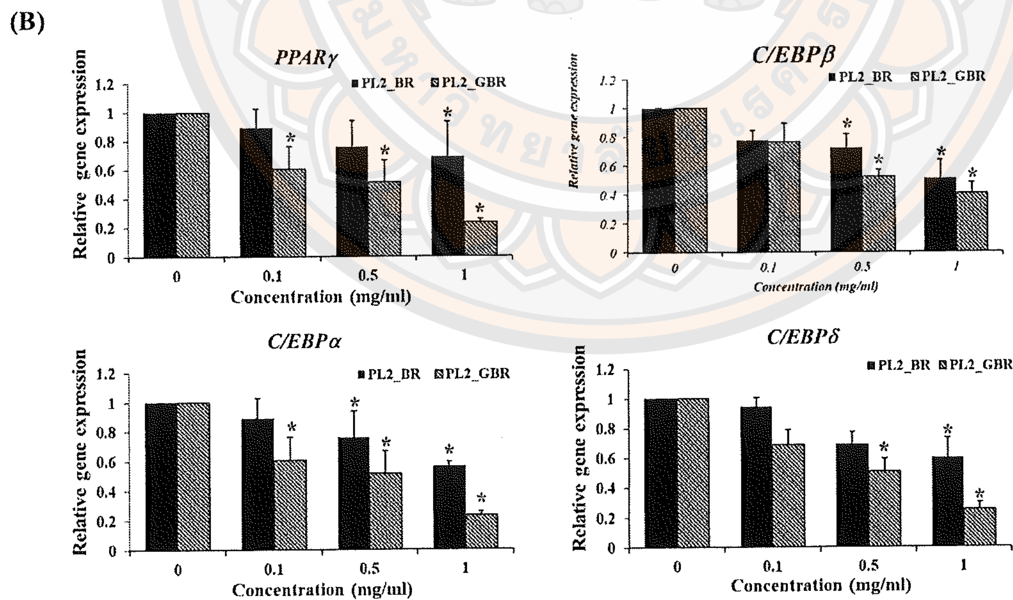


Figure 66 Effect of BR and GBR from PL2 cultivars on *adipogenic transcription factor* genes expression by qPCR. * *P*<0.05 compared to control. (N=6)

Effects of rice extracts on *lipogenic* genes expression in 3T3-L1 adipocytes by qPCR

The expression of lipogenic genes *SREBP-1c*, *FAS*, *LPL*, *aP2*, *ACC1,* and *ACC2* was also suppressed by the treatments (Figure 67 and 68) which were significantly decreased in the treated cells as compared to the untreated control cells ($P<0.05$).

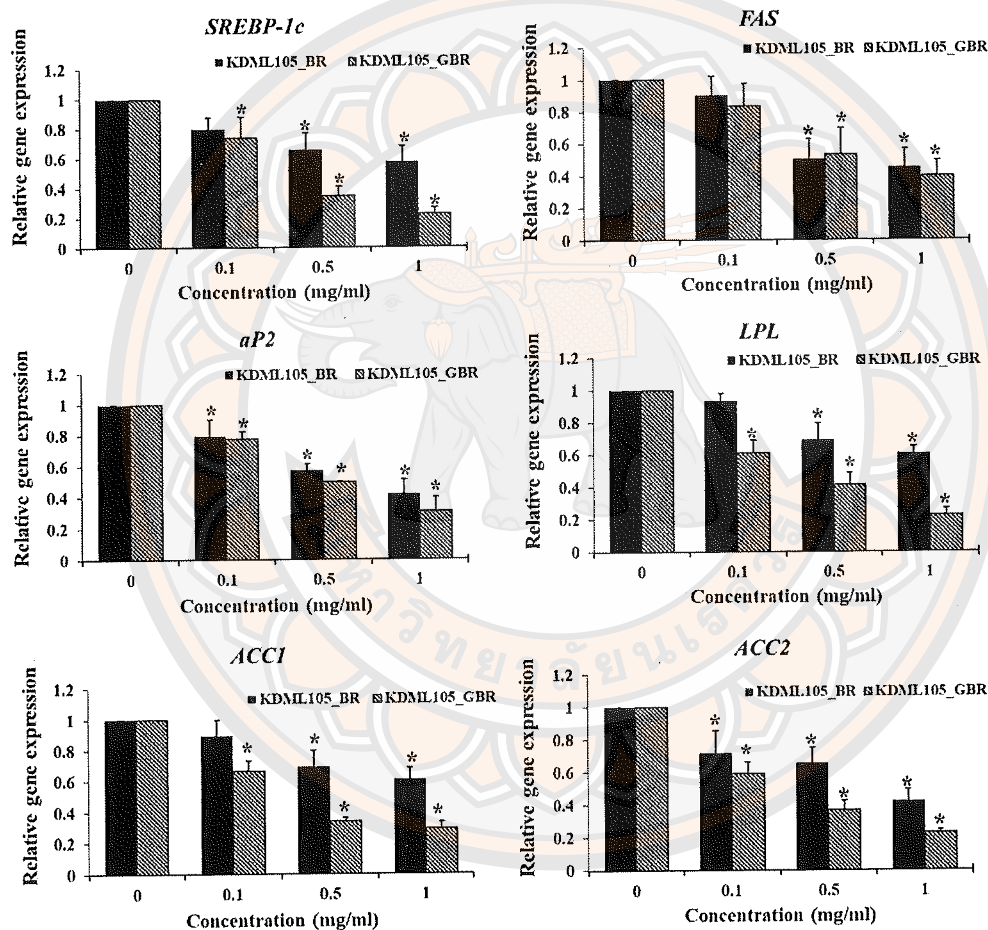


Figure 67 Effect of BR and GBR from KDML105 cultivars on *lipogenic* gene expression by qPCR. * $P<0.05$ compared to control. (N=6)

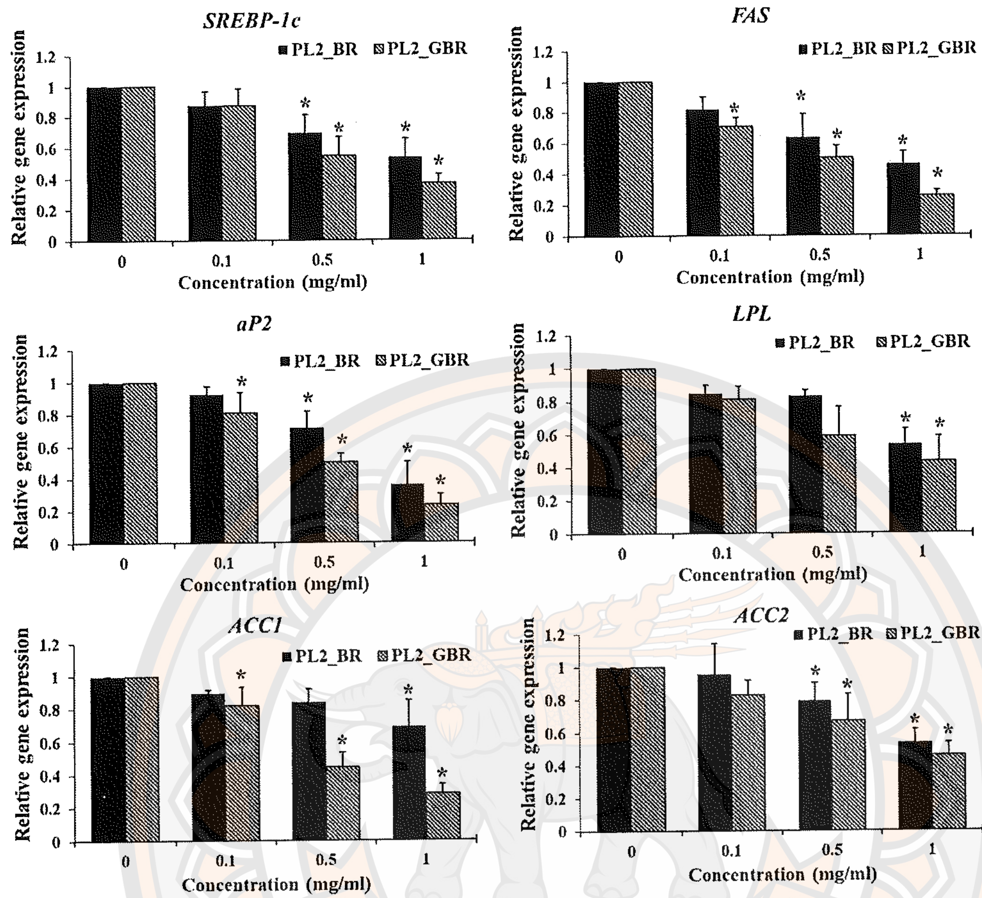


Figure 68 Effect of BR and GBR from PL2 cultivars on *lipogenic* genes expression by qPCR. * *P*<0.05 compared to control. (N=6)

Effects of rice extracts on *lipolytic* genes expression in 3T3-L1 adipocytes by qPCR

The expression of lipolytic genes *ATGL* and *HSL* significantly increased in the treated cells as compared to the untreated control cells ($P < 0.05$) (Figure 69).

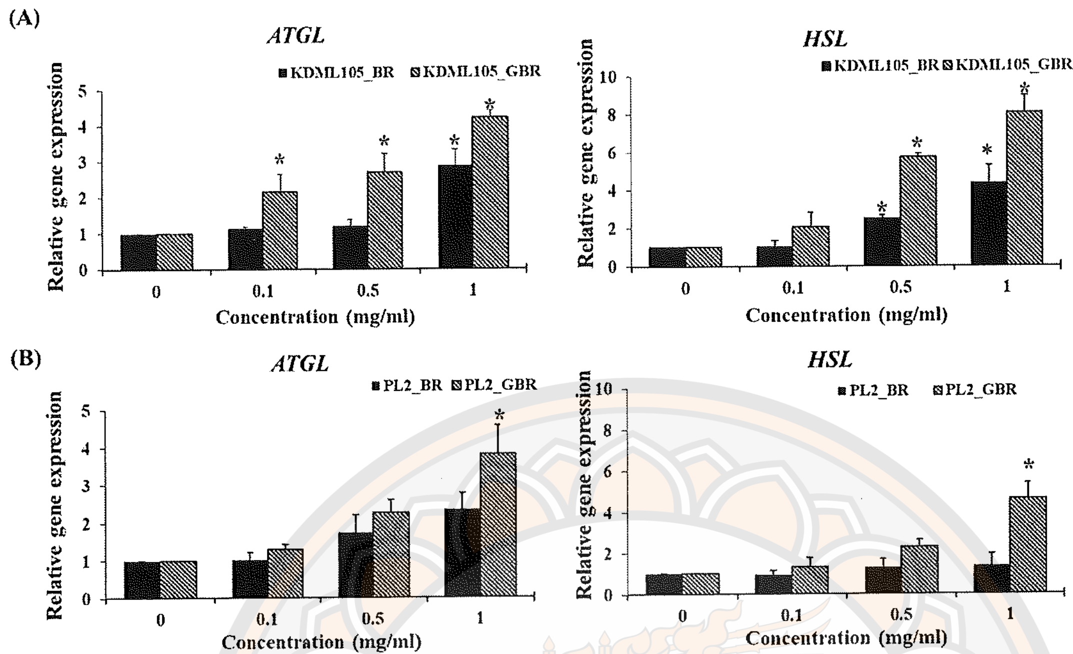


Figure 69 Effect of BR and GBR from KDML105 (A) and PL2 (B) cultivars on *lipolytic* genes expression by qPCR. **P*<0.05 compared to control. (N=6)

Effects of rice extracts on *pro-inflammatory cytokines* and *adiponectin* gene expression in 3T3-L1 adipocytes by qPCR

The expression of pro-inflammatory cytokines genes (*TNF-α* and *IL6*) and *adiponectin* significantly decreased in the treated cells as compared to the untreated control cells ($P < 0.05$) (Figure 70).

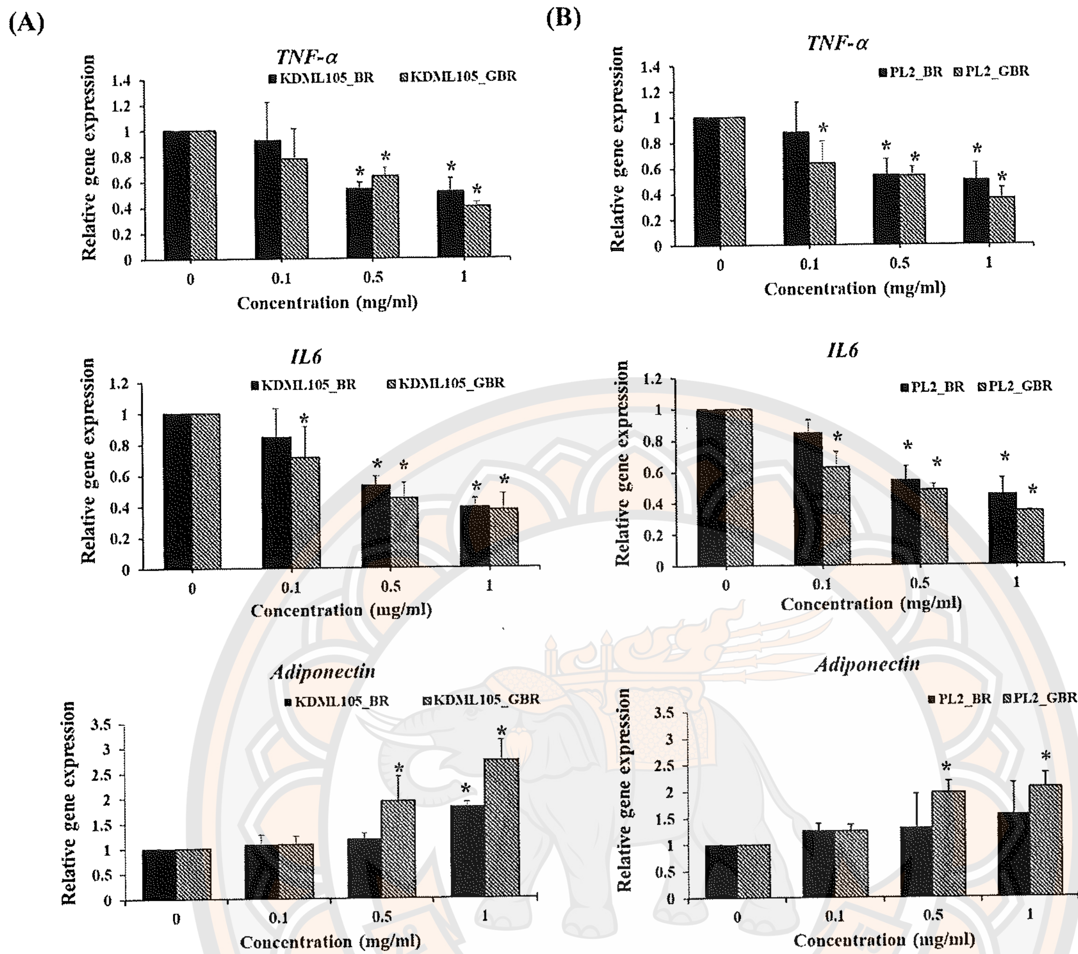


Figure 70 Effect of BR and GBR from KDML105 (A) and PL2 (B) cultivars on *pro-inflammatory cytokines* and *adiponectin* genes expression by qPCR. **P*<0.05 compared to control. (N=6)

Effects of rice extracts on *antioxidant* genes expression in 3T3- L1 adipocytes by qPCR

The expression of antioxidant genes (*SOD2, CAT,* and *GPx4*) significantly increased in the treated cells as compared to the untreated control cells (*P*<0.05) (Figure 71).

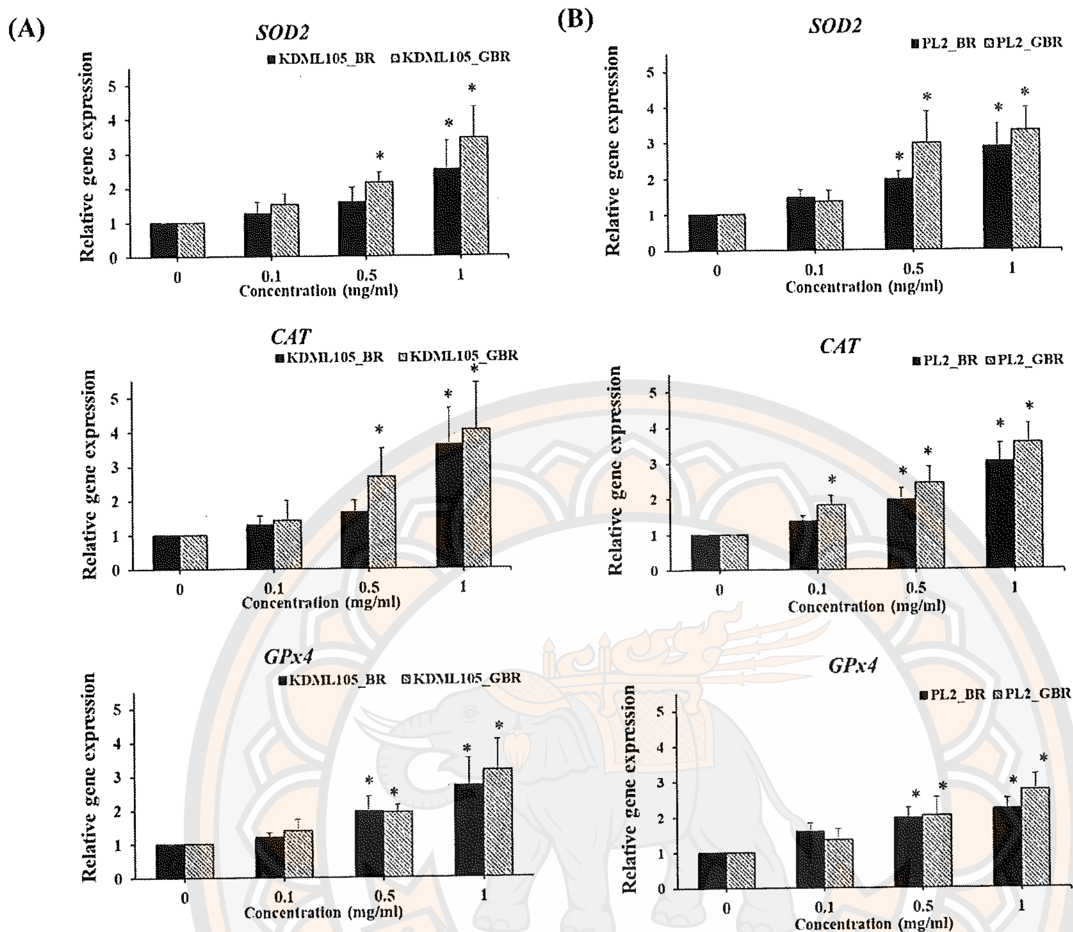


Figure 71 Effect of BR and GBR from KDML105 (A) and PL2 (B) cultivars on *antioxidant* genes expression by qPCR. * $P<0.05$, compared to control. (N=6)

Part II: H_2O_2-induced 3T3-L1 adipocytes treatments

Effects of H_2O_2 on cell viability in 3T3-L1 adipocytes

The effect of H_2O_2 on cell viability in 3T3-L1 adipocytes was evaluated by MTT assay, as shown in figure 72, H_2O_2 had no cytotoxic effect at 100-200 μM in both undifferentiated cells and differentiated cells.

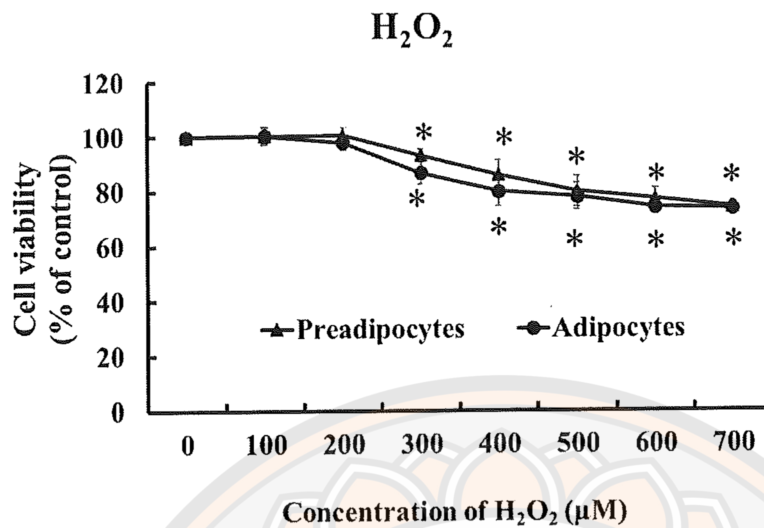


Figure 72 Effects of H_2O_2 on cell viability in 3T3-L1 adipocytes. * *P*<0.05, compared to undifferentiated cells (UD). Data represented as mean ± S.D. of duplicates per treatment. (N=16)

Effect of rice extracts on intracellular ROS level in H_2O_2-induced 3T3-L1 adipocytes

To investigate the effect of rice extracts on ROS levels in 3T3-L1 adipocytes, intracellular ROS was first induced by treating the 3T3-L1 adipocytes with H_2O_2 for 24 hours. The ROS levels were then measured using a DCFH-DA assay. As shown in figure 73, the intracellular ROS levels significantly increased ($P<0.05$) in the oxidative-stress induced 3T3-L1 cells in the comparison with both normal undifferentiated and differentiated adipocytes.

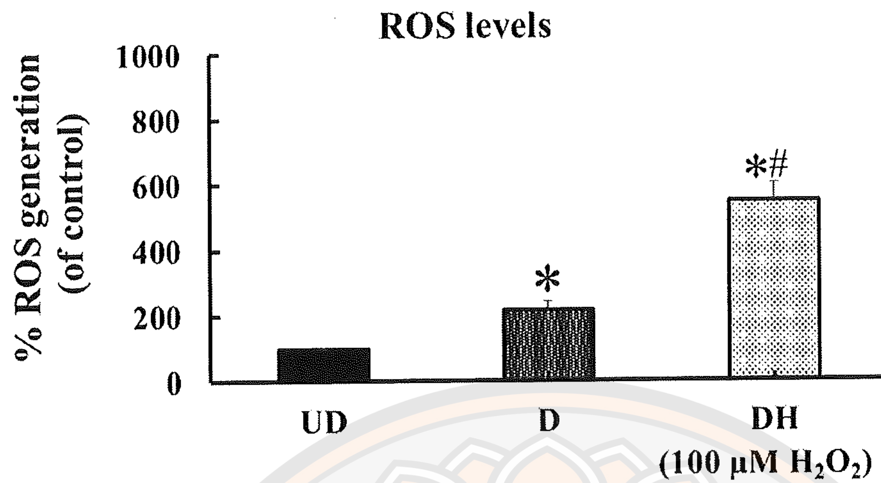


Figure 73 Effects of H_2O_2 on ROS levels in H_2O_2-induced 3T3-L1 adipocytes. * $P<0.05$, compared to undifferentiated cells (UD) and # $P<0.05$, compared to differentiated cells (D). Data represented as mean ± S.D. of duplicates per treatment. (N=16)

However, after treated with GBR and BR extracts (0.5 and 1 mg/ml) from both cultivars, the ROS levels significantly decreased ($P<0.05$) compared to the H_2O_2-induced 3T3-L1 adipocytes (Figure 74A and B). In contrast, after being treated with WR, there was no effect on intracellular ROS levels in H_2O_2- induced 3T3- L1 adipocytes.

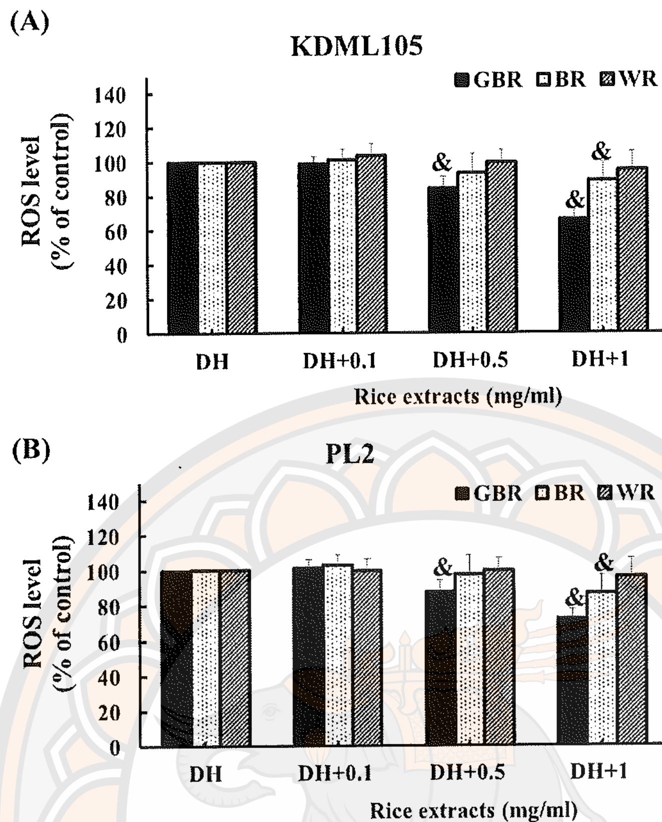
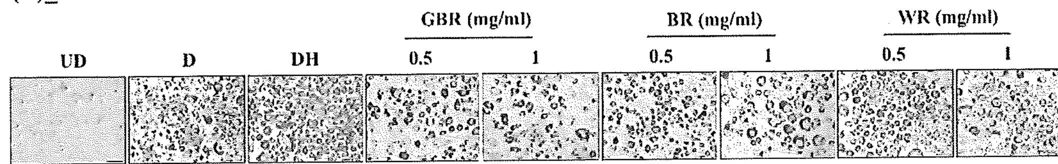


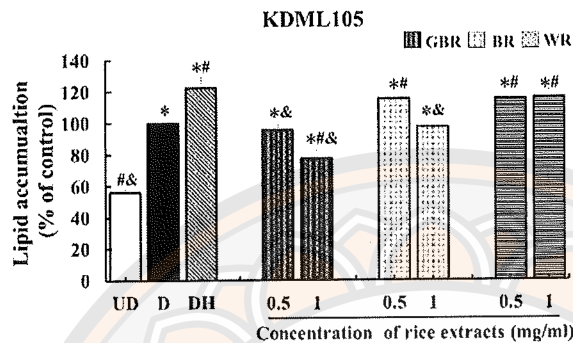
Figure 74 Effects of rice extracts (GBR, BR, and WR) from (A) KDML105 and (B) PL2 on ROS levels in H_2O_2-induced 3T3-L1 adipocytes. [&] $P<0.05$ compared to H_2O_2-induced cells (DH). Data represented as mean ± S.D. of duplicates per treatment. (N=16)

Effect of rice extracts on lipid accumulation in H_2O_2-induced 3T3-L1 adipocytes

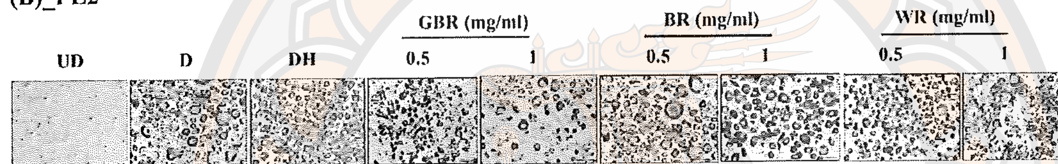
Intracellular lipid accumulation was determined by Oil-Red O staining in mature adipocytes. The result showed that the lipid accumulation was significantly increased ($P<0.05$) in the H_2O_2-induced 3T3-L1 adipocytes. 3T3-L1 cells were then induced with H_2O_2 for 48 hours and then treated with rice extracts (0.5 and 1 mg/ml) from KDML105 and PL2 cultivars for 24 hours, before the induction of oxidative stress. Oxidative stress caused significantly reduced ($P<0.05$) lipid accumulation in 3T3-L1 adipocytes as compared to the control group shown in (Figure 75).

(A)_KDML105





(B)_PL2



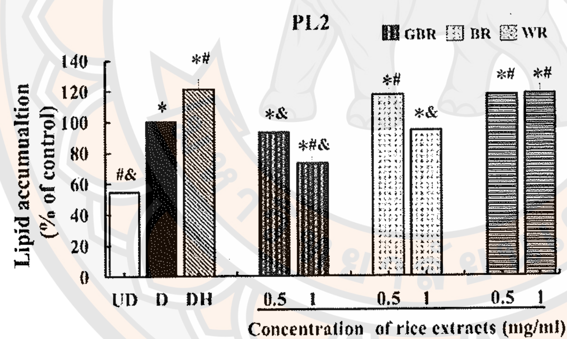
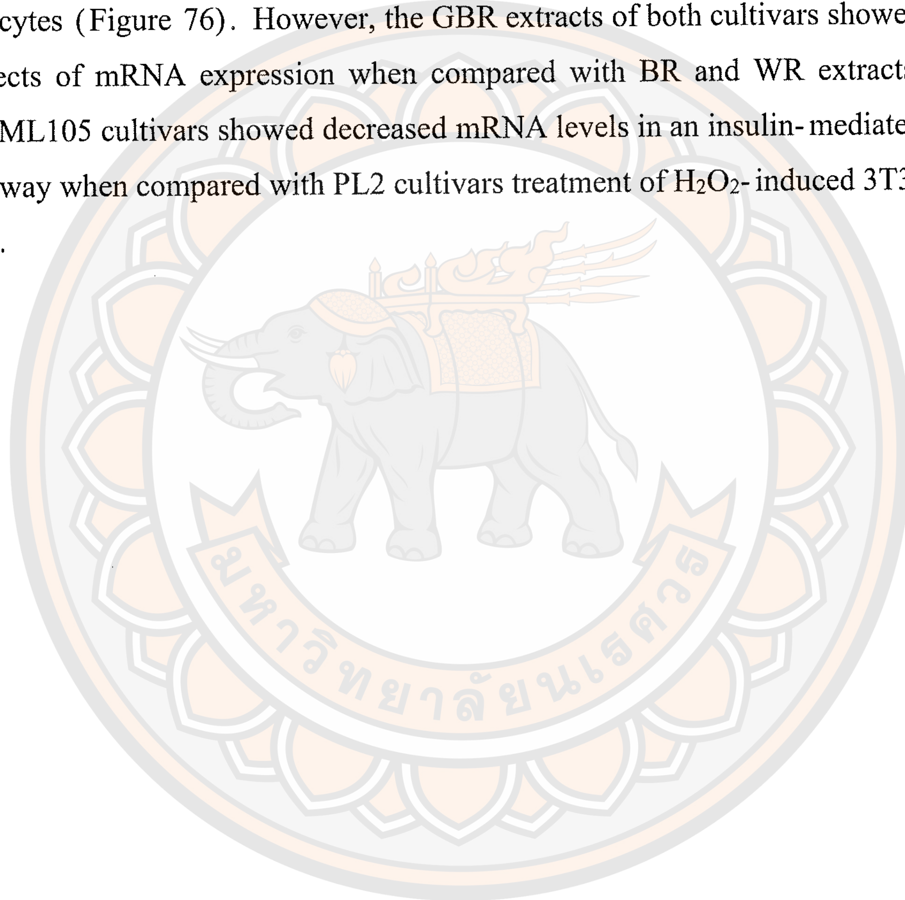


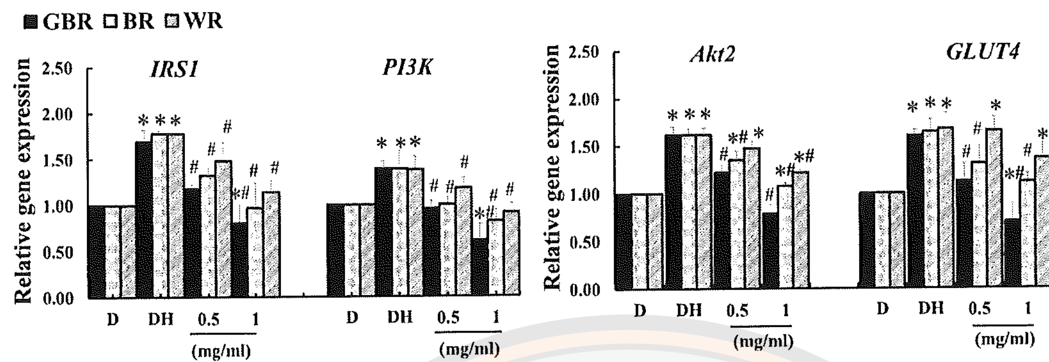
Figure 75 Effects of rice extracts from (A and C) KDML105 and (B and D) PL2 cultivars on lipid accumulation in H_2O_2-induced 3T3-L1 adipocytes. The lipid accumulation was examined by Oil-Red O staining and the absorbance was measured at 510 nm. * $P<0.05$, compared to undifferentiated cells (UD) and # $P<0.05$, compared to differentiated cells (D). & $P<0.05$ compared to H_2O_2-induced cells (DH). Data represented as mean ± S.D. of duplicates per treatment. (N=16)

Effect of rice extracts on mRNA expression of *insulin-mediated signaling pathway* genes in H_2O_2-induced 3T3-L1 adipocytes

qPCR analysis showed that the mRNA expression of *IRS1*, *PI3K*, *Akt2,* and *GLUT4* in H_2O_2-induced 3T3-L1 adipocytes significantly increased ($P<0.05$) as compared to normal differentiated cells (D). However, their expressions significantly decreased ($P<0.05$) after being treated with GBR and BR extracts (0.5 and 1 mg/ml) from both KDML105 and PL2 cultivars when compared to the untreated H_2O_2-induced 3T3-L1 adipocytes (Figure 76). However, the GBR extracts of both cultivars showed decreased effects of mRNA expression when compared with BR and WR extracts. Likewise, KDML105 cultivars showed decreased mRNA levels in an insulin-mediated signaling pathway when compared with PL2 cultivars treatment of H_2O_2-induced 3T3-L1 adipocytes.



(A)_KDML105



(B)_PL2

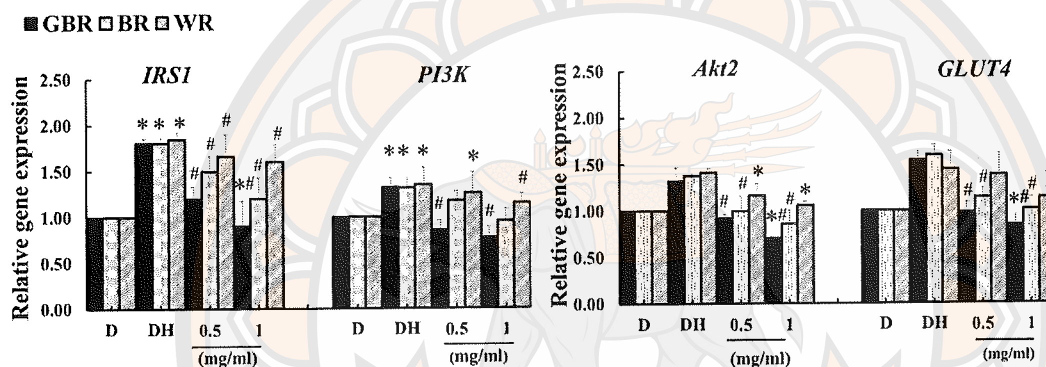


Figure 76 Effects of rice extracts from KDML105 (A) and PL2 (B) cultivars on genes in insulin-mediated signaling pathway in H_2O_2-induced 3T3-L1 adipocytes. The mRNA expression of the insulin-mediated signaling pathway *(IRS1, PI3K, Akt2, and GLUT4)* was quantified by qPCR. *β-actin* was used as an internal control. * $P<0.05$, compared to differentiated cells (D) and # $P<0.05$, compared to H_2O_2-induced 3T3-L1 adipocytes (DH). Data represented as mean ± S.D. of duplicates per treatment. (N=5)

Effect of rice extracts on mRNA expression of *adipogenesis* genes in H_2O_2-induced 3T3-L1 adipocytes

Our results showed that H_2O_2-induced 3T3-L1 adipocytes have significantly increased ($P<0.05$) mRNA expression of *PPARγ* and *SREBP-1c* compared to the differentiated cells. On the other hand, the GBR and BR extracts (0.5 and 1 mg/ml) from KDML105 and PL2 cultivars showed significantly decreased ($P<0.05$) *PPARγ* and *SREBP-1c* in H_2O_2-induced 3T3-L1 adipocytes compared to the differentiated cells and H_2O_2-induced 3T3-L1 adipocytes in a dose-dependent manner (Figure 77).

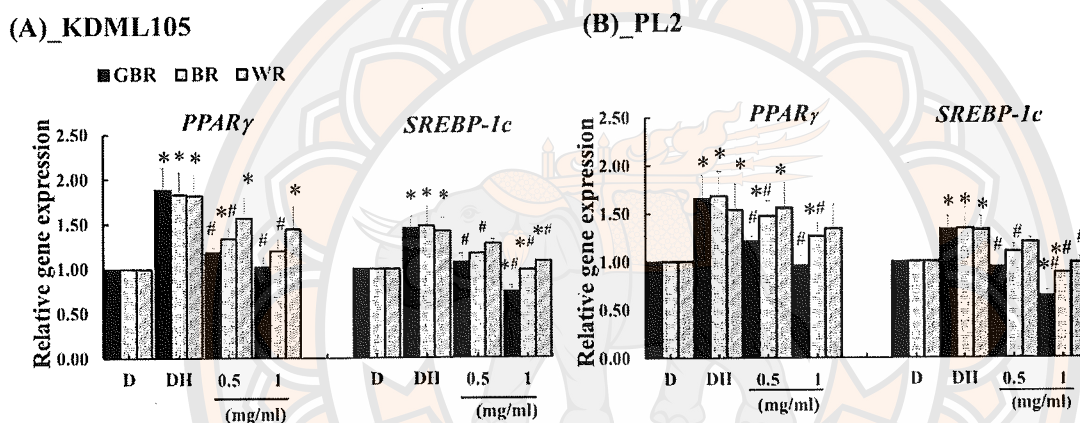


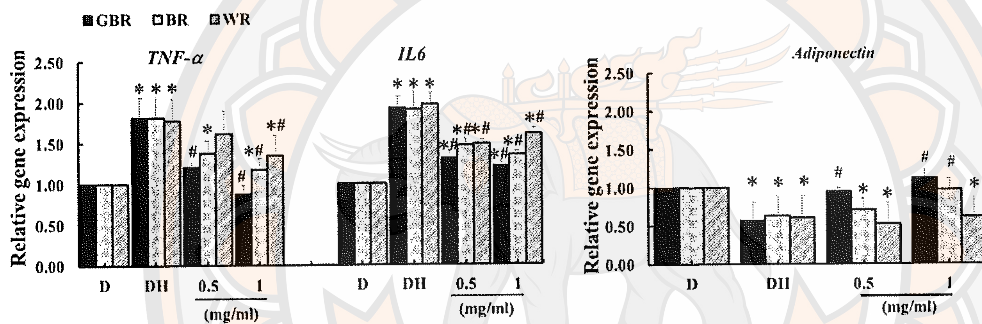
Figure 77 Effects of rice extracts from KDML105 (A) and PL2 (B) cultivars on genes in adipogenesis in H_2O_2-induced 3T3-L1 adipocytes. The mRNA expression of adipogenesis (*PPARγ* and *SREBP-1c*) was quantified by qPCR. *β-actin* was used as an internal control. * $P<0.05$, compared to differentiated cells (D) and $^{\#}P<0.05$, compared to H_2O_2-induced 3T3-L1 adipocytes (DH). Data represented as mean ± S.D. of duplicates per treatment. (N=5)

Effect of rice extracts on mRNA expression of *adipocytokines* genes in H_2O_2-induced 3T3-L1 adipocytes

The mRNA expression of pro-inflammatory cytokine (*TNF-α* and *IL6*) was significantly increased in H_2O_2-induced 3T3-L1 adipocytes. Moreover, the GBR and BR extracts (0.5 and 1 mg/ml) of both cultivars significantly decreased ($P<0.05$) mRNA expression of *TNF-α* and *IL6* compared to the differentiated cells and H_2O_2-induced

3T3-L1 adipocytes (Figure 78). In contrast, H_2O_2-induced 3T3-L1 adipocytes significantly decreased ($P<0.05$) the mRNA expression of *adiponectin*, an anti-inflammatory cytokine. Supplementation of H_2O_2-induced 3T3-L1 adipocytes with GBR and BR extracts (0.5 and 1 mg/ml) led to increased *adiponectin* expression in a dose-dependent manner ($P<0.05$) compared to the differentiated cells and H_2O_2-induced 3T3-L1 adipocytes (Figure 78). WR extracts from KDML105 and PL2 cultivars did not increase the *adiponectin* expression when compared to the H_2O_2-induced 3T3-L1 adipocytes.

(A)_KDML105



(B)_PL2

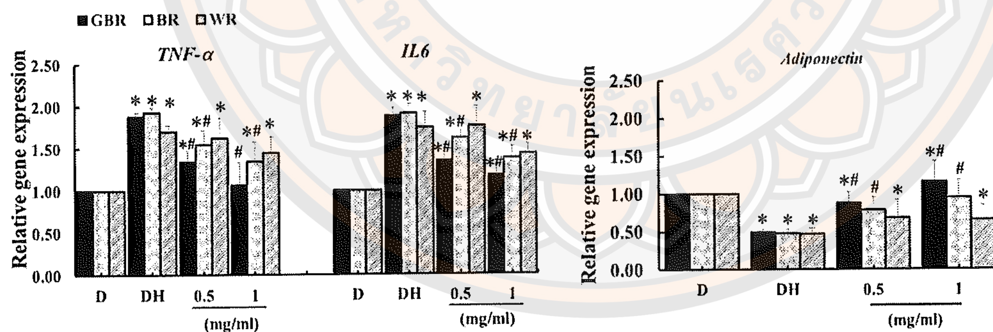


Figure 78 Effects of rice extracts from KDML105 (A) and PL2 (B) cultivars on mRNA expression of *TNF-α*, *IL6*, and *Adiponectin* in H_2O_2-induced 3T3-L1 adipocytes. The mRNA expression was quantified by qPCR. *β*-actin was used as an internal control. * $P<0.05$, compared to differentiated cells and # $P<0.05$, compared to H_2O_2-induced 3T3-L1 adipocytes. Data are represented as mean ± S.D. of duplicates per treatment. (N=5)

Effect of rice extracts on mRNA expression of *Antioxidant* genes in H_2O_2-induced 3T3-L1 adipocytes

We evaluated the effects of the rice (GBR and BR) extracts from KDML105 and PL2 cultivars on the mRNA expression of antioxidant genes in H_2O_2-induced 3T3-L1 adipocytes. As shown in Figure 79 and 80, the mRNA expression levels of *Nrf2* and the downstream genes *HO-1*, *SOD2*, *GPx4*, and *CAT* in oxidative stress-induced cells were significantly decreased ($P<0.05$) compared to differentiated cells. However, after being treated with the GBR and BR extracts at concentrations of 0.5 and 1 mg/ml, their expressions were significantly increased ($P<0.05$) in a dose-dependent manner compared to the differentiated cells and H_2O_2-induced 3T3-L1 adipocytes.

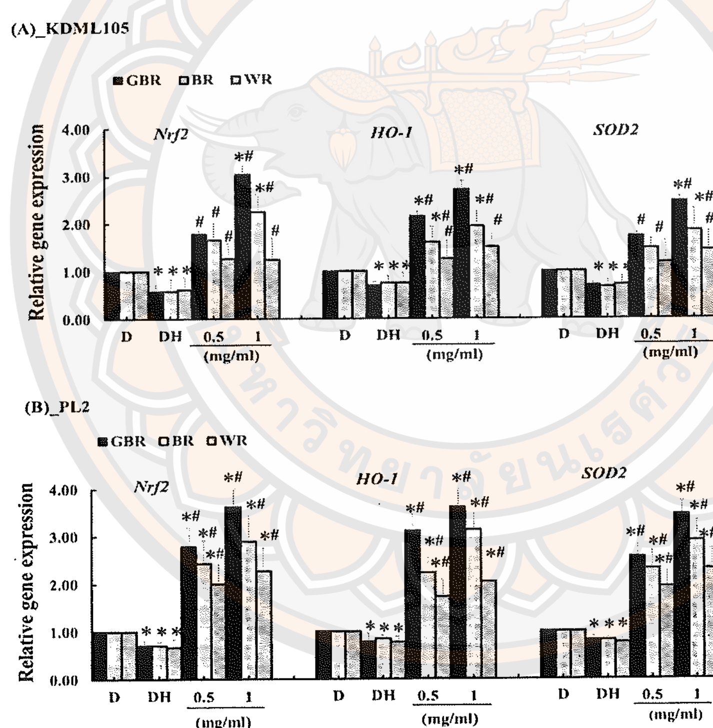
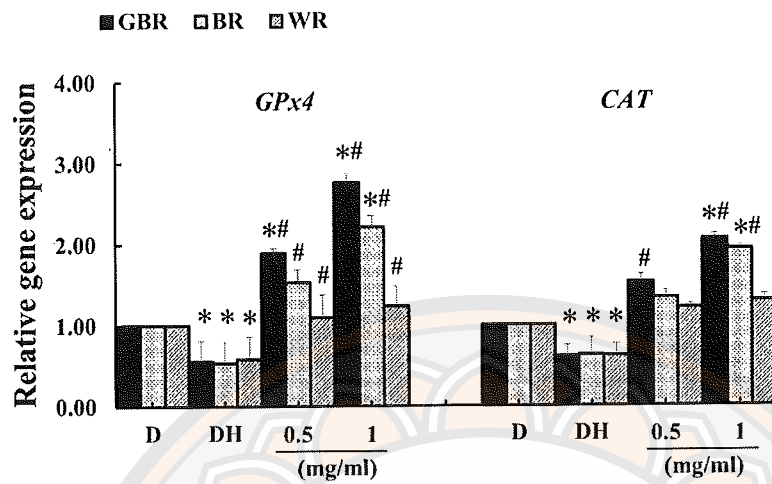


Figure 79 Effects of rice extracts from KDML105 (A) and PL2 (B) cultivars on mRNA expression of *Nrf2, HO-1, and SOD2* in H_2O_2-induced 3T3-L1 adipocytes. The mRNA expression was quantified by qPCR. *β-actin* was used as an internal control. * $P<0.05$, compared to differentiated cells and # $P<0.05$, compared to H_2O_2-induced 3T3-L1 adipocytes. Data are represented as mean ± S.D. of duplicates per treatment. (N=5)

(A)_KDML105



(B)_PL2

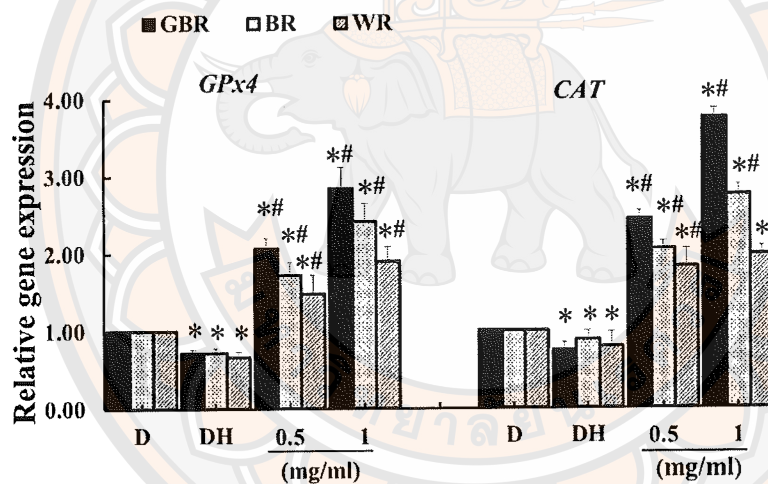
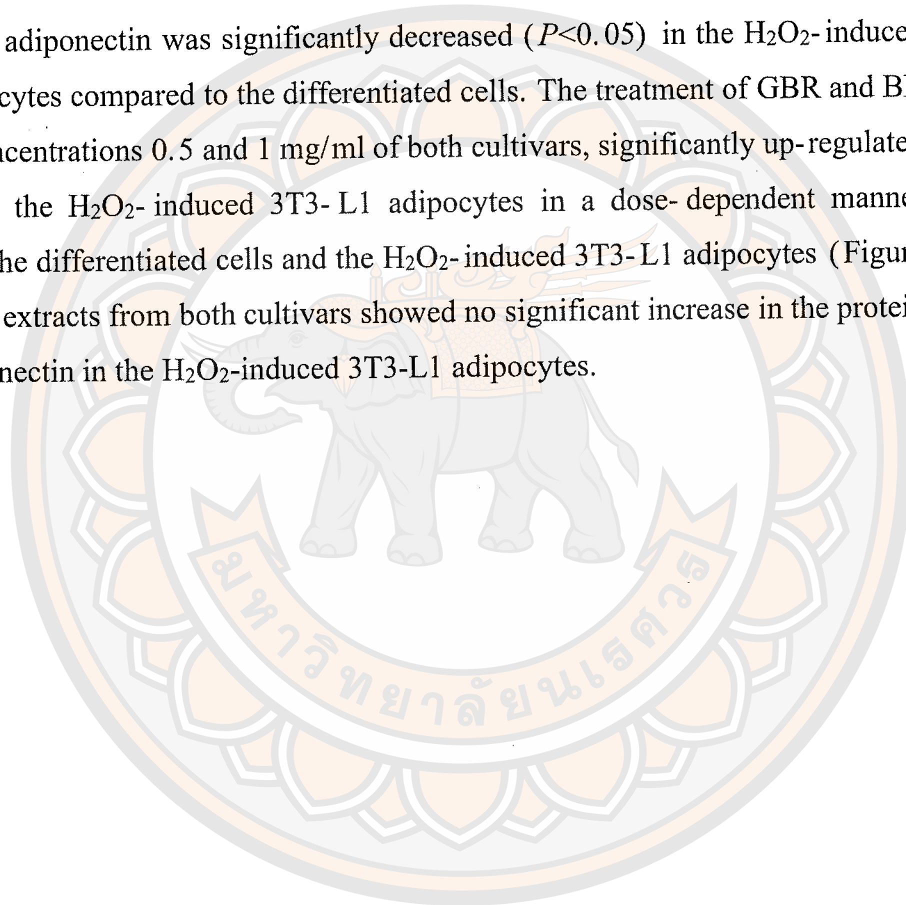


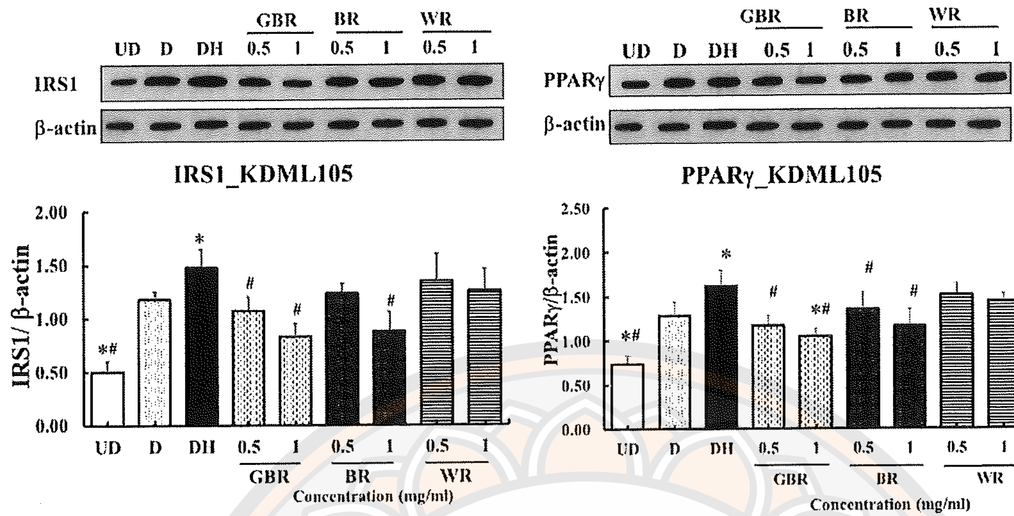
Figure 80 Effects of rice extracts from KDML105 (A) and PL2 (B) cultivars on mRNA expression of *GPx4* and *CAT* in H_2O_2-induced 3T3-L1 adipocytes. The mRNA expression was quantified by qPCR. *β-actin* was used as an internal control. * P<0.05, compared to differentiated cells and # P<0.05, compared to H_2O_2-induced 3T3-L1 adipocytes. Data are represented as mean ± S.D. of duplicates per treatment. (N=5)

Effect of rice extracts on protein expression of IRS1, PPARγ, and adiponectin in H_2O_2-induced 3T3-L1 adipocytes.

H_2O_2-induced 3T3-L1 adipocytes showed significantly increased protein levels of IRS1 and PPARγ compared to the differentiated cells, whereas the treatment of rice extracts (GBR and BR) of both cultivars were significantly down-regulated ($P<0.05$) IRS1 and PPARγ protein expression in H_2O_2-induced 3T3-L1 adipocytes compared to the differentiated cells and H_2O_2-induced 3T3-L1 adipocytes (Figure 81). In contrast, the protein of adiponectin was significantly decreased ($P<0.05$) in the H_2O_2-induced 3T3-L1 adipocytes compared to the differentiated cells. The treatment of GBR and BR extracts at concentrations 0.5 and 1 mg/ml of both cultivars, significantly up-regulated ($P<0.05$) in the H_2O_2-induced 3T3-L1 adipocytes in a dose-dependent manner compared to the differentiated cells and the H_2O_2-induced 3T3-L1 adipocytes (Figure 82). The WR extracts from both cultivars showed no significant increase in the protein level of adiponectin in the H_2O_2-induced 3T3-L1 adipocytes.



(A)_KDML105



(B)_PL2

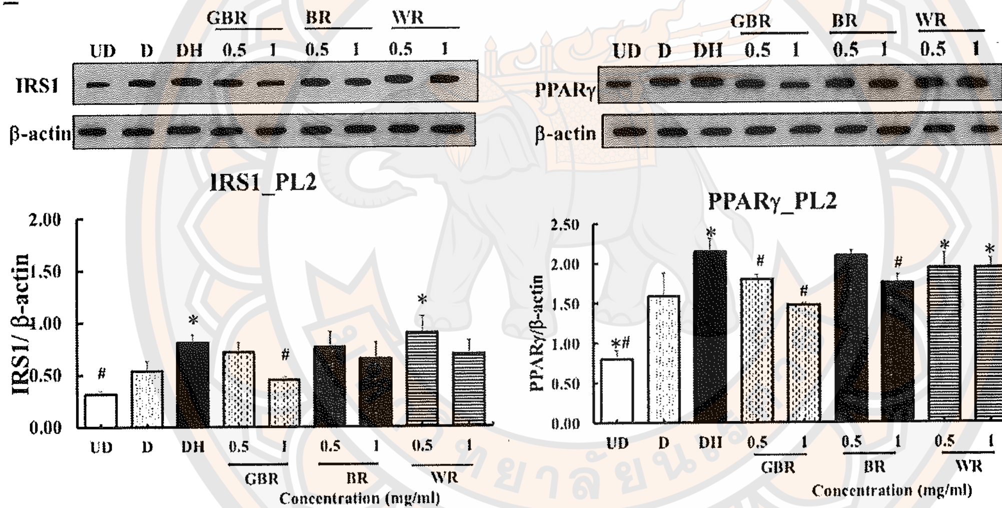


Figure 81 Effects of rice extracts from KDML105 (A) and PL2 (B) cultivars on the expression of IRS1 and PPARγ proteins in H_2O_2-induced 3T3-L1 adipocytes. Total protein was prepared, and the protein expression was analyzed by Western blotting. β-actin was analyzed as an internal control. * $P<0.05$, compared to differentiated cells and # $P<0.05$, compared to H_2O_2-induced 3T3-L1 adipocytes. Data are represented as mean ± S.D. of duplicates per treatment. (N=5)

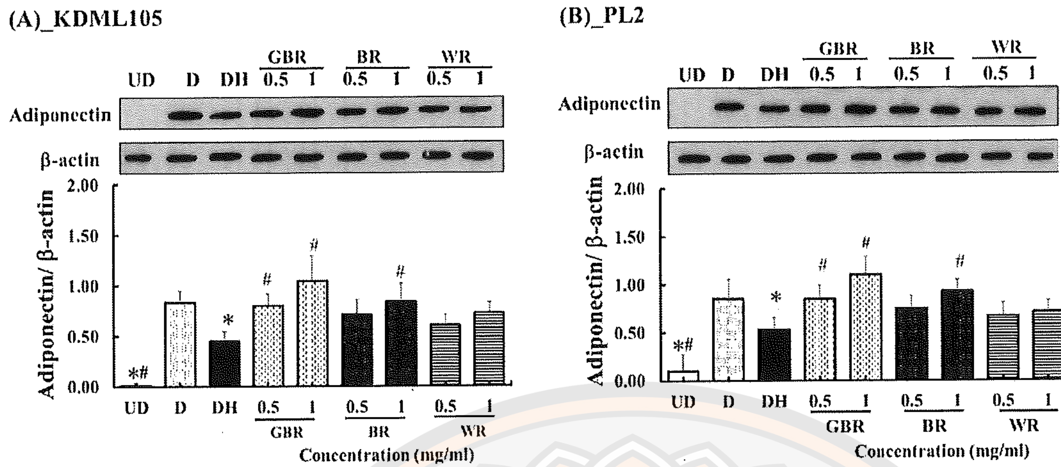
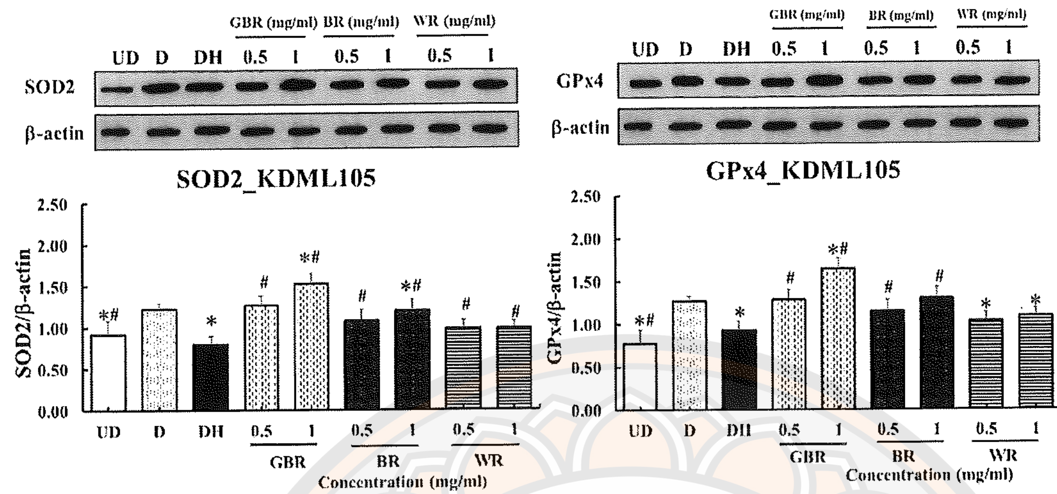


Figure 82 Effects of rice extracts from KDML105 (A) and PL2 (B) cultivars on the expression of adiponectin proteins in H_2O_2-induced 3T3-L1 adipocytes. Total protein was prepared, and the protein expression was analyzed by Western blotting. β-actin was analyzed as an internal control. * $P<0.05$, compared to differentiated cells and # $P<0.05$, compared to H_2O_2-induced 3T3-L1 adipocytes. Data are represented as mean ± S.D. of duplicates per treatment. (N=5)

Effect of rice extracts on protein expression of antioxidant in H_2O_2-induced 3T3-L1 adipocytes

We also found that the protein expression of SOD2 and GPx4 significantly decreased ($P<0.05$) in the H_2O_2-induced 3T3-L1 adipocytes compared to the differentiated cells. As expected, the treatment of the GBR and BR extracts (0.5 and 1 mg/ml) significantly up-regulated the expression of SOD2 and GPx4 protein in the H_2O_2-induced 3T3-L1 adipocytes in a dose-dependent manner compared to the differentiated cells and the H_2O_2-induced 3T3-L1 adipocytes (Figure 83). However, the GBR extracts of both cultivars showed a decrease in mRNA expression when compared with the BR and WR extracts. Likewise, KDML105 cultivars showed decreased mRNA levels in the insulin-mediated signaling pathway, when compared with the PL2 cultivars treatment of the H_2O_2-induced 3T3-L1 adipocytes. In addition, the treatment of WR showed that the protein expression of SOD2 and GPx4 did not increase when compared to H_2O_2-induced 3T3-L1 adipocytes.

(A)_KDML105



(B)_PL2

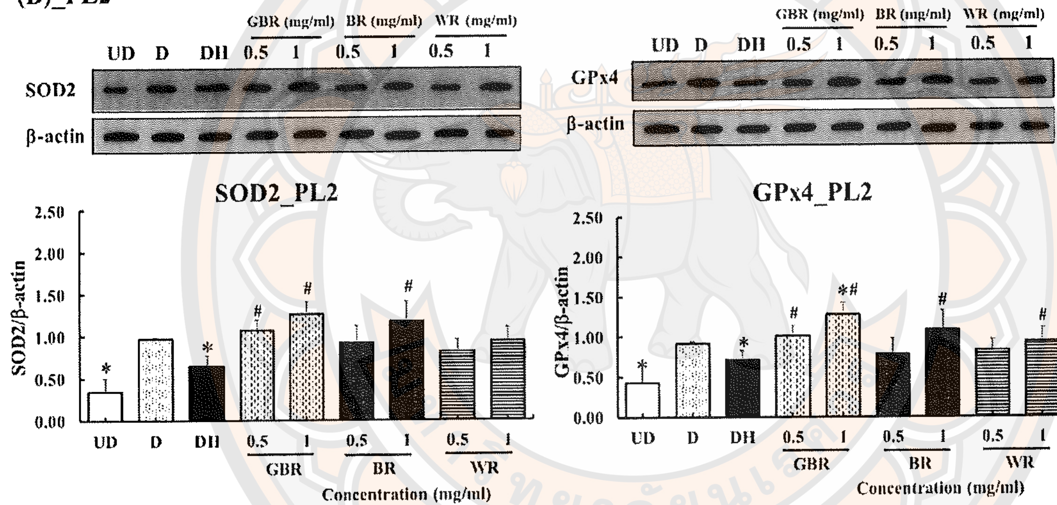


Figure 83 Effects of rice extracts from KDML105 (A) and PL2 (B) cultivars on protein expression in H_2O_2-induced 3T3-L1 adipocytes. Total protein was prepared and the protein expression of Adiponectin and IRS1 was analyzed by Western blotting. β-actin was analyzed as an internal control. * *P*<0.05, compared to differentiated cells and # *P*<0.05, compared to H_2O_2-induced 3T3-L1 adipocytes. Data are represented as mean ± S.D. of duplicates per treatment. (N=5)

CHAPTER V

DISCUSSION AND CONCLUSION

There were two experiments in this study: the first experiment was to measure antioxidant activity and the total phenolic content of the two Thai rice extracts cultivars, *O. sativa* L. Variety phitsanulok2 (PL2) and Kaw dokmali105 (KDML105). The second experiment was divided into two parts: the first part was to study the effect of the rice extracts on adipogenesis in 3T3-L1 adipocytes. The second part was to study the effect of the rice extracts on oxidative status in H_2O_2-induced 3T3-L1 adipocytes.

Experiment I

This study was to assess the antioxidant activity of the two Thai rice extracts from *O. sativa* L. Variety phitsanulok2 (PL2) and Kaw dokmali105 (KDML105) cultivars, which was determined by using ABTS and DPPH assay. In addition, total phenolic content was also measured by the Folin Ciocalteu assay. The hypothesis was that the Thai rice extracts exhibited high antioxidant activity and contained a high amount of total phenolic contents.

Experiment II

The first part of this study was studied in normal 3T3-L1 adipocytes. Firstly, the cytotoxicity effect of the rice extracts was determined by MTT assay. Then, the measurement of lipid contents was determined by using the Oil-Red O staining technique and TG kit assay. Furthermore, the expression of adipogenesis-related genes, including preadipocyte marker gene (*Pref-1*), adipogenic transcription factors (*PPARγ, C/EBPβ, C/EBPα, and C/EBPδ*), lipogenic (*SREBP-1c, FAS, ACC1, ACC2, aP2,* and *LPL*), lipolytic (*ATGL* and *HSL*), pro-inflammatory cytokines (*TNF-α* and *IL6*), *adiponectin*, and antioxidant (*SOD2*, *GPx4*, and *CAT*) genes by RT-PCR and qPCR techniques. The hypothesis in this part was that 1) intracellular lipid contents will decrease after the rice extract treatments; 2) GBR and BR extracts can down-regulate the expression of adipogenic and pro-inflammatory genes; 3) GBR and BR extracts up-regulate the expression of the preadipocyte marker gene, lipolytic, adiponectin, and antioxidant genes in normal 3T3-L1 adipocytes.

The second part of this work was studied in oxidative stress induced by H_2O_2 3T3-L1 adipocytes. The scope of this part was to investigate the effect of the rice extracts on ROS levels determined by DCFH-DA assay and lipid contents examined by Oil-Red O staining. The expression of the adipogenesis (*PPARγ* and *SREBP-1c*), insulin-mediated signaling (*IRS1*, *AKT2,* and *PIK3*), inflammation (*TNF-α and IL6*), *adiponectin,* and antioxidant systems (*Nrf2*, *SOD2*, *HO-1*, *CAT*, and *GPx4*) and protein expression were measured by qPCR and western blotting, respectively.

In this study, the two Thai rice cultivars: *O. sativa* L. Variety phitsanulok2 (PL2) and Kaw dokmali105 (KDML105) were selected. The PL2 cultivar is cultivated in Phitsanulok province. Its physical characteristics are long slender grain, relatively hard, and have no fragrance. Whereas the KDML105 cultivar is developed into Rice berry. Its physical characteristics are like a tapered seed, soft seeds, fragrant. The GBR, BR, and WR of both cultivars were extracted twice by 70% methanol. It was expected that the methanol extraction of GBR and BR will exhibit high antioxidant activity and total phenolic content when compared to WR. In addition, both GBR and BR extracts, which contain a higher number of bioactive compounds such as GABA, vitamin E, and γ-oryzanol than WR may play a role to affect adipogenesis and other related metabolic pathways such as insulin signaling, adipocytokine production, and oxidative status in normal and oxidative stress induced by H_2O_2 3T3-L1 adipocytes.

From the results, GBR and BR showed greater antioxidant activity than WR. This was agreed with a previous study (Mohd Esa et al., 2013), which showed that GBR extract had lower IC50 than BR and WR extracts. Moreover, Azmi and colleagues (2013) showed that the ethyl acetate GBR extract had higher antioxidant activity than BR.

For total phenolic content measurement by Folin-Ciocalteu assay, both GBR and BR showed higher total phenolic content than WR. This was consistent with a previous study, which was found that GBR had higher total phenolic content than BR extracts (Mohd Esa et al., 2013). These results suggest that the germination process and type of rice may affect the antioxidant activity and total phenolic contents in GBR, BR, and WR extracts.

In this study, the GABA and γ-oryzanol contents in GBR and BR extracted by 70% methanol from both PL2 and KDML105 cultivars were analyzed by the Central Laboratory (Thailand) Co., Ltd. using High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) techniques, respectively. The result showed that GABA content was higher in GBR than BR from both cultivars. It was agreed with a previous study, which found that the GBR extract extracted with methanol contained higher GABA and γ-oryzanol contents, in comparison to BR and WR (Imam et al., 2013). It has been shown that the germination process can increase the levels of oryzanol and GABA, which represented their antioxidant activity (Imam et al., 2014; Riant et al., 2009; Ravichanthiran et al., 2018). In addition, it was shown that the rice extracts with high antioxidant activity and total phenolic content can reduce lipid contents and adipogenesis (Ho et al., 2012; Bulanawichit et al., 2018). Therefore, the present study is interested to study the effect of the two Thai rice extracts from PL2 and KDML105 cultivars on adipogenesis and oxidative status in normal and oxidative stress induced by H_2O_2 in 3T3-L1 adipocytes.

Our results revealed that GBR and BR extracts can decrease intracellular lipid contents in 3T3-L1 adipocytes. Inconsistent with a previous study, GBR and BR of PL2 and KDML105 cultivars at a concentration of 1 mg/ml exhibited anti-adipogenesis via suppressing adipocyte cell growth and decreasing intracellular lipid contents in 3T3-L1 adipocytes (Kirdin and Boonsong, 2016). In addition, a study by Lim and colleagues (2014) demonstrated that the methanolic GBR extract reduced lipid accumulation in 3T3-L1 adipocytes compared to control.

To prove that GABA, which is one of the important compounds contained in GBR and BR extracts may play a role in anti-adipogenesis, GABA was then used as a positive control. The result showed that GABA can decrease intracellular lipid contents in 3T3-L1 adipocytes. It was reported that GABA can be converted into succinate by GABA transaminase (GABA-T) to activate GABAB receptors, which may exert direct roles on adipocytes (Hwang et al., 2019). The conversion of GABA might affect C/EBPα and PPARγ activity and could reduce intracellular lipid levels, which then may control proliferation, migration, differentiation, and reduction of lipid accumulation in adipocytes. This result suggests that GABA, which is contained in GBR and BR extracts

may play a role in the anti-adipogenic effect. From these results, the underlying mechanistic effects of GBR, BR, and WR were then further investigated.

In the present study, important genes and proteins which are involved in adipogenesis were examined. The first gene is a preadipocyte marker gene, *Pref-1*. Pref-1 is a novel member of the epidermal growth factor (EGF)-like family of proteins (Hudak and Sul, 2013) and it is used as a marker for preadipocyte. Its function is thought to be a negative regulator of adipocyte differentiation through MEK-ERK and SOX9 signaling pathways, leading to inhibition of adipogenesis (Wang et al., 2010). Our results showed that the basal expression of *Pref-1* was abundant in preadipocyte state and down-regulated at day 7 after adipocyte differentiation. However, when cells were treated with the GBR extract of KDML105 cultivar from the starting of differentiation, *Pref-1* mRNA expression was stably expressed throughout the differentiation. The results suggest that restoration of *Pref-1* mRNA expression by the GBR extract may affect adipocyte differentiation.

In addition, adipocyte marker genes were also investigated. The results showed that mRNA levels of adipogenic transcription factors *(C/EBPα, C/EBPβ, C/EBPδ,* and *PPARγ)* were significantly down-regulated in a dose-dependent manner in adipocytes treated with both GBR and BR. These adipogenic transcription factors can regulate each other (Evan et al., 2011) and control the expressions of lipogenic genes such as *SREBP-1c, FAS, ACC, aP2,* and *LPL* (Wu et al., 1996; Lee et al., 2007) which have been implicated in the differentiation of preadipocytes to mature adipocytes. As expected, mRNA expression of lipogenic genes, including *SREBP-1c*, *FAS*, *ACC1*, *ACC2*, *aP2*, and *LPL* were suppressed in a dose-dependent manner ($P<0.05$) in adipocytes treated with BR and GBR extracts.

Moreover, the present study also showed that BR and GBR to a greater extent GBR have significant effects in inhibiting mRNA expression of lipolytic genes (*ATGL* and *HSL*), which can cause a decrease in lipid contents in 3T3-L1 adipocytes. Ho et al., 2012 found that lipolytic genes were found to be up-regulated in the mice that were administrated with GBR. They hypothesized that the bioactive compounds including vitamin E, oryzanol, and GABA may improve lipid metabolism in obese mice.

From the results, the present study showed that GBR and BR extracts showed the inhibition of lipid contents in normal 3T3-L1 adipocytes. Both GBR and BR extracts were able to decrease the adipogenic and lipogenic genes in mature adipocytes, while GBR and BR extracts increased mRNA expression of lipolytic genes. It also suggests that because of bioactive compounds such as GABA and γ-oryzanol containing a high amount of the GBR and BR extracts may play a role in anti-adipogenic effects.

In addition to storage fat, adipose tissue can produce several adipocytokines including leptin, visfatin, resistin, adiponectin, and pro-inflammatory cytokines e.g., TNF-α and IL6 (Silva et al., 2011). It has been reported that TNF-α and IL6 have inhibitory effects on insulin signaling pathways, which contribute to insulin resistance (Paul and Wood, 2004). In this study, the mRNA expression of both *TNF-α* and *IL6* was decreased while adiponectin was increased in 3T3-L1 adipocytes treated with the GBR and BR extracts of both cultivars. Previous studies showed that TNF-a can activate IL6 production (Chiellini et al., 2002) and inhibit the synthesis of anti-inflammatory cytokines such as adiponectin and visfatin (Fernández-Sánchez et al., 2011). Therefore, decreasing pro-inflammatory and/or increasing anti-inflammatory cytokines may improve insulin sensitivity. A previous study also demonstrated that type 2 diabetic rats, which were administrated with the GBR and BR extracts increased adiponectin levels and reduced blood sugar levels (Wang et al., 2010). This could be a potential effect for the improvement of insulin sensitivity of the GBR and BR. Nevertheless, the effects of these rice extracts on insulin sensitivity are to be further evaluated.

It has been known that obesity-associated fat accumulation can lead to oxidative stress in adipocytes. This study was then interested to assess the effect of the rice extracts on oxidative status in 3T3-L1 adipocytes. In the present study, BR and GBR extracts can up-regulate mRNA levels of antioxidant enzymes, including *SOD2*, *GPx4*, and *CAT* in 3T3-L1 cells. A recent study demonstrated that GBR and BR extracts of Malaysian local rice can increase the activity of antioxidant enzymes (SOD and GPx) in rabbits (Mohd Esa et al., 2013). The active compounds found in the rice extracts may be involved in reducing oxidative stress in adipocytes (Patil and Khan, 2011; Kaukovirta et al., 2004).

The results from the first part of this study were summarized in a diagram shown below (Figure 84). These results showed the anti-adipogenic effect of GBR and BR extracts by reducing lipid contents and adipogenesis via inhibiting adipogenic-related genes (*C/EBPα*, *C/EBPβ*, *C/EBPδ* and *PPARγ*, *SREBP-1c*, *FAS*, *ACC1*, *ACC2*, *aP2*, and *LPL*) and inducing preadipocyte gene (*Pref-1*) and lipolytic genes (*ATGL* and *HSL*). In addition, the GBR and BR extracts also increase antioxidant genes (*SOD2*, *GPx4*, and *CAT*), which may lead to reduce intracellular oxidative stress. Moreover, GBR and BR also affect adipocytokines production by reducing pro-inflammatory cytokines (*TNF-α* and *IL6*) and enhancing *adiponectin* gene expression. The results suggest that GBR and BR may have beneficial effects for the prevention or treatment of obesity.

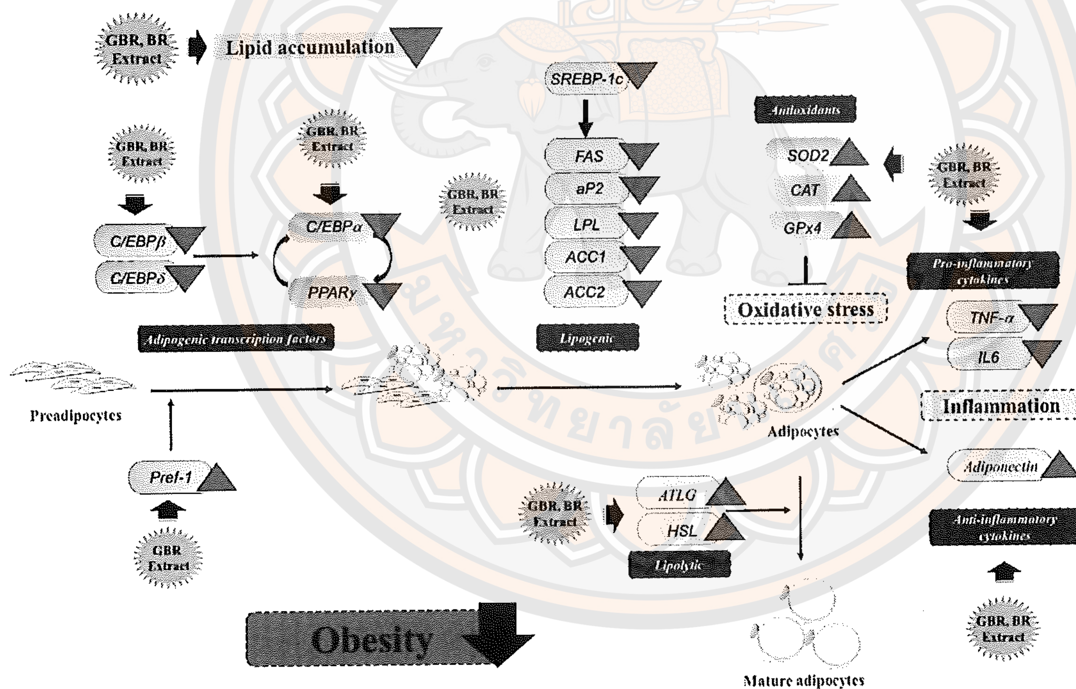


Figure 84 Schematic diagram showing proposed mechanistic effects of brown rice (BR) and germinated brown rice (GBR) extracts in 3T3-L1 adipocytes

Excessive lipid accumulation in obesity can induce cellular oxidative stress and ROS accumulation, leading to affect many metabolic pathways such as insulin-signaling (Keane et al., 2015), adipogenesis (Furukawa et al., 2004), antioxidant system (Masschelin et al., 2020), and pro-and anti-inflammatory cytokine levels (Furukawa et al., 2004; Marseglia et al., 2015). In this study, we treated mature 3T3-L1adipocytes with H_2O_2 to obtain the oxidative stress model resembling the obese conditions as described previously by Kim and Han, 2011, e.g., having high lipid and ROS levels. By using this cell model, we investigated the effects of rice extracts on ROS levels and lipid contents by DCFH-DA assay and Oil-Red O staining, respectively. In addition, the expression of genes and proteins involved in adipogenesis, insulin signaling pathway, pro-inflammatory and anti-inflammatory cytokine production, and antioxidant system were also determined by qPCR and western blot techniques, respectively.

To create an oxidative stress cell model, mature 3T3-L1 adipocytes were treated with 100 μM of H_2O_2 for 48 hours, which significantly increased the intracellular ROS levels, compared to normal undifferentiated and differentiated adipocytes. H_2O_2 is associated with increased oxidative stress and lipid accumulation in 3T3-L1 adipocyte, resulting in lipid peroxidation and cell membrane damage (Curtis et al., 2012; Hauck et al., 2018). In addition, Lee and colleagues (2009) demonstrated that H_2O_2 treatment can induce the G2/M phase leading to increase adipocyte differentiation, in a dose-dependent manner. Additionally, increasing H_2O_2 and decreasing the antioxidant system will increase lipid storage and adipocyte secretion, leading to an imbalance of energy homeostasis, insulin resistance, and type 2 diabetes (Masschelin et al., 2020). The present study demonstrated that H_2O_2-induced 3T3-L1 adipocytes treated with 0.5 and 1 mg/ml of GBR and BR extracts from KDML105 and PL2 cultivars reduced intracellular ROS levels and lipid contents in a dose-dependent manner. This result corresponds with a previous study, which reported that GBR extracts can reduce lipid contents in 3T3-L1 adipocytes (Ho et al., 2012). In addition, it was found that high-fat diet-induced obese rats fed by fermented brown rice extract can reduce body weight gain, improve lipid profiles, and white adipose tissue mass (Lim et al., 2016).

From the results of changes in gene expression, it was shown that the mRNA levels of antioxidants *Nrf2*, *HO-1*, *SOD2*, *GPX4*, and *CAT* were significantly down-regulated the mRNA levels in H_2O_2-induced 3T3-L1 adipocytes. However, after treating

with the GBR and BR extracts, the mRNA level of *Nrf2*, which is upstream of antioxidant enzymes such as *HO-1, SOD2, GPX4*, and *CAT* (Furukawa et al., 2004; Wang and Hai, 2016; Wang et al., 2012) was up-regulated., *HO-1, SOD2, GPX4*, and *CAT.* In addition, it was found that the GBR and BR extracts can induce SOD2 and GPX4 protein expression in a dose-dependent manner in H_2O_2-induced 3T3-L1 adipocytes. This result corresponds with previous studies that H_2O_2 was associated with the Nrf2-ARE antioxidant pathway (Schneider and Chan, 2013). Activation of Nrf-2 by the rice extracts will up-regulate the expression of downstream phase II detoxification enzyme, e.g., HO-1, SOD, CAT, and GPx, which may protect the cells from oxidative stress, inflammation, and insulin resistance (Jie et al., 2016; Buendia et al., 2016). A previous study also found that the rice extracts could considerably increase antioxidant enzymes such as SOD, GPx, and Vitamin E in hyperlipidaemic rabbits, which are the major mediators for reducing local levels of ROS (Esa et al., 2013).

Insulin plays an important role in regulating adipocyte differentiation, especially during terminal differentiation, which lipid synthesis is increased to accumulate intracellular lipid contents (Cignarelli et al., 2019). In adipocytes, insulin will bind to insulin receptor and promotes the phosphorylation of IRS1 by inducing the activation of the PI3K/Akt pathway, which is primarily responsible for the uptake of glucose and the suppression of gluconeogenesis (Bae et al., 2014) and GLUT4, that leads to glucose transport into adipocytes for adipocyte growth (Yu et al., 2020). This study showed that the expression of insulin-mediated genes, including *IRS1*, *PI3K*, *Akt2*, and *GLUT4* was up-regulated in H_2O_2-induced 3T3-L1 adipocytes, while treatment of GBR and BR extracts can significantly down-regulate these genes. Thus, the present study showed that GBR and BR extracts interfered with insulin action in H_2O_2 induced 3T3-L1 adipocytes, which then reduce cellular glucose uptake and inhibit the adipocyte differentiation process (Kim et al., 2008). The reduction of lipid contents and adipogenesis in obesity-linked oxidative stress adipocytes may help to improve insulin sensitivity in obese (Le Lay et al., 2014).

Oxidative stress can induce adipocyte differentiation processes (Taniguchi et al., 2006; Houstis et al., 2006; Choi et al., 2016). PPARγ and SREBP-1c are one of the adipogenic transcription factors that are associated with adipocyte differentiation and lipogenesis (Farmer, 2005; Rosen et al., 2002). In this study, *PPARγ* and *SREBP-1c*

mRNA expressions were up-regulated in H_2O_2-induced 3T3-L1 adipocytes, while the GBR and BR treatments were shown conflicting results. Previous studies have consistently shown that the GBR extract down-regulated *PPARγ* and *C/EBPα* expression in HFD-fed mice (Kim et al., 2020; Kim et al., 2008). Thus, this study demonstrated that the GBR and BR extracts of both cultivars could be used as a supplement to inhibit cell proliferation, adipocyte differentiation, and lipid accumulation in H_2O_2-induced 3T3-L1 adipocytes.

High oxidative stress-induced adipocytes can promote inflammation by producing pro-inflammatory cytokines (TNF-α, and IL-6), which cause insulin resistance (Dandona et al., 2004; Hotamisligil, 2017). In addition, oxidative stress can induce IKKβ activation leading to NF-κB translocation and increasing the expression of potential mediators of inflammation that can cause insulin resistance (Shoelson et al., 2006). This study demonstrated that the GBR and BR extract reduced mRNA expression of *TNF-α* and *IL6* in H_2O_2-induced 3T3-L1 adipocytes. Moreover, it was also shown that the treatment of GBR and BR extracts increased the expression of *adiponectin*, which is an anti-inflammatory cytokine and insulin-sensitizing molecule (Matsuura et al., 2007). As previously described, γ-oryzanol was presented in the GBR extract. It can directly influence lipid accumulation and triglyceride synthesis via inhibiting adipogenic-related genes e.g. *PPARγ* and *C/EBPs* (Ho et al., 2013). Minitel and colleagues (2016) also demonstrated that γ-oryzanol can activate adiponectin production in adipocytes, which will modulate the hepatic cells by activating peroxisome proliferator-activated receptors (PPAR-α) via AdipoR2 and thereby stimulating fatty-acid oxidation and decreasing triglyceride contents in liver tissue. In addition, γ-oryzanol can induce insulin production in pancreatic cells, which will then directly activate AMPK via AdipoR1 in hepatic cells. After AMPK activation, it will inhibit phosphoenolpyruvate carboxykinase (PEPCK) and Glucose 6-phosphatase (G6Pase), resulting in reducing gluconeogenesis in hepatic cells. Activated AMPK also induces β-isoform of coenzyme A carboxylase (ACC-β) phosphorylation, which inhibits acetyl coenzyme A carboxylase (ACC) and results in increased fatty acid oxidation in liver tissue. It suggests that the ability to activate adiponectin production in adipocytes, together with the high amount of γ-oryzanol content in the GBR and BR extracts can show a potential effect for the improvement of insulin sensitivity.

Following the rice extract treatments in H_2O_2-induced oxidative stress 3T3-L1 adipocytes model, the results were summarized in the diagram shown below (Figure 85). In H_2O_2-induced 3T3-L1 adipocytes, the GBR and BR extracts clearly reduced intracellular ROS levels and lipid contents. These inhibitory effects were associated with the down-regulation of genes and proteins involved in adipogenesis (*PPARγ* and *SREBP-1c*), insulin-mediated signaling pathway (*IRS1*, *PI3K*, *Akt2*, and *GLUT4*), and inflammation (*TNFα* and *IL-6*) as well as an anti-inflammatory cytokine (*adiponectin*), antioxidant system (*Nrf2, HO-1, SOD2, CAT,* and *GPx4*). These results revealed that the GBR and BR extracts exhibited anti-adipogenesis and antioxidative capacity. Particularly, it suggests that the GBR extract, which is obtained from the germination process, seems to produce a high amount of bioactive compounds e.g., GABA and γ-oryzanol, which are important for regulating adipogenesis and oxidative status in adipocytes. Thus, this study reveals the possibility of using the GBR and BR extracts as a supplement for the prevention or treatment of obesity or obesity-linked disease such as type 2 diabetes.

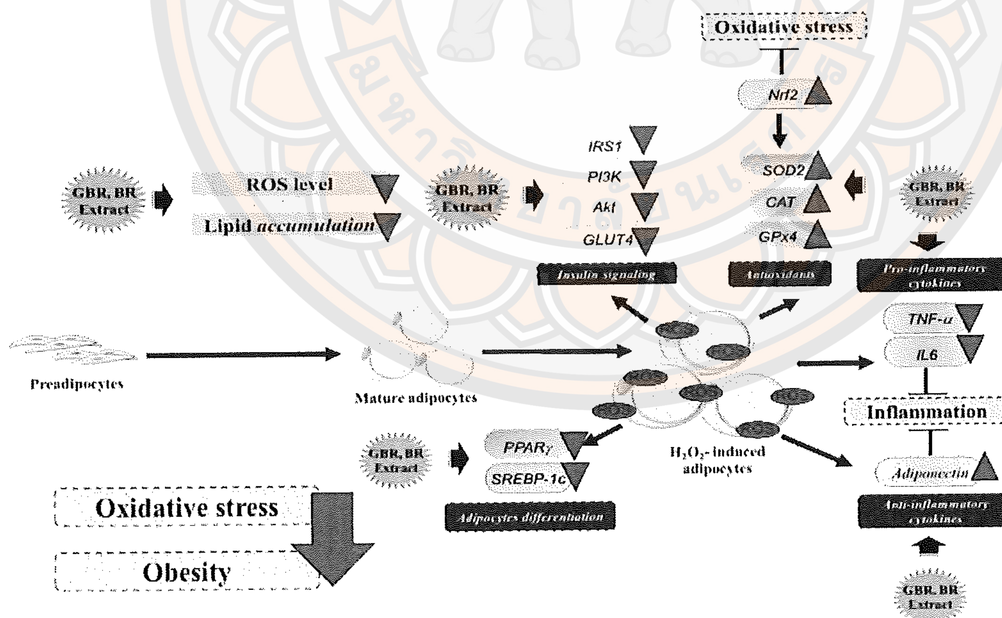


Figure 85 Schematic diagram showing proposed targets of germinated brown rice (GBR), brown rice (BR), and white rice (WR) extracts in H_2O_2-induced 3T3-L1 adipocytes

Conclusion

In this study, the two Thai rice extracts from *O. sativa* L. Variety phitsanulok2 (PL2) and Kaw dokmali105 (KDML105) cultivars display antioxidant activity and total phenolic content in GBR and BR greater than WR extracts. The GBR and BR extracts can suppress adipogenesis via maintaining the expression of the preadipocyte gene *Pref1*. In addition, they can inhibit the expression of adipogenic genes (*C/EBPα*, *C/EBPβ*, *C/EBPδ* and *PPARγ*, *SREBP-1c*, *FAS*, *ACC1*, *ACC2*, *aP2*, and *LPL*) and pro-inflammatory cytokine genes (*TNF-α* and *IL6*) as well as activating the expression of lipolytic (*ATGL* and *HSL*), *adiponectin*, and antioxidant (*SOD2*, *GPx4*, and *CAT*) genes in 3T3-L1 adipocytes. In addition, this study provides evidence that the GBR and BR extracts have the potential greater than WR extract to reduce adipogenesis and ROS levels in oxidative stress-induced 3T3-L1 adipocytes by H_2O_2. The underlying mechanistic effects occur via the decreasing of the expression of genes and proteins involved in adipogenesis (*PPARγ* and *SREBP-1c*), insulin signaling (*IRS1*, *AKT2*, and *PIK3*), and inflammation (*TNF-α* and *IL6*), as well as the up-regulation of the expression of anti-inflammatory cytokines (*adiponectin*), and antioxidant genes, and proteins. The health beneficial effects of the GBR and BR extracts from KDML105 and PL2 cultivars, seem to be at least attributed to the synergistic effects of bioactive compounds such as GABA, γ-oryzanol. Therefore, both GBR and BR extracts may provide beneficial health effects for the prevention and/or treatment of obesity and obesity-linked oxidative stress diseases (Figure 86).

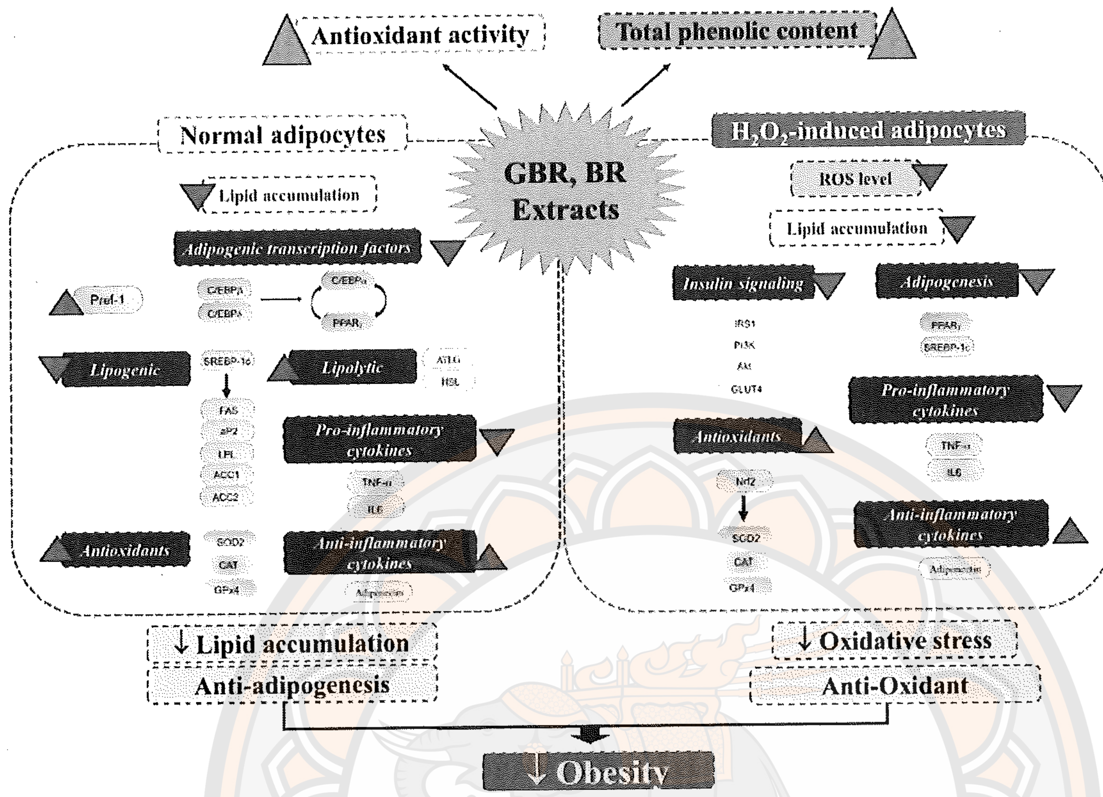


Figure 86 Schematic diagram showing proposed targets of rice extracts on adipogenesis and anti-oxidative status in 3T3-L1 adipocytes

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APPENDIX

Cell culture

Basal medium preparation

Dulbecco's Modification of Eagle's Medium (DMEM)	100	ml
Fetal bovine serum	10	ml
Antimycotic-antibiotic	1	ml

Freezing media

Basal medium	0.95	ml
5% DMSO	0.05	ml

5 mg/ml MTT

MTT	5	mg
Distilled water	1	ml

Mix and filtered with 0.22 μm of filter paper, storage at -20°C

Lipid content (Oil-Red O staining)

Oil-Red O stock solution (100 ml)

Oil-Red O powder	0.35	g
100% Isopropanol	100	ml

Stir overnight

Filtered with 0.22 μm of filter paper

Oil-Red O working solution (50 ml) (ratio 6:4)

Oil-Red O stock solution	30	ml
Distilled water	20	ml

Filtered with 0.22 μm of filter paper

10% Formalin (100 ml)

37% Formaldehyde	27	ml
PBS	10	ml
Distilled water	63	ml

Gene expression by RT-PCR

2% Agarose gel

Agarose	0.3	g
1X TBE buffer	15	ml

10X TBE buffer (500 ml)

Tris base	54	g
Boric acid	27.5	g
0.5M EDTA (pH 8.0)		
3.725 g		
Distilled water	500	ml

1X TBE buffer (500 ml)

10X TBE buffer	50	ml
Distilled water	450	ml

Protein expression by western blotting

1.5 M Tris-HCl pH 8.8 (75 ml)

Tris base	27.23	g
SDS	2.5	g
Distilled water	75	ml
Adjust pH to 8.8		

0.5 M Tris-HCl pH 6.8 (75 ml)

Tris base	4.55	g
SDS	2.5	g
Distilled water	75	ml
Adjust pH to 6.8		

10% SDS

SDS	10	g
Distilled water	100	ml

10% APS

Ammonium persulfate	0.1	g
Distilled water	1	ml

10X SDS-PAGE running buffer of 10X Transfer buffer (500 ml)

Tris base	15.14	g
Glycine	72.06	g
Distilled water	500	ml

1X SDS-PAGE running buffer (1000 ml)

10X SDS-PAGE running buffer	100	ml
10%SDS	10	ml
Distilled water	890	ml

1X Transfer buffer (1000 ml)

10X Transfer buffer	100	ml
10%SDS	10	ml
Methanol	200	ml
Distilled water	690	ml

Coomassie brilliant blue (R) staining (500ml)

Coomassie brilliant blue (R-250)	0.5	g
Methanol	175	ml
Acetic acid	50	ml
Distill water	275	ml

Destaing (1000 ml)

Methanol	350	ml
Acetic acid	100	ml
Distill water	550	ml

Ponceaus (5% W/V) (50 ml)

Ponceaus	2.5	g
Acetic acid	50	ml

10X TBS (1000 ml)

Tris base	24.33	g
NaCl	80.06	g
Distilled water	1000	ml

1X TBST (500 ml)

10X TBS	50	ml
Tween 20	5	ml
Distilled water	445	ml

5% Non-fat skim milk (Blocking buffer) (50 ml)

Non-fat skim milk	2.5	g
1XTBST	50	ml

3% Non-fat skim milk (0 ml)

Non-fat skim milk	0.3	g
1XTBST	10	ml

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