IN VITRO AND *IN VIVO* STUDIES OF PAPAYA ON ADIPOGENESIS IN 3T3-L1 ADIPOCYTES AND HEPATIC STEATOSIS IN OBESE RATS

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A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfilment of the Requirements
for the Master of Science Degree in Physiology
June 2019

Thesis entitled "*In vitro* and *in vivo* studies of papaya on adipogenesis in 3T3-L1 adipocytes and hepatic steatosis in obese rats"

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has been approved by the Graduate School as partial fulfillment of the requirements

for the Master of Science Degree in Physiology of Naresuan University

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ACKNOWLEDGEMENTS

First of all, I would like to acknowledge my major advisor, Assistant Professor Dr. Sakara Tunsophon, for her best advice, support, caring, and providing me with necessary materials and equipment while I was conducting my research.

I would like to extend special thanks to my co-advisor and special teacher, Assistant Professor Dr. Tantip Boonsong and Assistant Professor Julintorn Samran, MD for helpful suggestions and technical advice for my experiments.

I would like to thank Associate Professor Dr. Sirichai Adisakwattana of the external committee and Dr. Ittipon Phoungpetchara of the internal committee.

Moreover, I would like to thank Mr. Peter Barton and Mr. Kevin Roebl of the Division of International Affairs and Language Development (DIALD) for this thesis edition

This thesis is sponsored and supported by National Research Council of Thailand. Financial support from the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Ministry of Higher Education, Science, Research and Innovation is gratefully acknowledged.

Finally, I am very grateful to my friends, my laboratory team and my family for their love and support through the good and bad times.

Wanwisa Deenin

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Academic Paper	Thesis M.Sc. in Physiology, Naresuan University, 2018
Keywords	Lipogenic gene expression, Non-alcoholic fatty liver disease, Obesity, Oxidative stress, Papaya

ABSTRACT

Papaya is a source of natural dietary antioxidants, especially β-carotene, which accumulates in the liver, where it exerts biological effects. The aim of the present study is to determine the effect of papaya on biomarkers and gene expression related to lipid metabolism in obese rats. Adult male Sprague–Dawley rats were randomly grouped (n = 6 rats/group) in four experimental groups: C (normal diet), HFD (high fat diet), HFL (high fat diet with 0.5 ml papaya juice/100g BW) and HFH (high fat diet with 1 ml papaya juice /100g BW). After 12 weeks, the rats were euthanized, and plasma, faeces and liver were sampled and analysed for the biomarkers related to lipid metabolism, inflammation and oxidative stress. The results indicate that the high fat diet induced steatosis in the HFL and HFH groups, which was confirmed by the levels of alanine aminotransferase, aspartate aminotransferase and histological examination. Moreover, papaya decreased adipogenesis in 3T3-L1 adipocytes by reducing ROS production induced by H_2O_2. Papaya decreased the lipid accumulation in 3T3-L1 adipocytes indicated by reducing the staining with Oil red O and decreasing triglyceride levels. Papaya treatment suppressed the expression of pro-inflammatory cytokines genes such as tumour necrosis factor (TNF-α) and interleukin-6(IL-6) in 3T3-L1 adipocytes. These results suggest that papaya improves antioxidant gene expression such superoxide dismutase (SOD and catalase (CAT) both in vitro and in vivo. From the obtained results, it can be suggested that the mechanism of action of hepatoprotective effect of the papaya against the accumulation of hepatic fat was a result of the association of the anti-

lipogenic, anti-inflammatory and antioxidant activities revealed by the papaya. Therefore, the use of the papaya fruit may be a promising alternative dietary remedy to prevent or reverse the accumulation of fat in the liver, as found in NAFLD. However, future research should be performed using human trials to elucidate the intervention of papaya in clinical and public health implications.

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ABBREVIATIONS

ACC	=	acetyl-CoA carboxylase
BW	=	body weight
CAT	=	catalase
FAS	=	fatty acid synthase
FFA	=	free fatty acids
H&E	=	hematoxylin and eosin
HFD	=	high fat diet
MDA	=	malondialdehyde
NAFLD	=	non-alcoholic fatty liver disease
PBS	=	phosphate buffered saline
PCR	=	polymerase chain reaction
RNA	=	ribonucleic acid
RT-PCR	=	reverse transcriptase-PCR
SE	=	standard error
SOD	=	superoxide dismutase
SREBP-1c,	=	Sterol regulatory element-binding protein-1c
DMSO	=	dimetyl sulfoxide
TC	=	total cholesterol
TG	=	triglycerides

CHAPTER I

INTRODUCTION

Rationale of the study

Obesity is a condition caused by an imbalance of energy derived from food and energy used in everyday activities. Energy is extracted from the food. If too much food is consumed, too much energy is available to the body. Some of the available energy is used by the body, while the rest is stored in the form of fat.

Obesity has been considered to be a chronic disease that requires medical prevention and treatment (Hao et al., 2017). Obesity is a medical condition in which excess body fat has accumulated to the extent that it may have a negative effect on health. People are generally considered obese when their body mass index (BMI), a measurement obtained by dividing a person's weight by the square of the person's height, is over 30 kg/m^2, with the range 25–30 kg/m^2 defined as overweight. Obesity is a risk factor for hypertension, cardiovascular disease, type-2 diabetes, and Non-alcoholic fatty liver disease.

Non-alcoholic fatty liver disease (NAFLD) is a major public health issue due to its high prevalence worldwide. By 2014 the prevalence had increased to 58% in overweight people and was as high as 98% in non-diabetic obese people (Kleiner et al., 2014). The liver plays a major role in lipid metabolism, importing free fatty acids (FFAs) and manufacturing, storing, and exporting lipids. Irregularities in any of these processes can lead to the development of NAFLD. FAs are involved in many important cellular events, such as the synthesis of cellular membranes, energy storage, and intracellular signalling pathways. Additionally obesity increases tumour necrosis factor α (TNF-α) production in adipocytes, and increases the rate of lipolysis. Thus, the circulating pool of FFAs is increased in obese individuals and accounts for the majority of liver lipids in NAFLD.

Carica papaya, commonly known as papaya, is in the family of Caricaceae. Various parts of the papaya plant (e.g. fruit, leaves, bark, roots, flowers, seeds, and latex) have been used as extracts in medical treatment (Aravind G. et al., 2013). *C. papaya* is a nutraceutical plant with many medicinal properties. The fruits of *C. papaya* contain many nutrients such as fiber, vitamins, and minerals (Sadek KM, 2012).

However, obesity contributed to the increase of free radicals that lead to oxidative stress. In the body there is a balance between antioxidants and free radicals. The antioxidants in the cell consist of enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Non-enzymatic antioxidants contain glutathione (GSH), vitamin E and vitamin C (Rahman et al., 2017).

When energy intake exceeds energy expenditure, excessive cellular lipid accumulation occurs not only in adipose tissue but also in ectopic tissues such as liver (Boren, Taskinen, Olofsson, & Levin, 2013). Excessive ectopic lipid deposition often disrupts normal cellular and physiological function, which if allowed to proceed unchecked, will lead to pathological progression (Perry, Samuel, Petersen, & Shulman, 2014) . With the progress of obesity, increased hepatic lipogenesis and serum fatty acids lead to the excess accumulation of liver lipids which result in fatty liver, impaired liver function, and eventually liver failure(Donnelly et al., 2005).

Therefore, papaya may be used to prevent the development and progression of hepatic steatosis. However, the mechanism underlying the beneficial effect of papaya in liver has not been investigated. The purpose of this study was to examine whether papaya can decrease oxidative stress and improve hepatic steatosis in high fat diet rats and its possible mechanisms including both in vivo and in vitro study. Therefore, this study was divided into two parts; 1. *in vitro* study and 2. *in vivo* study.

Main objectives

This study aims to evaluate the effect of papaya on adipogenesis in 3T3-L1 adipocytes and hepatic steatosis in obese rats.

Specific objectives

1. To investigate the effects of papaya on the mechanism of lipid metabolism regulated hepatic steatosis in high fat diet rats.
2. To determine the effect of papaya on morphological changes of the liver in high fat diet rats.
3. To demonstrate the antioxidant activity of papaya in 3T3-L1 adipocytes.
4. To study the toxicity of papaya in 3T3-L1 adipocytes.

The scope of the study

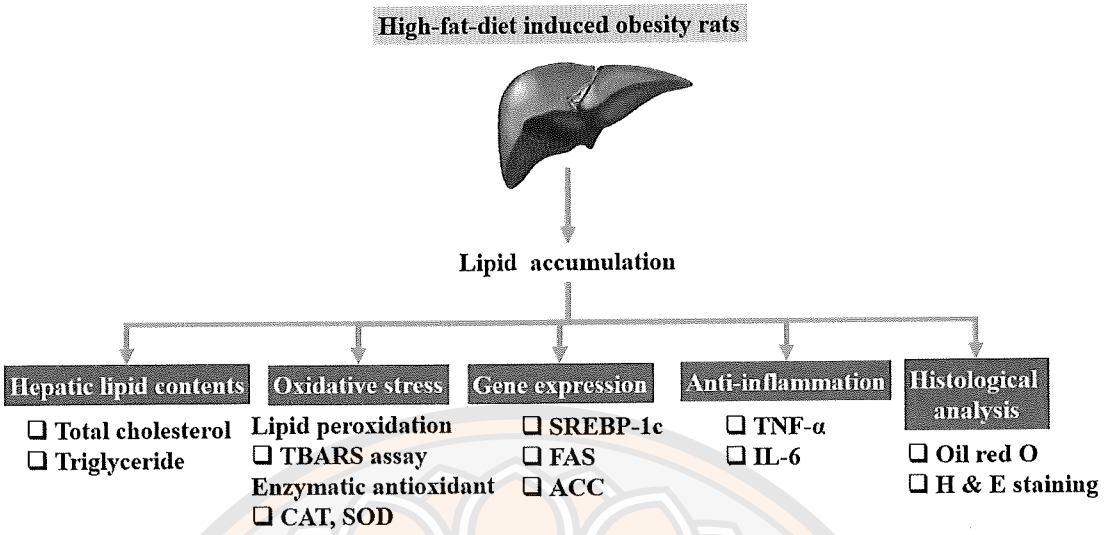


Figure 1 Scope of the study in high fat diet model

From the figure 1 is the *in vivo* study. The Sprague Dawley rats were induced with high fat diet. The liver tissue were determined lipid accumulation by detected hepatic lipid contents, oxidative stress, lipogenic gene expression, pro-inflammatory cytokine and histological analysis.

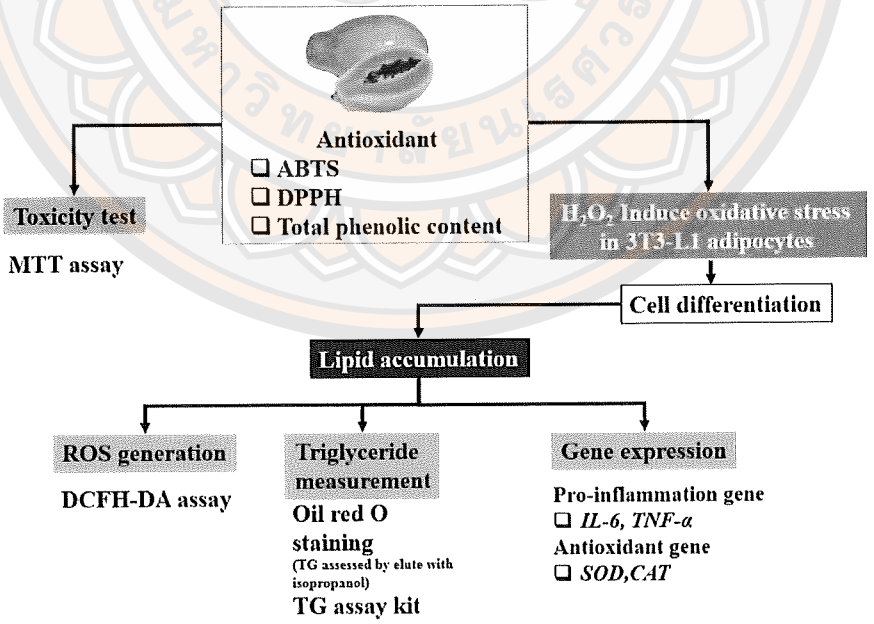


Figure 2 Scope of the study in 3T3-L1 adipocytes

From the figure 2 is the *in vitro* study. Papaya were detected antioxidant activity and toxicity test. The 3T3-L1 adipocytes were induced with H_2O_2. The cells were determined according to lipid accumulation by detected ROS generation, Triglyceride level, pro-inflammatory cytokine gene expression and antioxidant gene expression.

Hypothesis

1 . Papaya decreases lipid accumulation that leads to non-alcoholic fatty liver disease in the liver of rats by reducing hepatic lipid contents, oxidative stress, lipogenesis gene expression, pro-inflammatory cytokine.

2.Papaya decreases lipid accumulation in 3T3-L1 adipocytes by reducing ROS generation, triglyceride level, pro-inflammatory cytokine gene expression and antioxidant gene expression.

Keywords

Lipogenic gene expression, Non-alcoholic fatty liver disease, Obesity, Oxidative stress, 3T3-L1 adipocytes, Papaya

Expected outcomes of the research

Obesity is caused by excess adipose tissue mass, which is the major energy reserve in the body because it can progress to become non-alcoholic fatty liver disease. Therefore, the present study aims to investigate the *in vivo* and *in vitro* effect of papaya, its main active compounds that the high antioxidant content of papaya and can be used in preventative treatments for obesity. The results from this study provide beneficial information that could lead to the development of therapy for hepatic steatosis.

CHAPTER II

REVIEW OF RELATED LITERATURE AND RESEARCH

Obesity

Obesity is the imbalance between energy expenditure and energy intake. The energy excess leads to lipid accumulation in fat form. Causes of obesity are behaviour, environment and gene mutation which relate to fat cell formation(Tumova, Andel, & Trnka, 2016). From previous studies, it may be seen that there is a worldwide increase in obesity year-on-year (Lakshman, Elks, & Ong, 2012). Obesity relates to various metabolic syndromes, including hyperglycemia, hypercholesterolemia and hypertension. These characteristics can lead to other serious diseases such as cardiovascular disease, diabetes mellitus and cancer.

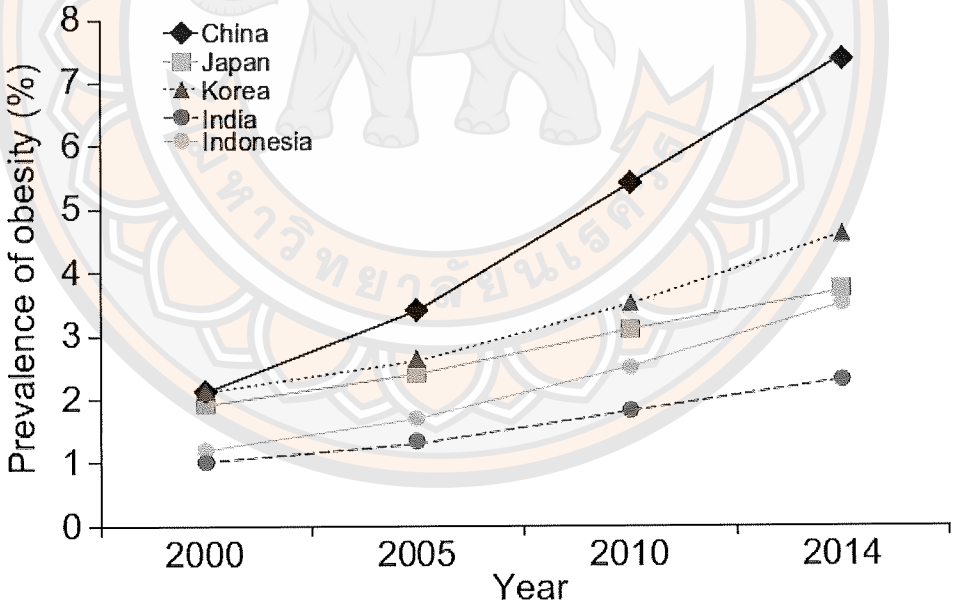


Figure 3 Prevalence of obesity

Source: Fan, Kim, & Wong, 2017

The World Health Organization (WHO) have given definition of overweight and obesity are the excess of lipid accumulation in the tissue. The lipid accumulation leads to risk factors for other diseases. The indicator for obesity is the Body Mass Index (BMI) which can be calculated by bodyweight (kg) divide by height (m) ^{2}. People who have a BMI of more than 30 kg/ (m)^2, are considered to be obese. Another indicator is waist circumference. Men who have a waist circumference more than 90 centimetres and women who have a waist circumference of more than 80 centimetres are also considered to be obese.

Table 1 Classification of obesity

Classification	**BMI(kg/m^2)** **Principal cut-off points**	**Additional cut-off points**
Underweight	<18.50	<18.50
Normal range	18.50-24.99	18.50-22.99
		23.00-24.99
Overweight	≥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)

Adipose tissue and lipid metabolism

Adipose tissue contains a lot of fat cells and it stores lipids in triglyceride form. Adipose tissue storage is in subcutaneous tissue, abdominal tissues, skeletal muscle, blood vessels and sebaceous glands where it helps to maintain the energy balance of the body (Wang et al., 2019).

Triglycerides are derived from fatty substances consumed in daily life. The digestion of fat uses bile salt which is produced by the liver and collected in the gall bladder. Bile salts are transferred to the small intestine where it is used to change fat to the micelle form. Triglycerides are digested with lipase enzyme within the small intestine to become monoacylglycerol, triacylglycerol, free fatty acid (FFA) and glycerol. These substances are absorbed through the small intestine and converted to triacylglycerol. Triacylglycerol combines with cholesterol and apolipoprotein to form chylomicrons (Dash, Xiao, Morgantini, & Lewis, 2015). Chylomicrons are transferred along the lymphatic system and blood circulation to the cells. Apolipoprotein with the element of chylomicrons activate lipoprotein lipase (LPL) in the blood vessel walls. The lipoprotein lipase digested triacylglycerol to become free fatty acids and glycerol. Free fatty acids are transferred to the cells, but chylomicron remnants are transferred to the liver for the synthesis of very low-density lipoprotein (VLDL). VLDL are broken with LPL to intermediate-density lipoprotein (IDL) and FFA. FFA are transferred to adipose tissue and other tissues where it is used. IDL are broken with LPL enzyme to become a low-density lipoprotein (LDL) which is transferred to LDL receptors or sent back to the liver (Bilheimer, Goldstein, Grundy, Starzl, & Brown, 1984).

FFA are transferred to adipose tissue for storage as energy reserves in triglyceride form, containing the glycerol 1 molecule and FFA 3 molecules. The fasting state of the body will break glycogen first. Triglycerides are broken after glycerol are used. In the adipose tissue, triglycerides are broken to get glycerol and FFA. Glycerol is used to synthesize gluconeogenesis, but FFA are used to break into the β-oxidation pathway to get energy(Liu et al., 2018).

In the normal state, the body receives fat in the diet which is used to synthesize triglycerides. Triglycerides are stored in adipose tissue with glycerol-3phosphate. Glycerol-3phosphate comes from glucose which is broken in the glycolysis pathway. Dihydroxyacetone phosphate is converted with L-glycerol 3-phosphate dehydrogenase enzyme while glycerol 3-phosphate are synthesized from glycerol. Glycerols are lysates from VLDL in the liver tissues with glycerol kinase enzyme. Next, L-glycerol 3-phosphate are converted to be phosphatidic acid 1, 2-diacylglycerol and triglyceride with phosphatidic acid phosphatase and acyl transferase enzyme respectively (Zingariello et al., 2019). If the body receives energy in excess, triglycerides are stored in other tissues such as muscle, liver, pancreas, kidneys and cardiac muscle.

Obesity patients have big bodies because they have lipid accumulation throughout the body. The shape of the obese body could be one of two types: the pear-shape obesity and the apple-shape obesity. Pear-shape obesity is mostly found in women with lipid accumulation in the hips and calves. Apple-shape obesity is central obesity which has a bigger waist circumference than hips. Central obesity occurs in lipid accumulation in abdominal and visceral tissue. Abdominal and visceral fat leads to serious diseases such as cardiovascular disease, diabetes mellitus and hypertension (Wu et al., 2018).

Adipocytes

Adipocytes are developed from stem cells of the fibroblast type. Stem cells are activated by hormones to become adipocytes. The differentiation of adipocytes takes place in three stages:

1. The preadipocyte stage where fibroblasts of the adipocytes type are found. The preadipocytes can develop into mature adipocytes when they are confluent until the proliferation arrest stage. It is activated by genes such as CCAAT/enhancer binding protein(C/EBP) type β and δ. So, preadipocytes can change to be early adipocytes stage.

2. The early adipocyte stage is the first of the differentiation to be adipocytes (Early differentiation). Preadipocytes which pass from the proliferation arrest stage, into early differentiation. Cell division is mitosis more than 1 cycle for increasing cell number, and are activated by genes such as C/EBPβ, C/EBPδ, C/EBPα and peroxisome proliferator-activated receptor gamma (PPARγ). Cell growth is stopped (growth arrest) and the cell changes shape from a spindle shape to spherical. Then, it has lipid accumulation in cells to become immature adipocytes.

3. The mature adipocyte stage is the last of the differentiation stages towards adipocytes (Terminal differentiation). The immature adipocytes increase their synthesis and are accumulations of fat in cells by genes such as C/EBPβ, C/EBPδ, C/EBPα and PPARγ until it is a big lipid droplet. The nucleus is squeezed near the cell membrane to become a mature adipocytes (Zuo, Qiang, & Farmer, 2006).

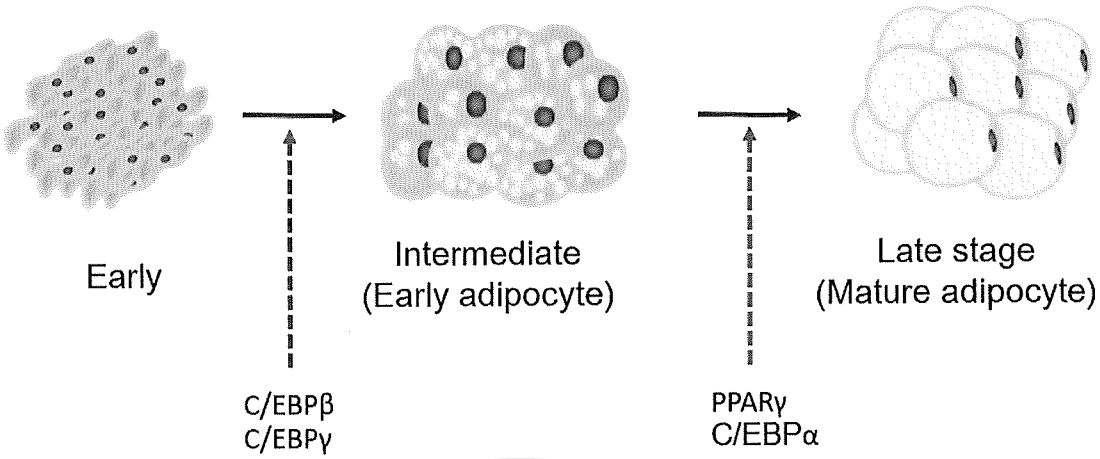


Figure 4 Stages of adipocyte cell development

Stage of adipocyte cell development

Obesity is a global health problem that affects other metabolic diseases such as cardiovascular disease. Adipose tissue resulting from the combination of triacylglycerols (TG) or the secretion of adipokines. Adipogenesis changes preadipocytes into adipocytes that through several maturation stages by insulin and adipocyte-specific transcription factors include CCAAT / enhancer-binding protein alpha (C / EBP-α) and peroxisome proliferator-activated receptor gamma (PPAR-γ) that work together to produce fat. Thus, the regulation of adipogenesis, which causes the accumulation of fat, is used to determine obesity prevention.

Fat cells can be divided into two types: white fat cells and brown fat cells. Brown fat cells are characterized by a large number of mitochondria that functions as a source of heat energy to the body which a protein called uncoupling protein (UCP1) is responsible for producing heat. Brown fat cells are found in rodents and other mammals in the infancy range and will decrease as they age. But for the white fat cell, it is important to have fatty deposits in large fat cells within the cell. It acts as a source of energy reserves of the body, prevents the loss of heat energy through the skin and protects the organs from physical force.

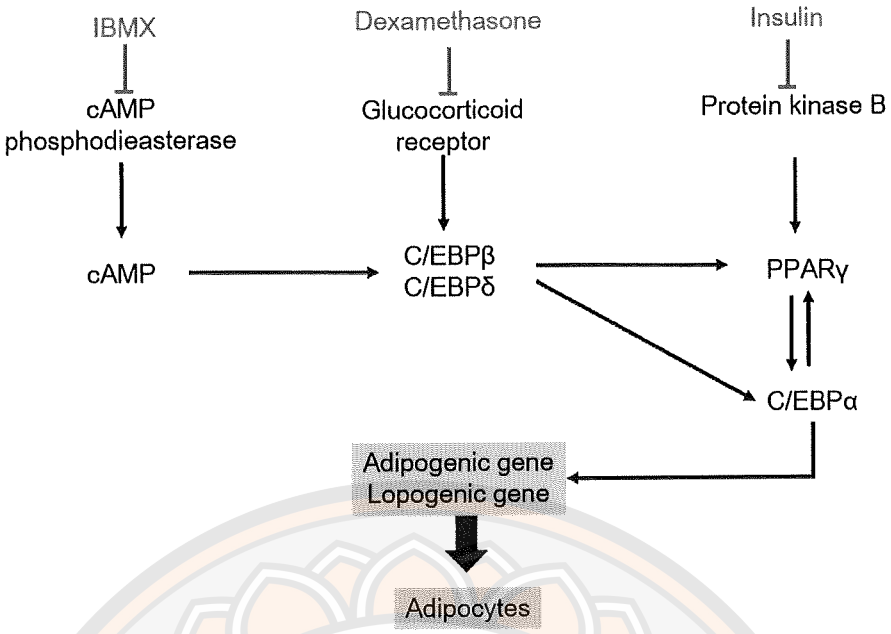


Figure 5 Genes relate to adipogenesis

Recently, fat cell studies are based on the study of fat cell cultures, namely 3T3-L1, which is derived from the fat cells of rats. It is difficult to isolate preadipocytes. The preadipocyte stage has a small volume and a shorter life span. It is essential to study the changes in fat cells using cell cultures. This study was carried out using preadipocyte rat cells.

Preadipocyte cell cultures are stimulated by chemicals including isobuthylmethylxanthine (IBMX), dexamethasone and insulin. Dexamethasone is a glucocorticoid agonist that can bind to Glucocorticoid receptors (GR). C/EBPβ and C/EBPδ are transcription factors that activate peroxisome proliferator-activated receptor gamma (PPARγ). C/EBPα regulates gene expression associated with changes to fat cells such as fatty acid synthase (FAS) and adipocyte protein 2 (aP2). IBMX is a cAMP phosphodiesterase inhibitor that inhibits cAMP phosphodiesterase activity and can increase cAMP. Increasing of cAMP from activation of C/ EBPβ and C/ EBPδ Moreover, insulin induced (PPARγ) via protein kinase B (Lee et al., 2018).

Hepatic de novo lipogenesis

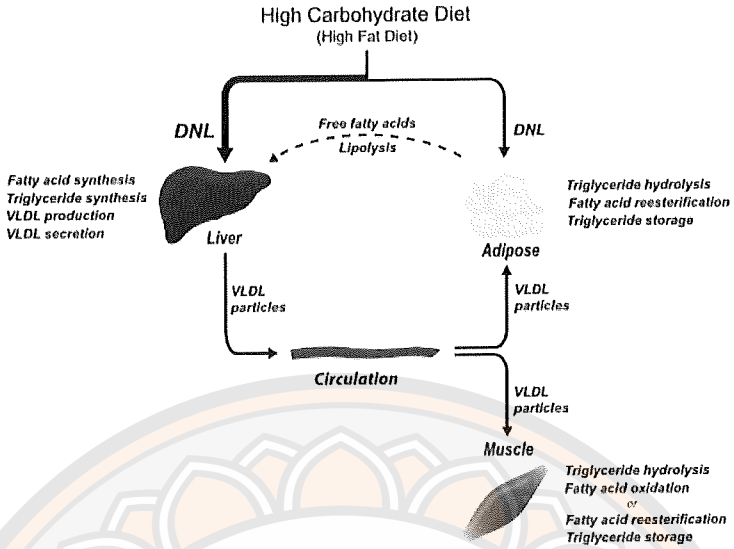


Figure 6 Lipid transport

Source: Strable, & Ntambi, 2010

Fat accumulation in the liver may be a product of lipogenesis in the liver. De novo lipogenesis is a process in which fat is synthesized externally from food sources, including carbohydrates or energy stores (Xu et al., 2015). Carbohydrates in foods contain starch and sugar, which are broken down into six types of carbon monoxide. Glucose or fructose is combined with the production of three types of carbon media: glyceraldehyde-3 phosphate (GA3P) and dihydroxyacetone phosphate (DHAP). These intermediates may be connected to pyruvate, which is the final product of carbohydrate metabolism in plasma.

Pyruvate may enter the mitochondria to be converted into acetyl-CoA using the tricarboxylic acid cycle (TCA). The accumulated TCA and citrate intermediates are transported back to the cytoplasm by the mitochondrial transport system. The Citrate is converted to acetyl-CoA by the action of adenosine triphosphate citrate lyase (ACL), which is the first step in the synthesis of external fatty acids. Citrate is an active cytoplasmic acetyl-CoA carboxylase (ACC) which has the function of converting acetyl-CoA to malonyl-CoA is to start producing lipogenesis. Malonyl CoA is the main carbon source used for the synthesis of external fatty acids.Fatty acid synthase (FAS)

uses malonyl-CoA to expand the chain of acetic acid, which is increased by two carbon monounsaturated 16 fatty acids. The main product of fatty acid synthesis. ACC enzyme action is an important regulatory process of body fat synthesis (Boyera et al., 1998).

Lipid transport

Lipids are transported in the blood circulation. It is packed in the form of lipoprotein. The liver is the center of control of cholesterol levels in the body. It synthesizes cholesterol for transport to other cells, but it also removes cholesterol from the body by converting it into bile salts and placing it in bile that can be eliminated in the stool. In addition, the liver also synthesizes many lipoproteins related to the transport of cholesterol and other lipids throughout the body (Feldstein et al., 2004).

The synthesis of cholesterol in the liver is under negative feedback regulation. Increased cholesterol in liver cells leads to reduced activity of HMG-CoA reductase HMG-CoA reductase is a rate-limiting enzyme in cholesterol synthesis.

Lipoproteins include triacylglycerol (TAG), cholesterol, phospholipids and amphipathic proteins called apolipoproteins. Lipoprotein can make a difference on the basis of their density. The level of lipoprotein fat affects its density - the lower the density of lipoprotein, the more fat is compared to protein. Four major lipoprotein types are chylomicrons, very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) (Ramasamy, 2014)

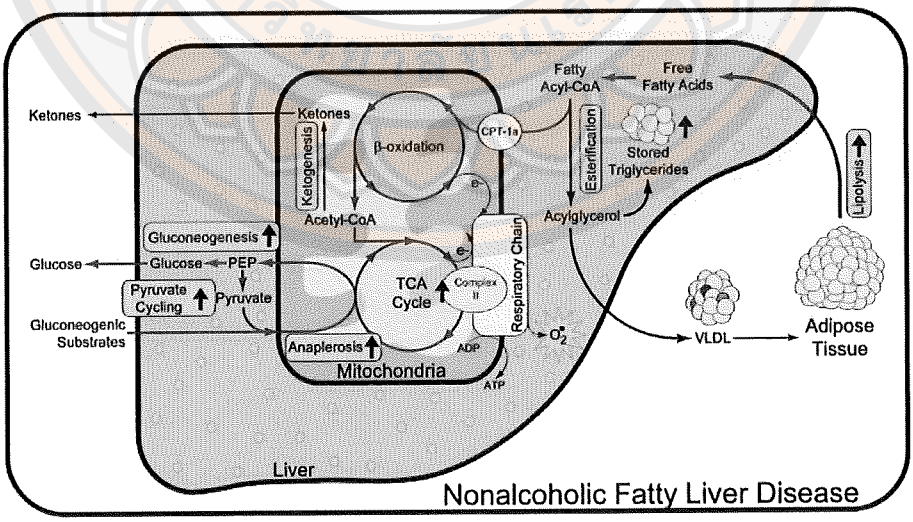


Figure 7 Non-alcoholic fatty liver disease

Source: Sunny, Parks, Browning, & Burgess, 2011

Chylomicrons and VLDL send TAG to the cells in the body. Two types of lipoprotein are rich in triglycerides. Chylomicrons are synthesized by enterocytes from lipids that are absorbed in the small intestine. VLDL is synthesized in the liver. The function of these lipoproteins is to deliver. Triacylglycerol (TAG) rich in energy to the cells in the body. TAG is packed from chylomicrons and VLDL through the action of lipoprotein lipase.The lipoprotein lipase is an enzyme that is found on the surface of endothelial cells. This enzyme digests the TAG to fatty acids and monoglycerides, which can then diffuse into the cell to be oxidized, or in the case of an adipose cell, to be re-synthesized into TAG and stored in the cell (Dash, Xiao, Morgantini, & Lewis, 2015).

Low density lipoprotein (LDL) transports cholesterol to the cells in the body. Because the VLDL particles contain triacylglycerol. These particles have been redesigned in the liver and converted to LDL. The function of LDL is to transport cholesterol to cells. Cholesterol-receptor cells by accepting endocotoxins LDL binds to specific LDL receptors and is made into zones in an endocytic vesicle (Yuan et al., 2018).

High density lipoprotein (HDL) is related to the return of cholesterol. Excess cholesterol is removed from the body through the liver, which will bile cholesterol in the bile or convert to bile salts. The liver will eliminate LDL and other lipoprotein from the blood circulation by endocytosis that is received by mediation. In addition, excess cholesterol from the cells is brought back to the liver by HDL in a process called reverse cholesterol transport. HDL is synthesized and secreted by the liver and small intestine(Zhou, Li, Gao, & Wang, 2015).

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease. NAFLD demonstrates broad liver damage from common steatosis to steatohepatitis without alcohol, advanced fibrosis, and cirrhosis or even liver cancer. Insulin resistance may be a factor that causes NAFLD (Chitturi et al. 2002). Mitochondrial dysfunction, increased oxidative stress (Musso, Gambino, Cassader, & Pagano, 2010) and changes in the content of glutathione (Videla et al. 2004), cytokine and adipokine imbalanced also plays an important role in the occurrence of NAFLD. These factors may increase the sensitivity of the liver to acute toxic injury.

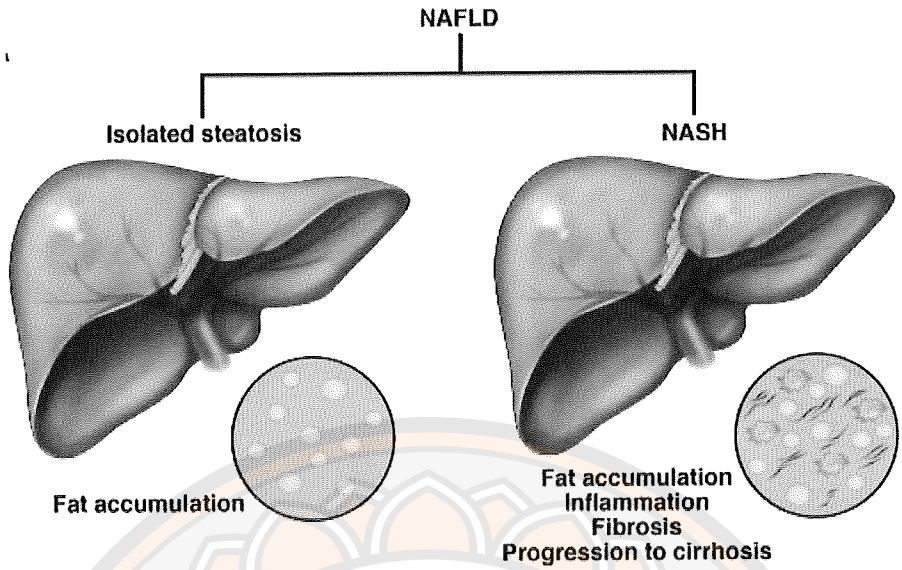


Figure 8 Hepatic steatosis and steatohepatitis

Source: Camilleri, Malhi, & Acosta, 2017

Lipid metabolism

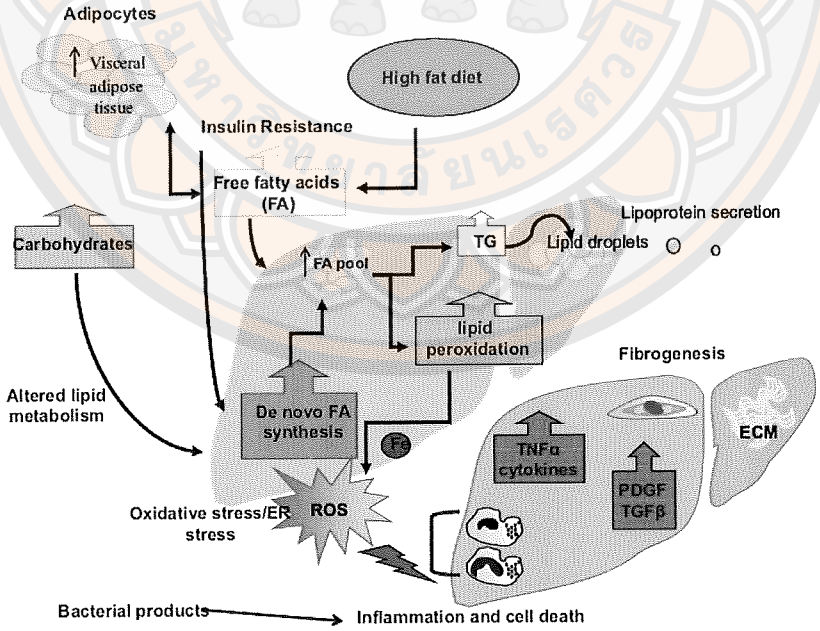


Figure 9 Lipid accumulation in liver

Source: Dongiovanni, Anstee, & Valenti, 2013

NAFLD is characterized by the accumulation of fat in the liver, which is the result of an imbalance between triglycerides and removal. Most free fatty acids (FFAs) that are stored as triglycerides during the liver steatosis are derived from the breakdown of fat related to insulin resistance in adipose tissue, followed by lipogenesis de novo caused by hyperinsulinemia and food. In the liver, FFA can be catabolized through β-oxidation, re-esterification to triglycerides and stored as fat droplets or exported to very low density lipoprotein (VLDL). The ability to excrete deficiency of lipoprotein and reduce oxidation due to damage of Mitochondria The (Especially in the presence of NASH) may play a role in fat accumulation in the liver Food factors also play an important role in NAFLD (Chen et al., 2017).High dietary fructose, trans-fatty acids, and cholesterol have all been found to contribute to the development of dyslipidemia, hepatic fat accumulation and subsequent disease progression. FFA and cholesterol not only induce hepatic lipid deposition, but may also have injurious effects in the liver, in part by inducing hepatocyte apoptosis, lipotoxicity, mitochondrial dysfunction and endoplasmic reticulum stress with subsequent ROS formation and inflammation (Bedossa, 2017). Furthermore, activation of hepatic stellate cells and Kupffer cells by hepatocyte apoptosis and ROS, also appear to promote disease progression through the secretion of TNFα, IL6, transforming growth factor β and collagen, which induces inflammation and fibrosis. Indeed, increased generation of ROS and lipid peroxidation are believed to be key pathogenic components in NAFLD, perpetuating inflammation, fibrogenesis, and development of cirrhosis and hepatocellular carcinoma. FFA and cholesterol is taken up by hepatocytes and processed to be exported from the liver by VLDL. The hepatic VLDL export is saturated and lipids are accumulated in hepatocytes. NAFLD progression is increased oxidative stress and inflammation, promoting activation of hepatic stellate and kupffer cells, which alongside an increased hepatic production of TNFα and IL6. Combined with key pathogenic changes taking place during the advancement from simple steatosis (NAFLD) to steatohepatitis (NASH) include hepatocyte ballooning, formation of Mallory bodies and fibrosis. NASH may progress even further to hepatic cirrhosis and hepatocellular carcinoma(Sanchez-Valle, Chavez-Tapia, Uribe, & Mendez-Sanchez, 2012). A number of investigators have found that markers of oxidative damage such as malondialdehyde (MDA) and protein carbonyls are elevated, while antioxidants like catalase and SOD are decreased in the

plasma and livers of animals and humans with NAFLD(Sakaguchi, Takahashi, Sasaki, Kumagai, & Nagata, 2011).

Oxidative stress

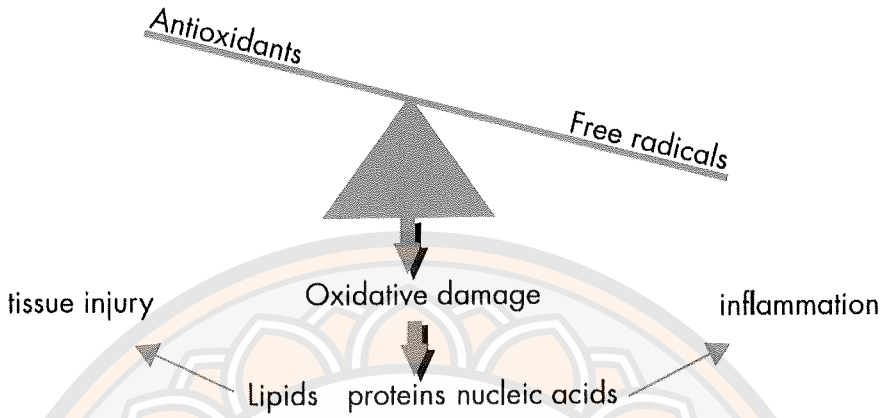


Figure 10 Oxidative stress exists when there is an excess of free radicals

Source: Kelly, 2003

From the figure show a consequence where free radicals attack and oxidize other cell components such as lipids (particularly polyunsaturated lipids), proteins, and nucleic acids. This leads to tissue injury and in some cases, the influx of inflammatory cells to the sites of injury. Oxidative stress is an imbalance between reactive oxygen species (ROS) and antioxidants (antioxidants), with a greater number of reactive oxygen species (ROS) than antioxidants (antioxidants). Therefore, oxidative stress is a result of increased ROS production. The reduction of antioxidants will affect the protection system (Enzymatic and Non enzymatic)(Roberts & Sindhu, 2009).Oxidative Stress is also responsible for destroying various biomolecules such as nucleic acids, membrane lipids, and proteins, as well as disrupting normal body functions (Ozata et al., 2002).

Excess ROS from obesity will destroy the protein, fat, nucleic acid and cell damage (cell damage), causing the cells to die. Because obesity affects the enzyme antioxidant. The system includes superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx), as well as non-enzymatic oxidative systems such as Thiol or GSH, vitamins and minerals (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012).

Oxidative stress is defined as an imbalance between the oxidant and the antioxidant systems of the organism, in favour of the oxidants. The oxidant systems are represented by free radicals, i.e. molecules containing unpaired electrons, mainly constituted by the so-called reactive oxygen species (ROS). Targets for the free radicals are lipids, DNA, and proteins. If a fatty acid is damaged by the free radicals, then it becomes a free radical itself by setting up a chain reaction of lipid peroxidation, which destroys the cell membranes, inducing apoptosis and necrosis processes. Mitochondria are the major source of ROS; in particular, an increased food supply may cause a boost in mitochondrial ROS production, triggering possible oxidative stress in the liver.

Inflammation

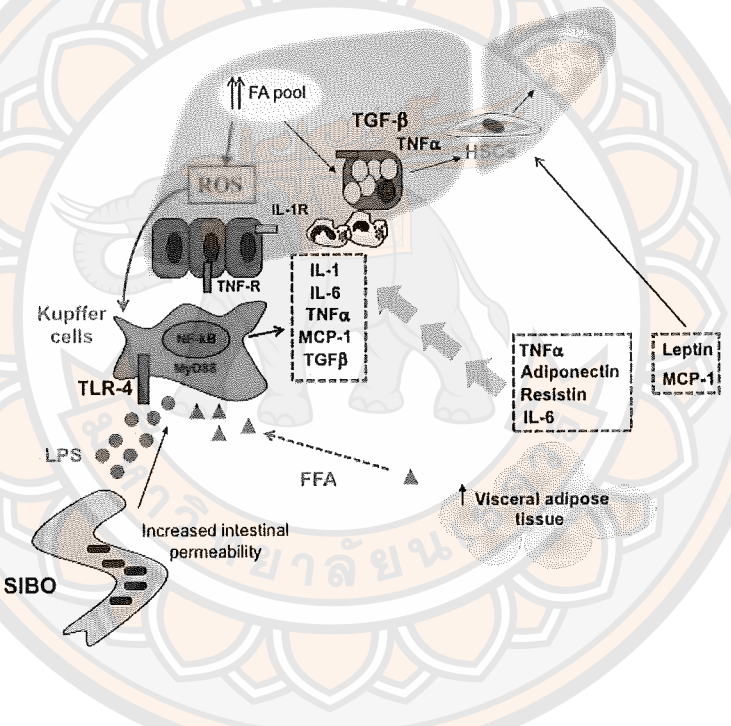


Figure 11 The process of inflammation

Source: Dongiovanni et al., 2013

Obesity and NAFLD are directly related to the activation of inflammatory pathways. Hypertrophic adipocytes release chemokines and proinflammatory cytokines, including TNF-α, IL-6, resistin and MCP-1. Chemokines recruit large, especially in organ fat tissue. Inside the fatty tissue macrophages it produces inflammatory cytokines such as TNF-α, IL-6 and IL-1.These inflammatory changes in fat tissue cause

adipocytokine dysregulation: reduction of insulin sensitizing and anti-adipocytokine inflammation as adiponectin and increase in pro-inflammatory cytokines such as TNF-α, interleukins and resistin-free fatty acids (FFAs), including endotoxins from bacteria. Activated Kupffer cells, by attracting Toll-like receptor 4 receptors (TLR4) produce inflammatory cytokines such as TNF-α and IL-1, chemokines such as MCP-1 and ROS that lead to liver damage.

The loss of acute liver cells stimulates the growth of surviving liver cells. However, in a chronic fatty liver, many liver cells receive sustained oxidative damage that inhibits development into the cycle and regeneration. Moreover, fatty liver cells reduce production capacity. Damaged liver cells release many factors, including ROS, cytokines, chemokines that receive inflammatory cells into the liver. Once in the liver, these inflammatory cells release toxic cell factors that cause liver cells to die.

Papaya

Figure 12 Papaya

Source: Wei, & Wing, 2008

Papaya (Carica papaya) is well known for its medicinal properties throughout the world. The whole papaya plant including its leaves, seed, ripe and unripe fruit and their juice is used in traditional medicine. The fruit is large, oval in shape, with yellowish-green skin and yellow fresh. The medicinal properties of papaya include anti-hypertensive(Koffi N et al., 2009) , hypolipidemic and hypoglycemic(Adeneye AA et al., 2007), anti-fungal(Nwinyi et al. , 2010), anti-bacterial (Doughari JH et al. , 2007), anti-tumour (Otsuki N et al. , 2010) and anti-oxidant (Srikanth G et al. , 2010).

Papaya fruit is a good source of bioactive phytochemicals, including carotenoids (β-carotene, α-carotene, β-cryptoxanthin, α- cryptoxanthin, lutein, 9-cis-β-carotene), phenolic compounds (ferulic acid, caffeic acid, p-coumaric acid, rutin, quercetin, kaempferol) and glucosinolates (benzyl glucosinolate, benzyl isothiocyanate).

Carotenoid

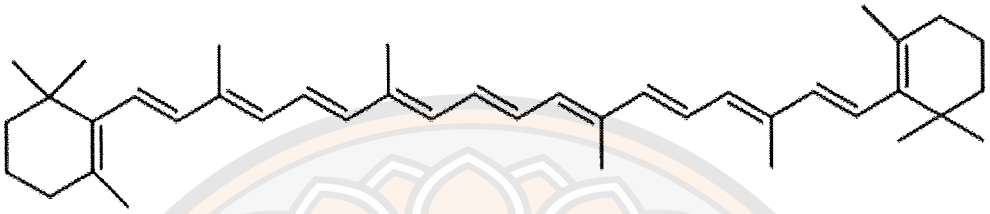


Figure 13 Structure of β-carotene

Source: Gmoser, Ferreira, Lennartsson, & Taherzadeh, 2017

Carotenoids are a group of more than 600 fat soluble pigments. Animals can't synthesize carotenoids, so they must be fed through supplementary foods (Sandmann, 2015). The carotenoids, including carotene, lycopene, lutein and zeaxanthin (Rodrigo, Cilla, Barbera and Zacarias, 2015). β-carotene can protect cells from damage by inhibiting lipid peroxidation by radicals. Independent and single oxidation oxygen (Tan et al., 2014)

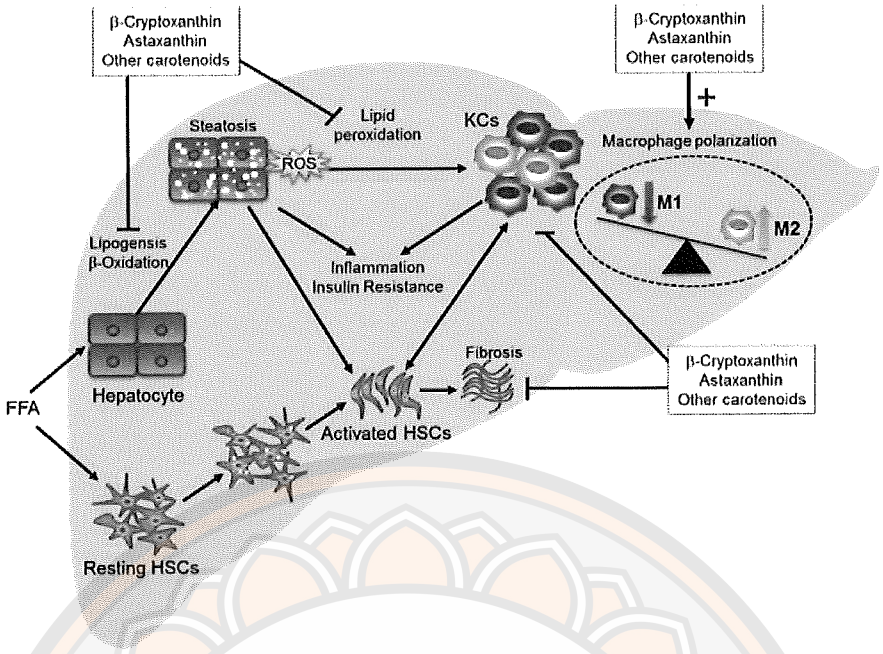


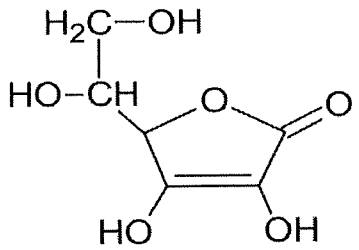
Figure 14 Carotenoids relate to hepatic steatosis

Source: Ni, Zhuge, Nagashimada, & Ota, 2016

Carotenoids storage in the liver and are incorporated into lipoproteins to help with blood circulation. Carotenoids that are ingested may lead to an antioxidant defense system when high levels of free radicals in the liver and the physiological functions of carotenoids can inhibit the development of liver disease (Sugiura, Ogawa, & Yano, 2014)

Carotenoids are severely depleted in the liver tissue of patients with liver injury or chronic liver disease. Therefore, the lack of antioxidants may lead to the development of obesity, insulin resistance and NASH. The concentration of low serum carotenoids such as α-carotene, β-carotene and vitamin E are associated with obesity. In addition, the higher serum carotenoids are associated with lower serum ALT levels and the risk of NAFLD decreases.

Vitamin C (Ascorbic acid)



ascorbic acid

Figure 15 Structure of ascorbic acid

Source: Kocot, Luchowska-Kocot, & Kurzepa, 2017

Vitamin C is a water-soluble compound. Bodily concentrations are maintained through the consumption of vitamin C. Humans cannot synthesize ascorbic acid de novo. Oxidation of vitamin C produces dehydroxy ascorbic acid, which is transported into our cells through glucose transporters and then reduced back to ascorbic acid for cellular use(Lv H. et al., 2018). Vitamin C is a powerful antioxidant and free radical scavenger that protects our tissues, cell membranes, and DNA from oxidative damage. It also serves as an essential cofactor and electron donor during collagen hydroxylation, encouraging the maturation of intracellular and extracellular collagen(Boyera, Galey, & Bernard, 1998).

Vitamin C is a co-factor for the 7α-hydroxylase catalysing the conversion of Cholesterol to 7α-hydroxycholesterol, constituting the rate-limiting step in bile acid formation, and thus, Vitamin C deficiency results in reduced excretion of cholesterol in animals. Vitamin C is involved in the regulation of both circulating and hepatic lipid homeostasis, supporting Vitamin C as an important factor in the development of NAFLD.

CHAPTER III

RESEARCH METHODOLOGY

Chemicals and reagents

Table 2 The chemical of this study

Agents	Company
Triglyceride assay kit	(HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany)
Cholesterol assay kit	(HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany)
Mounting medium optimum cutting temperature(OCT) FSC 22® Clear frozen Section compound	(Leica Biosystems)
Ethanol	(ACI Labscan)
Isopropanol	(ACI Labscan)
Coverslip	(MENZEL-GLASER)
Centrifuge	(Biofuge , Germany)
RIPA	(Sigma-Aldrich,MO ,USA)
Protease inhibitor	(Millpove, CA, USA)
BCA kit	(Thermoo scientific,USA;Cat.No.23225)
DMEM	(Gibco,USA;Cat.No. 10270-098)
FBS	(Gibco,USA;Cat.No. 10270-098)
CO_2 incubator	(SANYO,Japan)
IBMX	(Sigma-Aldrich, MO ,USA;Cat.No.I7018-250 mg)
Dexamethasone	(Sigma-Aldrich, MO ,USA;Cat.No.D2915-250 mg)

Table 2 (CONT.)

H_2O_2	(EDM Millipore, MA, USA)
2′,7′-Dichlorofluorescin diacetate(DCFDA)	(Sigma-Aldrich, MO ,USA)
Insulin	(Santa cru2 Biotechnology,Flask(SPL,Korea) USA; Cat.No.11061-68-0)
DMSO	(Bio Basic, Canada ;Cat.No.D0231)
Oil red O	(Sigma, Canada; Cat.No. O0625)
Isopropanol	(RCI labscan, Bankok, Thailand Cat.No. 67-63-0)
Real-time Premi (SYBR Green)(2X)	(Cat.No. RT009)(RBCBioscience,Taiwan)
Everted light microscope	(Leica, USA)
Enhancer	(TOYOBO ;NKB-101T)
Microplate reader	(Biotek, Bankok, Thailand0
Trolox	(Sigma-Aldrich,MO ,USA)
DPPH	(Sigma-Aldrich,MO ,USA)
Methanol	Labscan
NaCl	(Ajax Finechem,New Sath wales, Australia)
NaH_2PO_4	(Ajax Finechem,New Sath wales, Australia)
Cholesterol	(Ajax Finechem,New Sath wales, Australia)
Heat block	(Julabo)
Ethanol	(Sigma-Aldrich,MO ,USA)
Mastermix	(Bolis biodyne)
RNase inhibitor	(Cat.No.Bio 65028,Bioline)
RiboZol	AMRESCO, USA&Canada
Chloroform	(Cat. No. A 3505 E)(Lab scan, Thailand)
Isopropanol	(Cat.No. 67-63-0)(ACI lab scan,Thailand)
DEPC water	(Cat.No. 1609-47-8)(Bio Basic, Canada)
PCR Red Mix(2x)	(Bioline,USA)
PCR Forward Primer(10 mM)	(Integrated DNA Technologies,Singapore)
PCR Reverse Primer(10 mM)	(Integrated DNA Technologies,Singapore)

Table 2 (CONT.)

TBE buffer	(Bioline,USA)
Red safe dye	(Cat.No.21141)(Biotechnology,Korea)
100 bp DNA ladder	(Cat.No. DM003-R500)(Genedirex, Germany)

Instruments

Laminar flow class II biohazard (Sanyo MCV-B131F)

CO_2 incubator (Sanyo mco-20aic)

Inverted microscope (Olypus, 1x71)

High Speed Refrigerated Centrifuge (ScanSpeed, Model 2236R)

Microplate reader (Synergy HT Multi-Mode, BioTek Instruments Inc., USA)

Methods

1. Preparation of papaya

Papaya fruit (*Carica papaya* L.) breed 'Holland' were purchased from local market at Naresuan University. Papaya juice was prepared for feeding to rats. The dose of papaya fruit are 0.5 ml/100 g BW and 1 ml/100 g BW.

2. Animal model (*In vivo* study)

Male Sprague Dawley (SD) rats weighed between 100 and 120g were used in this study. Sprague Dawley rats were bought from Mahidol University. Animal ethics were approved by centre for animal research Naresuan University (NU-AE 580714).

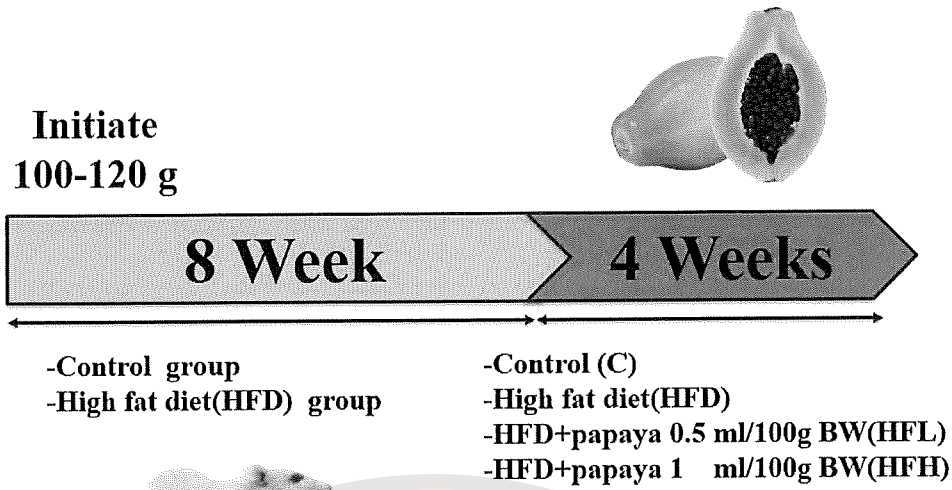


Figure 16 Animal model

3. Determination of hepatic lipid content

3.1 Triglyceride

Liver tissue homogenized and hepatic concentrations of triglycerides were measured using commercial kits (HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany) after lipid extraction according to Folch et al. (Folch, et al., 1957).

3.2 Cholesterol

Liver tissue homogenized and hepatic concentrations of total cholesterol were measured using commercial kits (HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany) after lipid extraction according to Folch et al. (Folch, et al., 1957).

4. Determination of Lipid peroxidation

TBARS (Thio barbituric acid reactive substance) exmamined lipid peroxidation from malondialdehyde. The solutions were prepared such as 500 mM TMP, 0.25N HCl (Sigma-Aldrich,MO,USA) and 0.37% 2-thiobarbitulic acid(POCH sowinskiego , Poland) with 1: 1: 1 ratio. Then diluted TMP (POCH sowinskiego, Poland). These solutions were mixed and added 200 µl in each eppendorf. After that, each eppendorf were incubated in heat block 95°C for 15 minutes. The solution was

centrifuged 3,500 g for 25 minutes and supernatant was pipetted to 96 well plate. Finally, the samples were measured for absorbance at 535 nm.

5. Determination of Enzymatic antioxidant

5.1 Assay of catalase (CAT)

The CAT catalyses the reduction of Hydrogen peroxide(H_2O_2) to oxygen and water. Thus, H_2O_2 levels were used as a quantitative measure of CAT by commercial assay kit.

5.2 Determination of SOD

The SOD play a role in scavenging O_2^- and catalyses the reduction of O_2^- to H_2O_2. In the first step of assay, O_2^- is generated by xanthine oxidase and xanthine in the presence of oxygen. In the second step, O_2^- can react with ferricytochome C and generate reducing cytochome C. The reduction of cytochrome C was inhibited by the presence of SOD and measured by commercial assay kit.

6. Histopathology

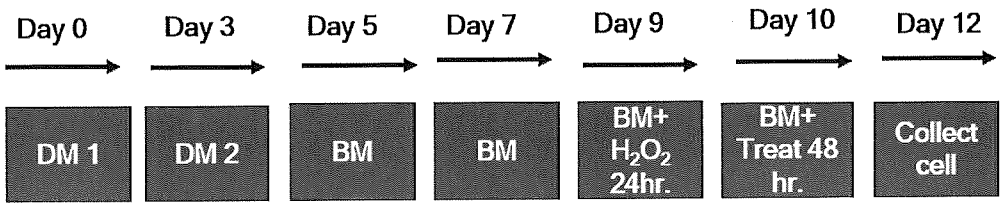
6.1 Oil red O

Another part of frozen tissue sections was stained with oil-red O to reveal the lipid droplet contents. The liver specimens were frozen in a freezer at -20 °C, sectioned by a cryostat, and stained for lipids using Oil Red O staining. Then, the sections were assessed for the degree of hepatic steatosis by comparing the degree of accumulation of red lipid droplets and photographed under a light microscope.

6.2 H&E staining

Liver tissue were fixed with 10% neutral formaldehyde for 24 hrs. Then, tissues will be dehydrated in series of graded alcohol and embed in paraffin wax. The tissues were cut into 5 μm thickness and stained with H&E as show in table. All section were examined for histological change under light microscope.

7. Cell culture model (*In vitro* study)



1. Undifferentiation
2. Differentiation(control)
3. Differentiation +H_2O_2
4. Differentiation +H_2O_2 +Papaya
5. Differentiation +H_2O_2+ Positive control(B-carotene)

Figure 17 Cell model

3T3-L1 cells were grown in DMEM, supplemented with 10% foetal bovine serum and 100U/mL penicillin at 37 °C in a 5% CO_2 atmosphere. 3T3-L1 cells followed by incubation in the presence or absence of Papaya extract (0.0625, 0.125, 0.25, 0.5, 0.75, 1 mg/ml) for 24 hrs. The effects of papaya extract on cell viability were determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). Cells were seeded in a 96-well plate and incubated with various concentrations of Papaya. The cells were washed and incubated with 180 µL of MTT and DMEM without foetal bovine serum for an additional 2 hrs. Following additional washing, the insoluble formazan products in each well were dissolved in 100µL of dimethyl sulfoxide, and the absorbance of each well at 590 nm was measured spectrophotometrically.

8. Determination of cell viability by MTT assay

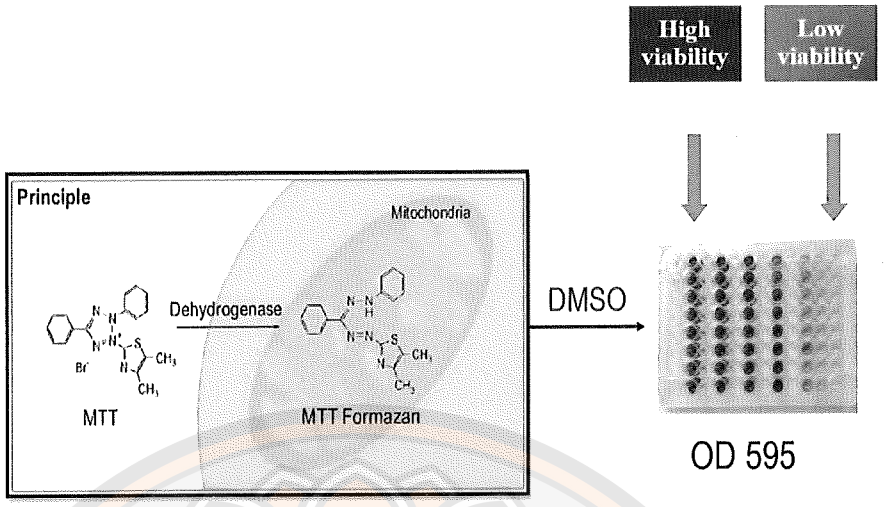


Figure 18 Principle of MTT assay

The principle of determination of cell viability was measured by MTT assay, where cell viability used enzyme succinate dehydrogenase in mitochondria. The cells reacted with MTT solution and appeared yellow, and formed formazan crystals. DMSO solution was added to the solution, making it violet in colour. The absorbance was measured at 540nm.

In this study, cell culture was done in a 96-well plate for 24-48 hrs. After that the DMEM media was discarded. Papaya was added to DMEM without foetal bovine serum each concentration was 0.1, and 0.5 mg/ml. Control groups did not get papaya (0 mg/ml). Cell cultures were incubated for 24 hrs. in CO_2 incubator, 37°C, 95% air/ 5% CO_2. The cell-culture and solutions were discarded. DMEM was added to the 96 well plates, each well was added 100 µl/well. The wells were shaken for 30 minutes. The solution was measured absorbance 540 nm. The cell viability were calculated by A_{540} (Cells was treated with papaya)/A_{540}(Control) x 100 soluted by MTT solution 20 µM(50 mg/ml) got MTT solution final concentration 5 mg/ml. Incubation in a CO_2 incubator, 37°C, 95% air/ 5% CO_2 for 2 hrs. The MTT solution was discarded. The formazan crystal in cell was dissolved with 100 µl/well of DMSO solution. It was shaken by a shaker for 30 minutes. After, the solution was measured absorbance 540 nm and calculated %cell viability by:

$$\% \text{ Cell viability} = [A_{540} \text{ (Cell were treated by papaya)}/A_{540} \text{ (Control)}] \times 100$$

9. Antioxidant activity tests

9.1 ABTS(2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) assay

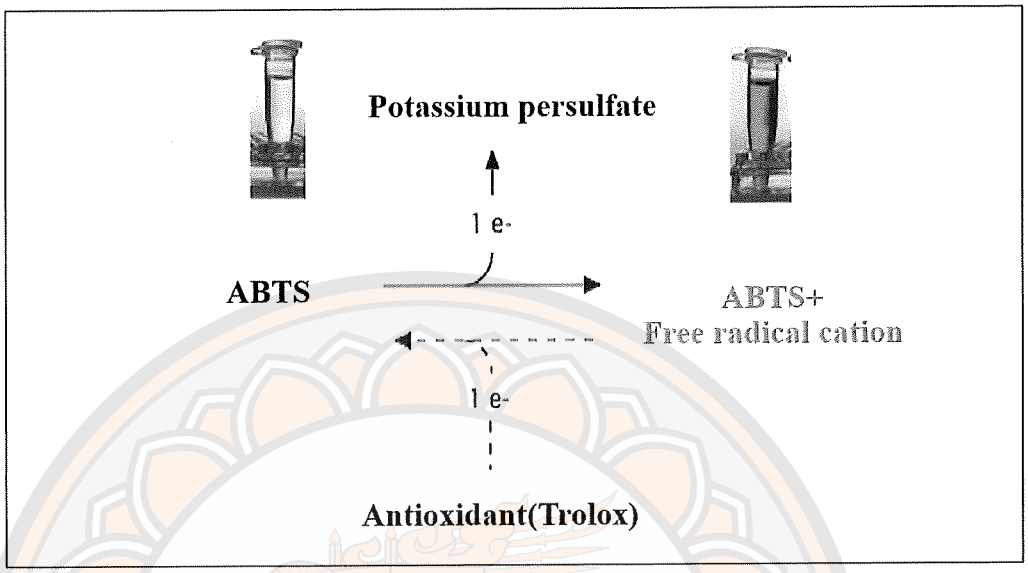


Figure 19 Principle of ABTS assay

Trolox equivalent antioxidant capacity (TEAC).Antioxidant activity was measured by the TEAC method. Briefly, the radical cation ABTS (2,20- azinobis 3-ethylbenzothiazoline 6-sulfonate, Sigma) was prepared 12-16 hrs. prior to the assay. ABTS 0.0192 g dissolved in DW 5 ml (stock 7 mM). Potassium persulfate 0.038 g dissolved in DW 1 ml (stock 140 mM). Radical production occurred by mixing an aqueous solution of 7 mM ABTS in 140 mM potassium persulfate. The radical was diluted with water until the absorbance reached a value of 0.70±0.02. Then the ABST radical has to be diluted with distilled water until absorbance 734 nm. Trolox 0.1 g dissolved in 100% methanol 10 ml (stock 50 mM). Dilute 50 mM Trolox in 100% methanol to various concentration. Incubate 30 minutes and measure absorbance 734 nm. Percentage (%) of reduction power = $[(A_{blank} - A_{sample}) / A_{blank}] \times 100$

By A_{blank} is Absorbance of control (ABTS radical)

A_{sample} is Absorbance of sample

9.2 DPPH (2,2-diphenyl-1-picrylhydrazy) assay

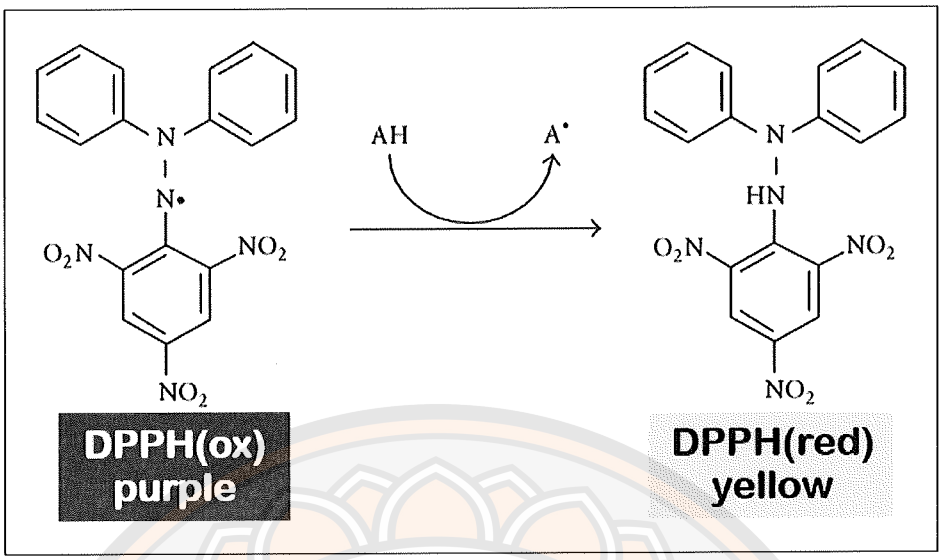


Figure 20 Principle of DPPH assay

DPPH 0.0253 g dissolve in 100% Methanol 100 ml (stock 0.6 mM).Prepare working 0.12 mM (0.6 mM 2 ml dissolve in ethanol 8 ml).Vitamin C 1 g dissolved in DW 1000 ul (stock 1000 mg/ml) Incubated 30 min.Measured absorbance 515 nm. DPPH scavenging activity (%) = $[(A_{blank} - A_{sample}) / A_{blank}] \times 100$

By A_{blank} was Absorbance of control (DPPH radical)

A_{sample} was Absorbance of sample

9.3 Total phenolic content

The total phenolics of the extracts were determined using the Folin and Ciocalteu reagent. Sample and standard readings were made using a spectrophotometer at 765 nm. Folin reagent 500 ul were mixed with 20% (W/V) of sodium carbonate 500 ul and then, add distilled water 9 ml keep in dark 30 minutes got working reagent. Gallic acid 0.17 g was dissolved in DW 10 ml (stock 100 mg/ml). The reaction was kept in the dark for 30 min and after centrifuging the absorbance of blue color from different samples was measured at 765 nm. The phenolic content was calculated as gallic acid equivalents GAE/g of dry plant material on the basis of a standard curve of gallic acid. All determinations were carried out in triplicate.

10. DCFH-DA assay (ROS)

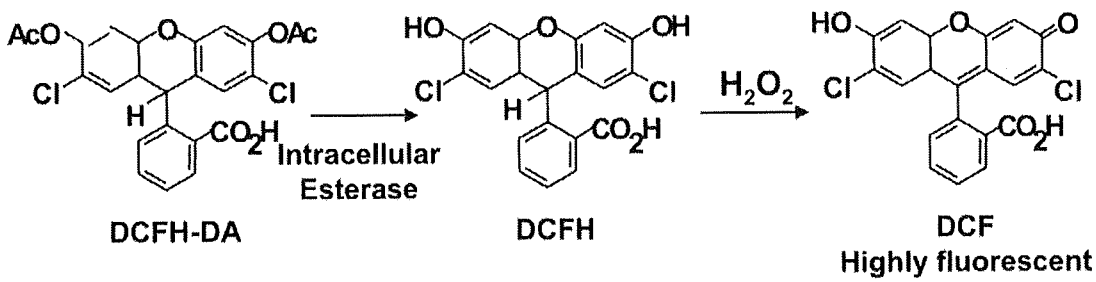


Figure 21 Principle of DCFH-DA assay

Source: Dikalov, Griendling, & Harrison, 2007

The principle of the ROS level in cells may be detected by DCFH-DA assay. The esterase enzyme may be used to change DCFH-DA to DCFH. DCFH is non-fluorescent and cannot be used to detect ROS levels within a cell. Once cellular oxidation occurs, within a cell then DCFH becomes DCF, which is fluorescent and may be used to detect ROS levels. Thus, fluorescent light intensity is related to ROS level. High fluorescence light intensity showed high ROS level and low fluorescent light intensity shows low ROS levels. Cell culture was used in 96 well/plate for 24-48 hrs. Then, various concentrations of papaya, 0.1, 0.5 1 mg/ml by 100 µl/well. Cell culture was kept in CO_2 incubator, 37°C , 95% air/ 5% CO_2 for 24 hrs. The control group was treated in DMEM without foetal bovine serum and without papaya. Positive control was treated in DMEM without foetal bovine serum and added 500 µM H_2O_2 solution. Then all solutions were discarded and DCFH-DA solution with DMEM 100 µM, 100 µl/well was added to the cell culture. The cell culture was kept in a CO_2 incubator for 30 minutes. The solution in the 96 well/plate was discarded and washed with PBS. Finally, this 96 well/plate is used to measure the fluorescence wavelength 485±10 nm(excitation) and 530±12.5 nm(emission) each 5 minutes for 30 minutes by Microplate reader (Labsystems, Finland). Calculation % of DCF fluorescence by:

% DCF fluorescence = $(F_{t30}-F_{t0})/F_{t0} \times 100$

When F_{t30} = Fluorescence at 30 minutes, F_{t0} = Fluorescence at 0 minute

11. Lipid accumulation

11.1 Oil red O

Seeding 3T3-L1 adipocytes in 24-well plate until 100% confluence and differentiation for 9 days. Day 0 was treated by DMEM, 0.5 mM IBMX and 0.25 µm Dexamethasone with papaya 0.1,0.5, 1 mg/ml. Day 3 was treated by DMEM and 0.001 mg/ml insulin with papaya 0.1, 0.5, 1 mg/ml. Day 5, 7 and 9 were treated by DMEM with papaya 0.1, 0.5, 1 mg/ml.

11.2 Triglyceride assay

Seeding 3T3-L1 adipocytes in 12-well plate until 100% confluence and differentiation for 9 days. Day 0 was treated with DMEM,0.5 mM IBMX and 0.25 µm Dexamethasone with papaya 0.1,0.5, 1 mg/ml. Day 3 was treated by DMEM and 0.001 mg/ml insulin with papaya 0.1, 0.5, 1 mg/ml. Day 5,7 and 9 were treated by DMEM with papaya 0.1, 0.5, 1 mg/ml. After the cell culture was collected from the 12-well plate. Centrifuge 10,000 g for 20 minutes, twice. Then, supernatant were measured using commercial kits (HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany).

12. Determine the effect of papaya on gene expression in 3T3-L1 adipocytes

12.1 Extraction total RNA

Total RNA were extracted by RiboZol (AMRESCO, Canada) and added RiboZol 1 ml in a tube with a pellet of isolated cells from 6 well/plate. The cell was broken down by a mixed solution and incubated at room temperature for 5 minutes. Then, chloroform 0.2 ml was added and incubated for 2-3 minutes and centrifuge 12000xg at 4 °C for 15 minutes. The supernatant was put in a 1.5 ml tube and isopropanol 0.5 ml was added. It was centrifuged 12000x g at 4 °C for 8 minutes. Then the supernatant was discarded and the lower layer of RNA was retained. Pellet RNA was washed twice with 75% ethanol and then dried at room temperature. Total RNA was combined in solution with diethylpyrocarbonate (DEPC) water 20 µl and brought to a temperature of 55 °C for 10 minutes by heat block. The total RNA was measured in concentration and purified by nanodrop spectrophotometer wavelength 260/280 nm. Finally, the total RNA were collected and stored at -80 °C.

12.2 cDNA synthesis (Complementary DNA) by Reverse transcription(RT)

Reaction mixture were prepared by adding 5X RT Buffer 4 µl, dNTP mixture(10 mM) 2µl, RNase inhibitor(10 U/µl) 1 µl, Random Primer(25 pmol/ µl) 1 µl, Reverse Ace reverse transcriptase 1 µl (Bioline, USA) and total RNA 500 ng were adjusted by RNase free water until a total volume 20 µl was obtained. Then, it was incubated at 30 °C for 20 minutes and 42 °C for 20 minutes. Finally, it was collected cDNA solution at -20 °C for RT-PCR.

12.3 Determine gene expression of IL-6, TNF-α, SOD, CAT, PPARγ

cDNA reacted to PCR containing the master mix (1X) (Cat no.Bio-25047, Bioline, USA) 12.5 µl, forward primer and reverse primer (10 µM) each 0.75 µl, cDNA solution 2 µl and adjusted with distilled water without nuclease enzyme for a total volume of 25 µl. Then, it was put in a PCR machine. The PCR was set for thermo-cycling. The PCR product was measured by agarose gel electrophoresis technique with 2% agarose gel. The running buffer is 1X TBE buffer (1M Tris, 0.9 M boric acid and 1 mM EDTA). Stained DNA were measured by fluorescence colour (Biotechnology, Korea). DNA was measured and the target gene expression compared with β-actin as the housekeeping gene. Finally, it was analysed DNA bar by Image J.

Data analysis

Results were presented as the mean ± SEM. Statistical analyses were performed using IBM SPSS version 23. Group difference was assessed by a one-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons. A p-value < 0.05 was considered statistically significant.

CHAPTER IV

RESULTS

Effect of papaya correlation between body weight and liver weight

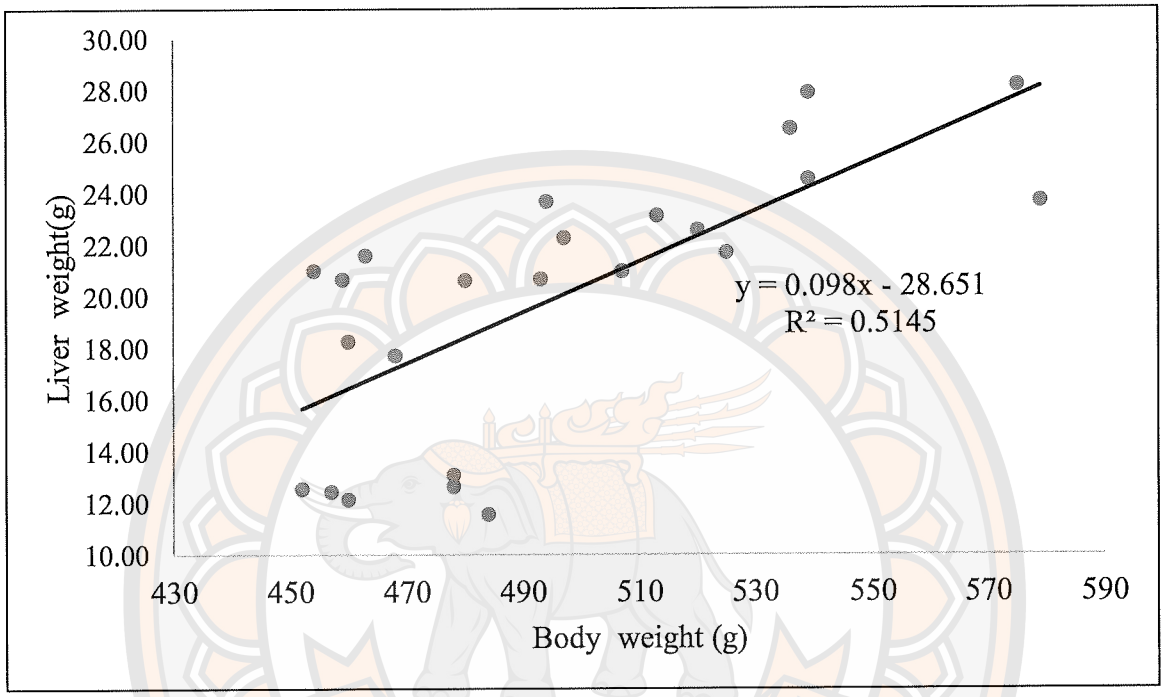


Figure 22 Relation between body weight and liver weight

The results of the relationship between liver weight and body weight showed that increasing liver weight can relate to increasing body weight (r^2= 0.5145). The high fat diet rats had a greater liver weight than normal diet rats.

Effect papaya on hepatic lipid content in rats

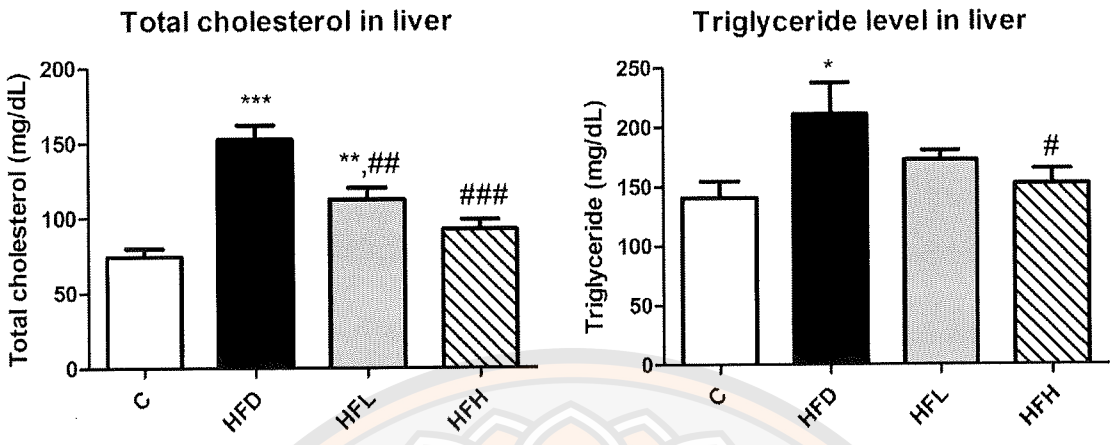


Figure 23 The effect of papaya on hepatic lipid content in rats. Data are expressed as mean ± SEM (n = 6-7). $^{*}p < 0.05$, $^{**}p < 0.01$ versus C , and $^{\#}p < 0.05$ versus HFD group C, Control; HFD, High fat diet; HFL, High fat diet treated with 0.5 mL of papaya juice/100g BW; HFH, High fat diet treated with 1 mL of papaya juice/100g BW

The result showed that papaya improved hepatic lipid contents in high fat diet-fed rats. The HFD group showed significantly increased hepatic TG and cholesterol levels when compared with the C. The TG levels were significantly decreased in the HFH ($p < 0.05$), while TC were significantly decreased in both the HFL ($p < 0.01$) and HFH ($p < 0.001$) when compared with the HFD group. The result indicated that papaya markedly reduced the hepatic TG and TC contents.

The effect of papaya on cecal lipid content in rats

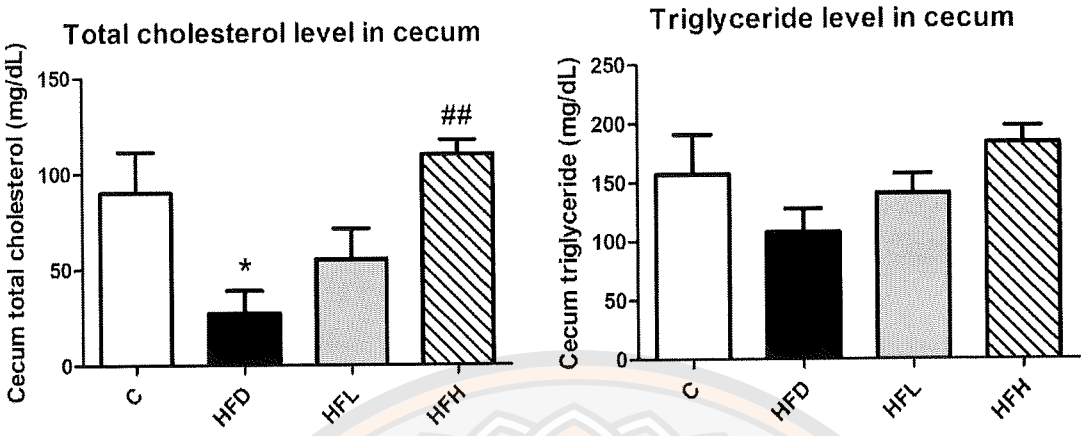


Figure 24 The effect of papaya on cecal lipid content in rats. Data are expressed as mean ± SEM (n = 6-7). $^{*}p < 0.05$ versus C , and $^{\#\#}p < 0.01$ versus HFD group C, Control; HFD, High fat diet; HFL, High fat diet treated with 0.5 mL of papaya juice/100g BW; HFH, High fat diet treated with 1 mL of papaya juice/100g BW

The result showed that papaya improved cecal lipid contents in high fat diet rats. The HFD group showed significantly decreased cecal cholesterol levels when compared with the HFH($p < 0.01$) , while the cecal triglyceride content in high fat diet rats trended to decrease when compared with the HFH. Also, papaya can increased lipid excretion to cecum.

Effect papaya on lipid peroxidation in rats

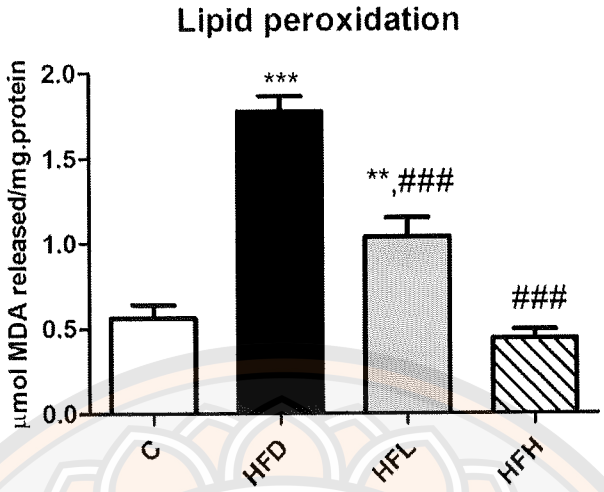


Figure 25 Effect papaya on lipid peroxidation in rats. Data are expressed as mean ± SEM (n = 6-7). $^{**}p < 0.01$ and $^{***}p < 0.001$ versus C , and $^{\#\#\#}p < 0.001$ versus HFD group C, Control; HFD, High fat diet; HFL, High fat diet treated with 0.5 mL of papaya juice/100g BW; HFH, High fat diet treated with 1 mL papaya juice/100g BW

The result showed that papaya improved lipid peroxidation in high fat diet-fed rats. The HFD group showed significantly increased lipid peroxidation when compared with the C ($p < 0.001$)

Effect papaya on enzymatic antioxidant in rats

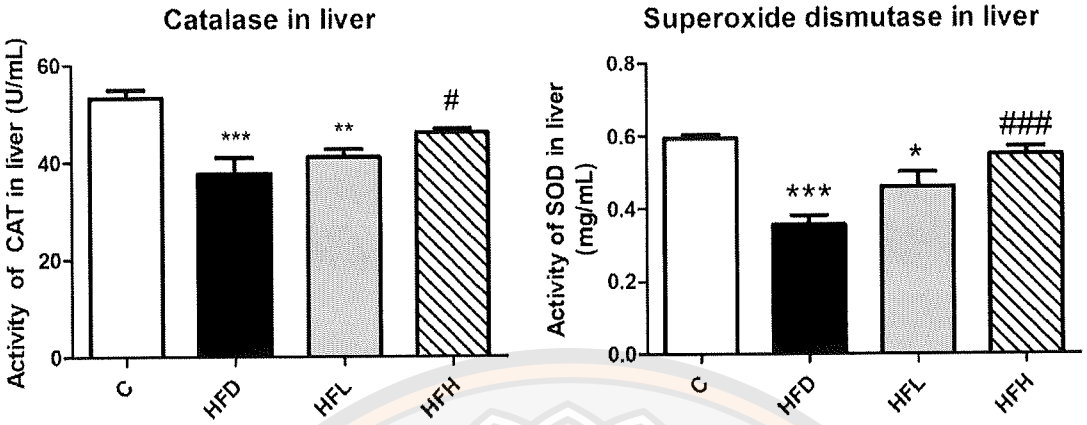


Figure 26 The effect papaya on enzymatic antioxidant in rats. Data are expressed as mean ± SEM (n = 6-7). $^{*}p < 0.05$,$^{**}p < 0.01$ and $^{***}p < 0.001$ versus C , and $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ versus HFD group C, Control; HFD, High fat diet; HFL, High fat diet treated with 0.5 mL of papaya juice/100g BW; HFH, High fat diet treated with 1 mL of papaya juice/100g BW

The lipid peroxidation were significantly decreased in the HFD treated with papaya 0.5 and 1 mL/100 g body weight ($p < 0.001$) when compared with the HFD group. In contrast, the CAT and SOD activities were found to decrease in the HFD group whereas those significantly increased in HFL and HFH groups.

The effect of papaya on inflammation in rats

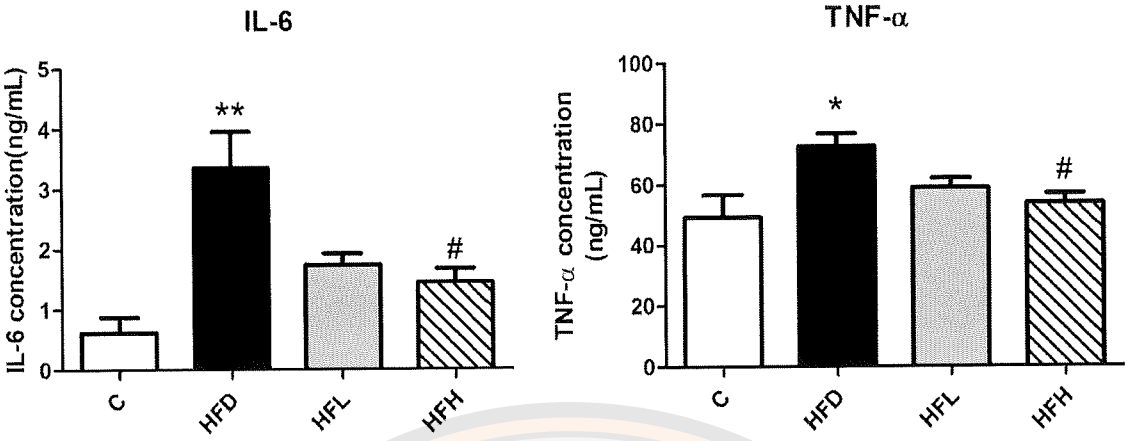


Figure 27 Effect papaya on inflammation in rats. Data are expressed as mean ± SEM (n = 6-7). $^{*}p < 0.05$,$^{**}p < 0.01$ and $^{***}p < 0.001$ versus C , and $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ versus HFD group C, Control; HFD, High fat diet; HFL, High fat diet treated with 0.5 mL of papaya juice/100g BW; HFH, High fat diet treated with 1 mL of papaya juice/100g BW

The presence of proinflammatory cytokines in liver tissues were determined by ELISA. The results showed that high fat diet rats significantly increased the serum levels of TNF- α and IL-6 while these two cytokine levels significantly decreased in the HFD treated with papaya 1 mL/100 g body weight ($p < 0.05$).

Effect papaya on liver function test

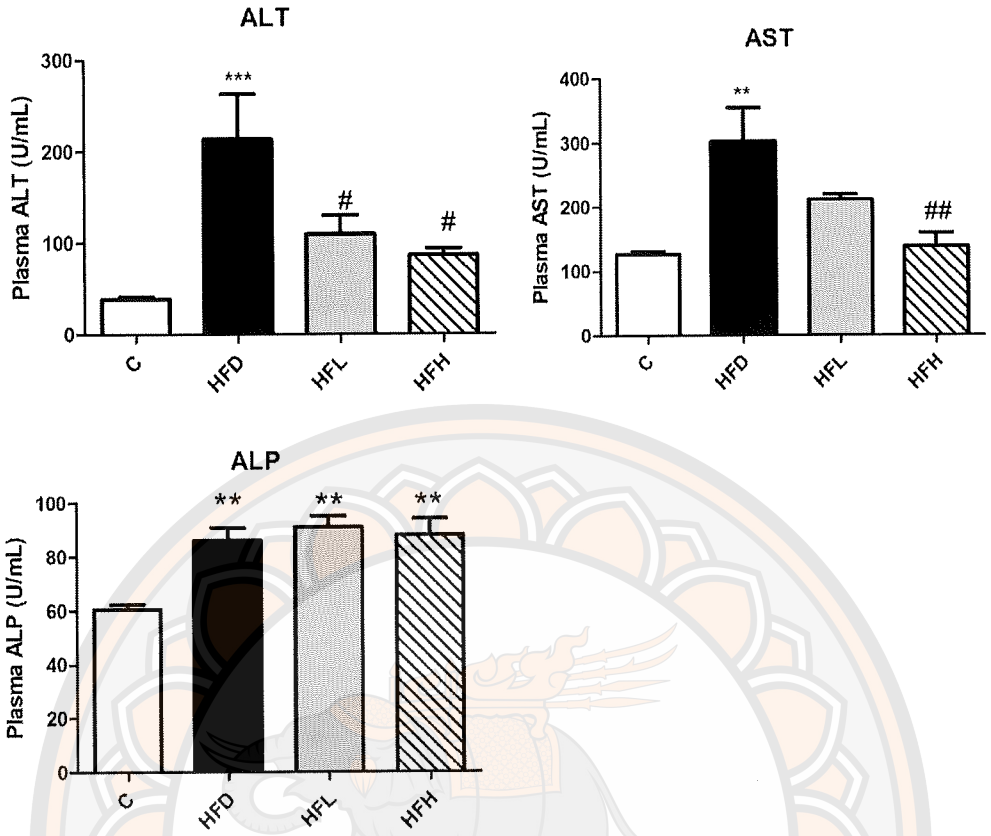


Figure 28 Effect papaya on liver function test in rats. Data are expressed as mean ± SEM (n = 6-7). $^{**}p < 0.01$ and $^{***}p < 0.001$ versus C , and $^{\#\#}p < 0.01$ versus HFD group C, Control; HFD, High fat diet; HFL, High fat diet treated with 0.5 mL of papaya juice/100g BW; HFH, High fat diet treated with 1 mL of papaya juice/100g BW

The serum levels of AST, ALT and ALP were significantly increased in rats fed with a high fat diet. Higher levels of those enzymes suggest that high fat diet can induce liver inflammation or liver damage. Moreover, the liver damaged indices also significantly decreased in the papaya treated group when compared to the HFD group. This result suggests that papaya administration may improve liver injury that found in NAFLD.

Histological analysis by Oil rad O staining

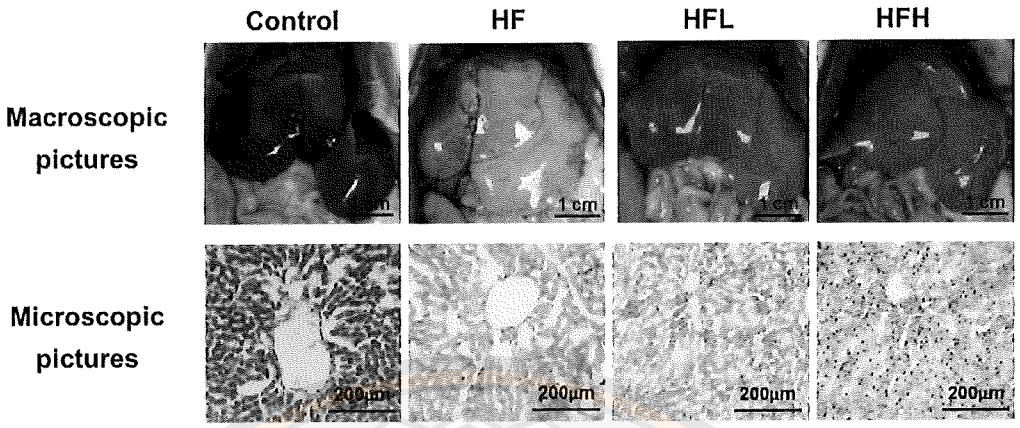


Figure 29 Effect papaya on lipid accumulation in rats when staining by oil red O

Histological analysis by haematoxylin & eosin staining

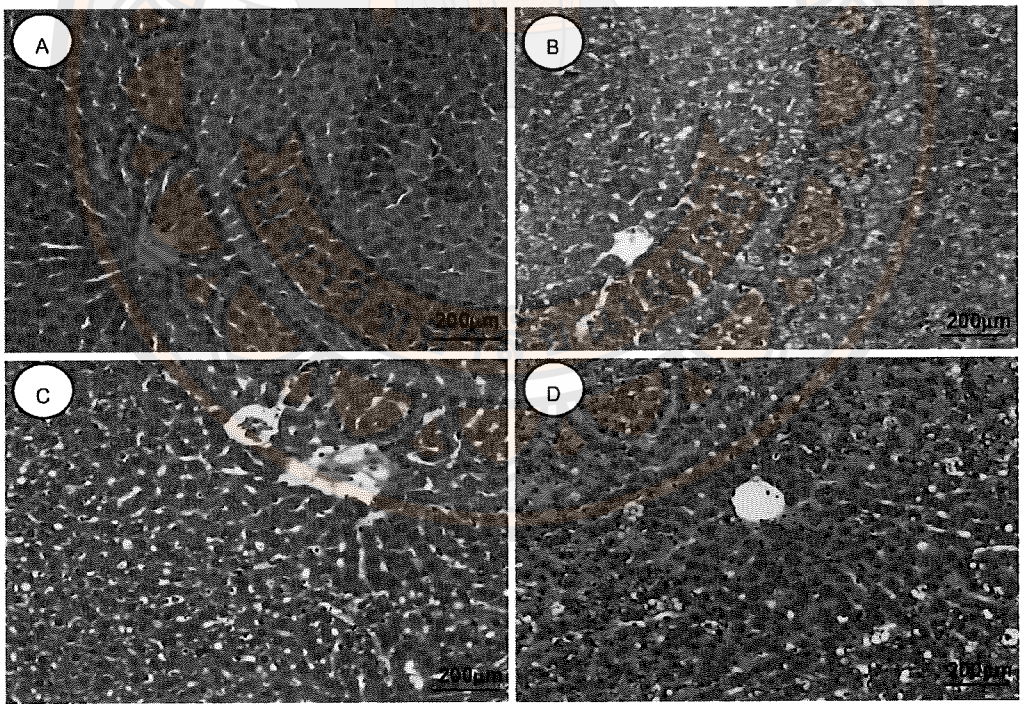


Figure 30 Effect papaya on lipid accumulation in rats when staining by H&E
A) Control group, B) HFD group, C) HFL group, D) HFH group

Table 3 Analysis NAS scores for all groups

Experimental groups	Steatosis	Lobular inflammation	Hepatocyte Ballooning	NAS
Control	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
HFD	2.17±0.75***	2.00±0.63***	1.83±0.41***	6±1.79***
HFL	1.67±1.21**	1.33±1.03*	1.33±0.52***	4.33±2.76***
HFH	1.17±0.75	1.00±0.89	1.50±0.55***	3.67±2.19***,#

Data are expressed as mean ± SEM (n = 6-7). $^{**}p < 0.01$ and $^{***}p < 0.001$ versus C , and $^{\#\#}p < 0.01$ versus HFD group C, Control; HFD, High fat diet; HFL, High fat diet treated with 0.5 mL of papaya juice/100g BW; HFH, High fat diet treated with 1 mL of papaya juice/100g BW

Effect papaya on gene expression in liver tissue (Lipogenic gene)

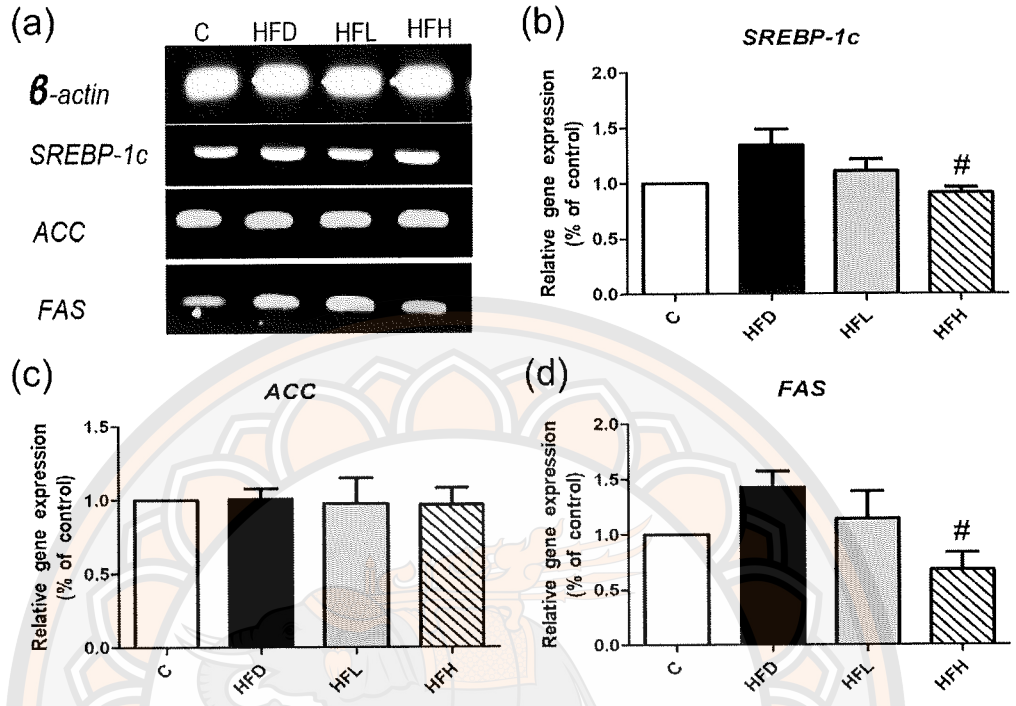


Figure 31 The effect of papaya on lipogenesis gene expression in rats when measure by PCR

SREBP-1c, ACC and *FAS* are the important genes involved in the regulation of hepatic lipogenesis. The mRNA expression of *SREBP-1c* and *FAS* had a tendency to increase in HFD rats as compared with the control, whereas the significant decreased expression of those genes were observed in HFH rat ($p < 0.05$). In contrast, the expression of *ACC* was not different among the groups.

Antioxidant activity of papaya

Table 4 Antioxidant activity of papaya

	Antioxidant of papaya
ABTS	93 mM Trolox/g of papaya
DPPH IC50	12.84 mg/ml of papaya
Total phenolic content	56 ± 0.01 mg GAE/100 g

Results are means ± SEM of three independent experiments with triplicate analysis.

The results obtained with ABTS method were presented on Table 4 had 93 mM Trolox/g of papaya. The free radical scavenging potential of papaya at different concentrations was tested by the DPPH method. Papaya had scavenging effects against DPPH radicals, IC50 12.84 mg/ml of papaya (Table 4). Total phenolic content (mg GAE/100 g) of the papaya are shown in the Table 1, papaya had total phenolic content 56 ± 0.01 mg GAE/100 g.

Effect of papaya to cytotoxicity test

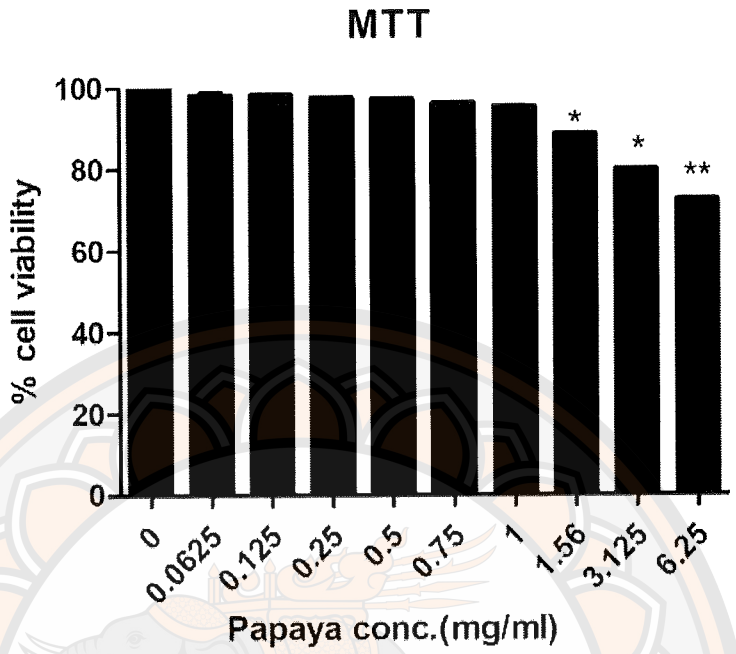


Figure 32 Effect of papaya to cytotoxicity test. * $p < 0.05$ and ** $p < 0.01$ when compare with control group.

The effect of papaya to cytotoxicity test can check whether this anti-adipogenic effect of papaya is due to its cytotoxicity, the effect of papaya on cell viability was measured by MTT assay. Our results showed that papaya does not exhibit cytotoxicity up to 1 mg/ml of papaya concentration suggesting that anti-adipogenic of papaya is not from cytotoxicity, but through the regulation of another molecular mechanism. Together, these results suggest that papaya has antiadipogenic effects in 3T3-L1 preadipocytes.

Effect of papaya to ROS production in 3T3-L1 adipocytes

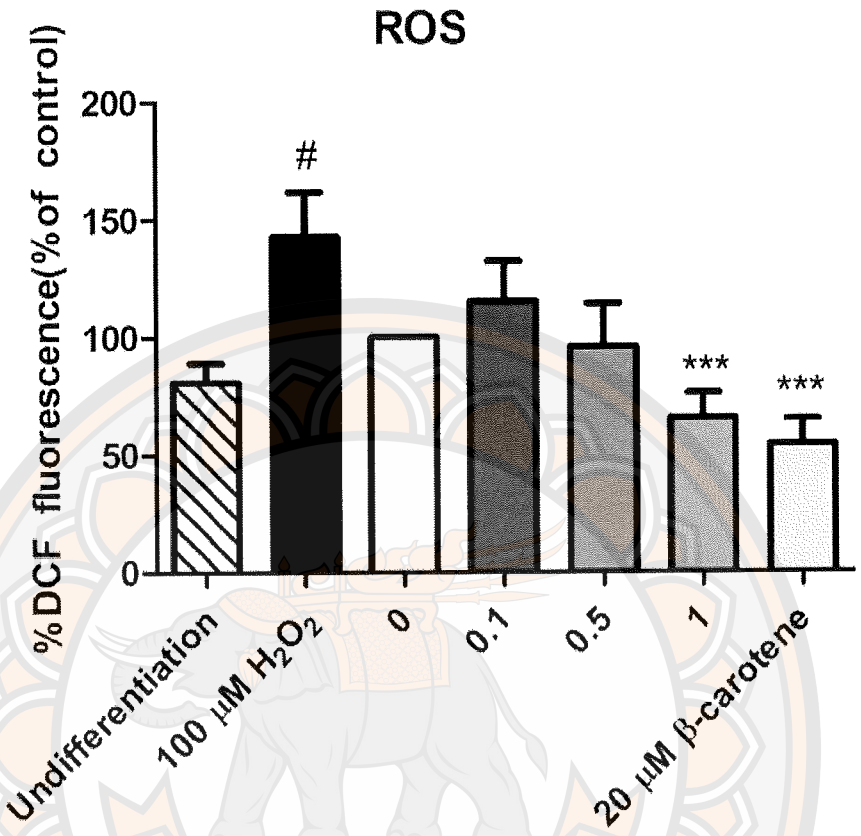


Figure 33 ROS measurement by DCFH-DA assay. # $p < 0.05$ when compared with an undifferentiated group, *** $p < 0.001$ when compare with 100 μM H_2O_2 group.

The effect of papaya to ROS production in 3T3-L1 adipocyte (Figure33) found that papaya to reduce H_2O_2 induced ROS production, we use the fluorescence probe DCFH-DA. Our results showed that the adipocyte cells exposed to H_2O_2 showed an increase in the intracellular level of ROS compared to the control. However, treated cells with papaya reduced significantly ($p < 0.001$) the ROS level of about 65.6% DCF fluorescence (% of control) at 1 mg/ml of papaya concentration.

Effect papaya on lipid accumulation by using Oil red O staining in 3T3-L1 adipocytes

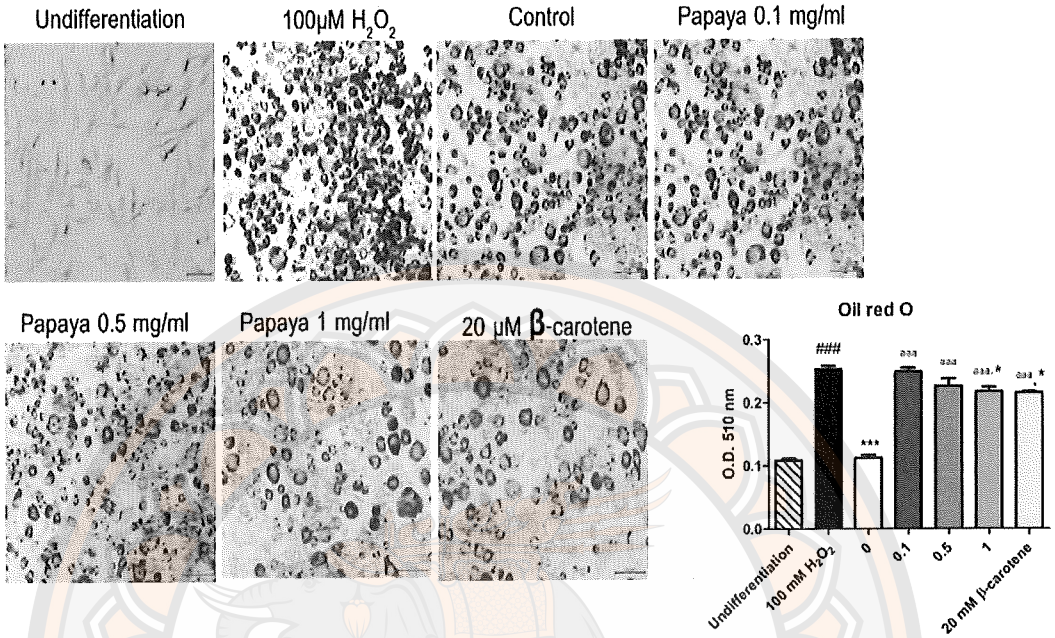


Figure 34 Effect papaya on lipid accumulation by using Oil red O staining in 3T3-L1 adipocytes. Graph of Oil red O staining in 3T3-L1 adipocytes. ### is $p < 0.001$ when compared with undifferentiation group, aaa is $p < 0.001$ when compare with control group.* is $p < 0.05$, * is $p < 0.001$ when compare with 100 μM H_2O_2 group.**

The effect papaya on lipid accumulation by using Oil red O staining in 3T3-L1 adipocytes(Figure 34) for investigate the anti-adipogenic effect of papaya, 3T3- L1 preadipocytes were induced to differentiate with H_2O_2 in the presence or absence of papaya, and cells were stained with oil red O solution. Our results showed that papaya dose-dependently inhibits adipogenesis of 3T3- L1 preadipocytes. Intracellular lipid accumulation was reduced after papaya treatment by 0.1, 0.5 and 1 mg/ml of papaya respectively compared to control group.

Effect of papaya to triglyceride level in 3T3-L1 adipocytes

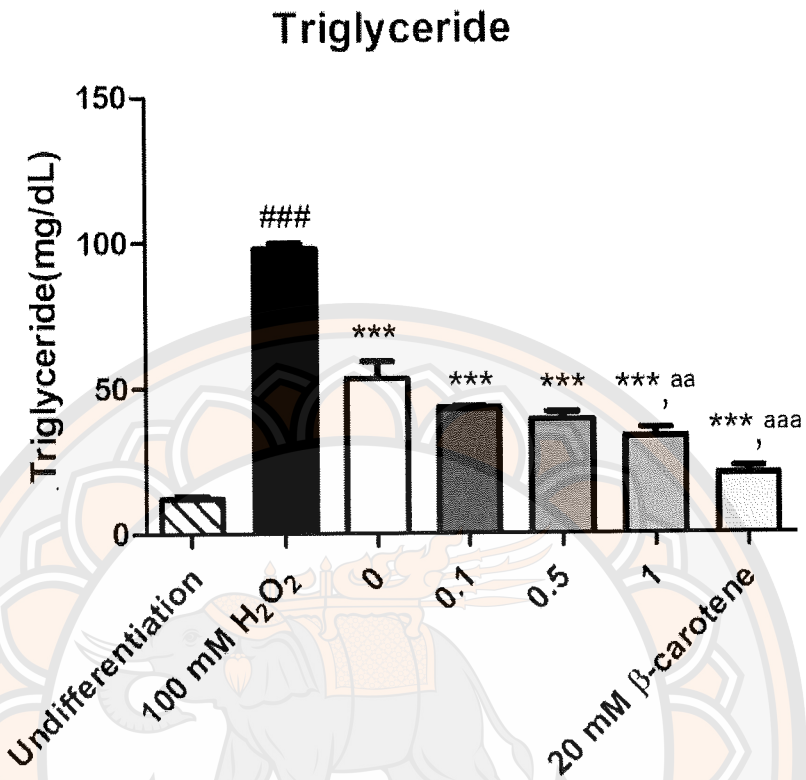


Figure 35 Effect of papaya to triglyceride level in 3T3-L1 adipocytes. Graph of triglyceride level in 3T3-L1 adipocytes. ### is $p < 0.001$ when compared with undifferentiated group, aa is $p < 0.01$, aaa is $p < 0.001$ when compare with control group. * is $p < 0.001$ when compare with 100 µM H_2O_2 group.**

The effect of papaya to triglyceride level in 3T3-L1 adipocytes (Figure 35) about the anti-adipogenic effect of papaya, 3T3- L1 preadipocytes were induced to differentiate with H_2O_2 in the presence or absence of papaya, and the lipid accumulation of cells were measured with triglyceride assay kits. The results showed that papaya dose-dependently inhibits adipogenesis of 3T3- L1 preadipocytes. Intracellular lipid accumulation was reduced after papaya treatment by 1 mg/ml of papaya compared to control group ($p<0.01$).

Effect of papaya to inflammation gene expression in 3T3-L1 adipocytes

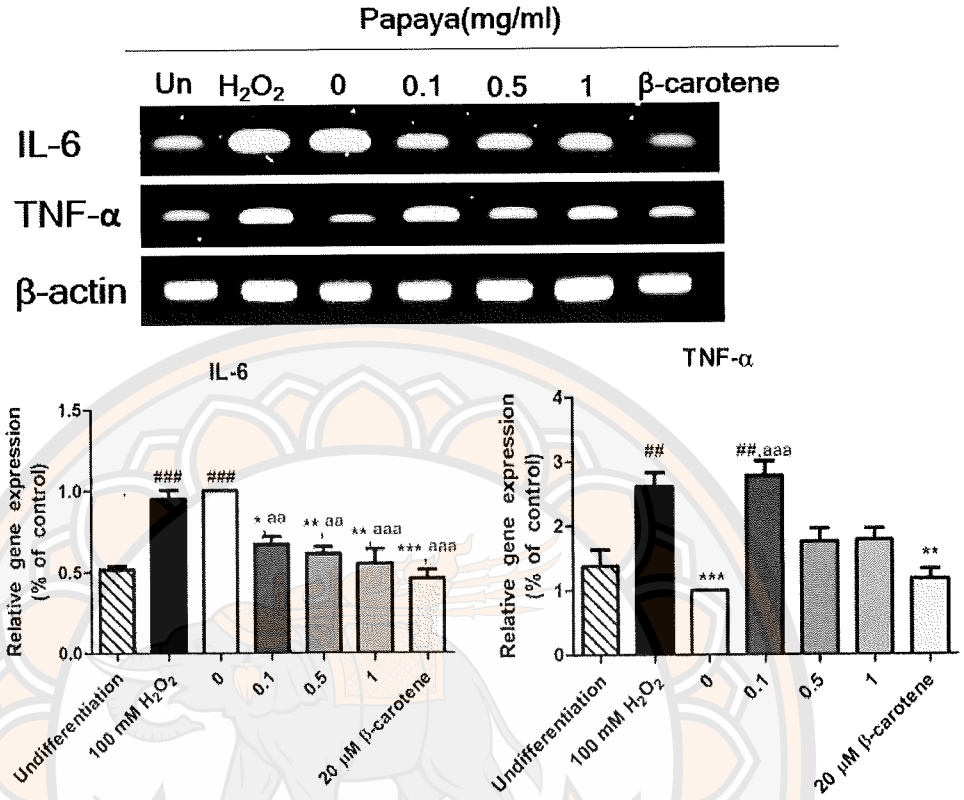


Figure 36 The effects of papaya on pro-inflammation (*IL-6 and TNF-α*) gene gene expression in 3T3-L1 adipocytes. ### is $p < 0.001$ when compared with undifferentiation group, a is $p < 0.05$, aa is $p < 0.01$, aaa is $p < 0.001$ when compare with control group. *is $p < 0.05$, ** is p < 0.01, * is p < 0.001 when compare with 100 mM H_2O_2 group.**

IL-6 and TNF-α are important genes involved in the inflammation gene. The mRNA expression of *IL-6 and TNF-α* had increase in 100 mM H_2O_2 group as compared with the control. A significantly decreased expression of *IL-6* genes were observed in treat papaya (0.1,0.5, and 1mg/ml)group ($p < 0.05$ and $p < 0.01$) as shown in Figure5a. In contrast, the expression of *TNF-α* was not different among the groups (Figure 36).

Effect of papaya to antioxidant gene expression in 3T3-L1 adipocytes

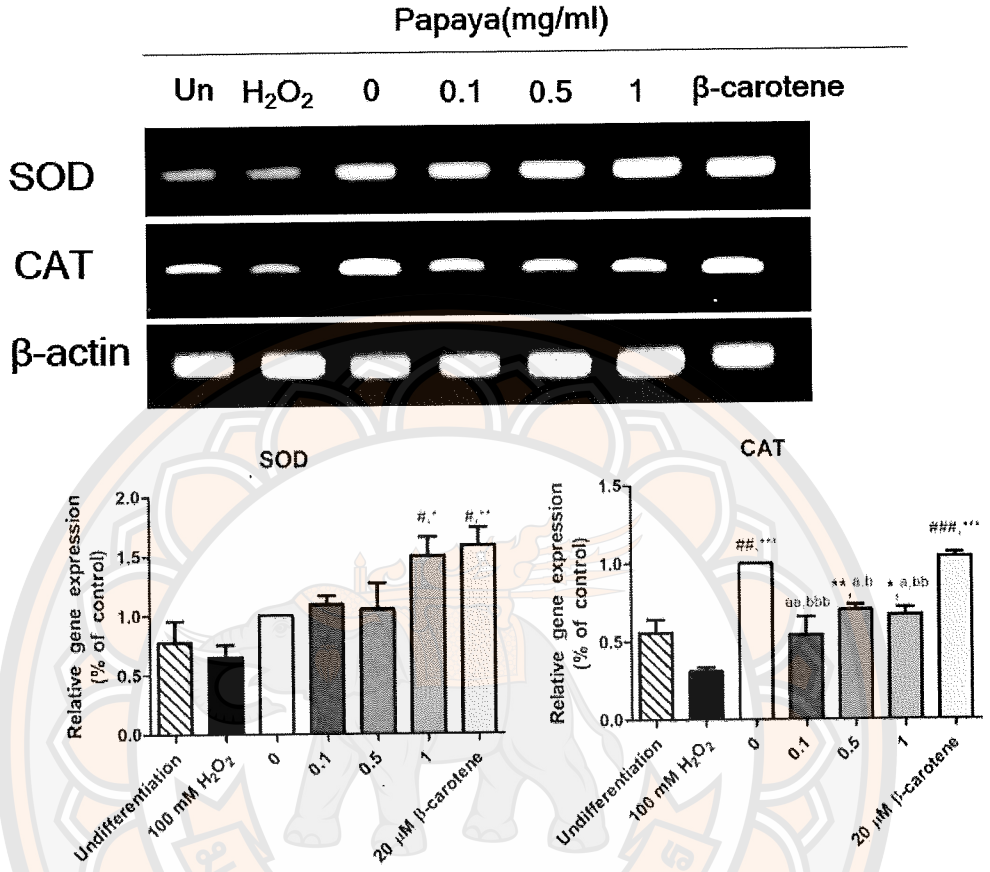


Figure 37 The effects of papaya on antioxidant (*CAT and SOD*) gene expression in 3T3-L1 adipocytes. ### is $p < 0.001$ when compare with undifferentiated group, a is $p < 0.05$, aa is $p < 0.01$, aaa is $p < 0.001$ when compare with control group. *is $p < 0.05$, ** is $p < 0.01$, * is $p < 0.001$ when compare with 100 μM H_2O_2 group.**

CAT and *SOD* are important genes involved in the antioxidant gene. The mRNA expression of *CAT and SOD* had decrease in 100 mM H_2O_2 group as compared with the control. The mRNA expression of *CAT* and *SOD* had a tendency to increase in treat papaya (0.1,0.5, and 1mg/ml)group as compared with the control. The result showed that papaya improved antioxidant gene in the *CAT* and *SOD* activities (Fi 37).

CHAPTER V

DISCUSSION AND CONCLUSION

Fat accumulation in the liver of non-alcoholic fatty liver disease (NAFLD) is mainly in the form of triglycerides (Simon et al., 2018). Triglyceride accumulation is a mechanism to balance free fatty acid (FFA) excess. Previous evidence suggests that liver steatosis generates toxic metabolites, lipotoxicity, which could result in liver injury (Bilgic, Gelecek, Akgun, & Ozmen, 2014). This result shows that papaya can significantly reduce hepatic triglyceride content with high doses of papaya treatment (1 ml/100g bodyweight) and both low doses (0.5 ml/100g bodyweight) and high doses (1 ml/100g bodyweight) of papaya decrease hepatic total cholesterol level. It is related to the study of (Pan, Lai, Tsai, & Ho, 2014) that β-carotene can prevent and improve NAFLD by decreasing triglyceride accumulation in liver tissue. Hepatic steatosis is a simple form of fatty liver disease, and the accumulation of triglyceride-rich lipid droplets within hepatocytes. The high-fat diet is a model that induces obesity and NAFLD(Nakamura & Terauchi, 2013). Hepatic triglyceride formation is related to diet, de novo lipogenesis, and adipose tissue lipolysis (Moore, Gunn, & Fielding, 2014). In this study, the papaya significantly decreased the level of hepatic triglyceride. Accumulating studies revelled that a high fat diet which induced obesity is associated with abnormal hepatic lipid metabolism (Cordeiro, Pereira, Saboya, & Ramalho, 2015). The high fat diet causes an increased lipid intake into the liver (Moro et al., 2016). We found that papaya can decrease hepatic triglyceride and total cholesterol levels in high fat diet rats with a significant decrease in liver weight, indicating papaya reduced high fat diet induced hepatic steatosis by decreasing lipid transport into the liver. Obesity is also related to non-alcoholic fatty liver disease (NAFLD). It increases the triglyceride content in the human body that causes steatosis, and is also associated with inflammation and fibrosis causing steatohepatitis (McPherson et al., 2015). In this current study, high fat diet fed rats showed that the development of NAFLD was further ameliorated by papaya.From the results, papaya at 1 ml/100 g bodyweight was shown to increase lipid excretion of total cholesterol. Papaya demonstrated that it could lower the absorption of cholesterol and triglyceride in rats. The micellar solubilization of

hydrolyzed lipids, is a critical process which facilitates the uptake and absorption of them by enterocytes.

Papaya was shown to decrease cholesterol absorption by forming insoluble co-precipitates of it, and decreasing bile acid-induced micellar solubility by binding them together, thereby increasing fecal excretion (Coreta-Gomes, Vaz, Wasielewski, Geraldes, & Moreno, 2016).

Oxidative stress, results from an imbalance between ROS, prooxidant, and antioxidant chemical species, that leads to cellular damage (Scott, Rangaswamy, Wicker, & Izumi, 2014). The NAFLD group decreases antioxidant enzyme activities, which include catalase (CAT) and superoxide dismutase (SOD). In our study, CAT and SOD enzyme activity was higher in the HFH group, and was in agreement with a previous report also using a high fat diet (Feksa et al., 2018). NAFLD increased liver oxidative stress as evidenced by increasing the MDA level and the inhibition of antioxidant enzymes in the liver (Surapaneni & Jainu, 2014). Treatment with papaya was able to significantly decrease MDA levels, consequently lipid peroxidation, and oxidative stress. Moreover, increased activities of plasma ALT, AST and ALP enzymes were also observed in high fat diet fed rats. Oxidative stress causes tissue damage in the liver, which may increase the markers enzyme activities in the plasma, and this increases the leakage of ALT and AST from the liver into the blood stream (Tahaei, Mohebbi, & Zali, 2012). Papaya improved the liver function by normalizing its weight and the plasma activities of ALT, AST and ALP. The local macrophages which are also known as Kupffer cells, can recruit monocytes in the injured area of the liver. In this study, high fat diet feeding of rats showed a massive recruitment of inflammatory cells in the liver, followed by an increase in the extracellular matrix deposition which supports the notion of hepatic injury, but the use of papaya prevented it.

The protective effect is mediated due to the restoration of the antioxidant capacity and lipid lowering effect of papaya. Cytokines as TNF-α and IL-6 play a key role in the pathogenesis of many metabolic diseases through either its inhibitory or pro-inflammatory action(Assuncao et al., 2018). Because of the anti-inflammatory properties of papaya, it is assumed that it might be effective in managing NAFLD and restraining its progression. Previous animal studies showed that papaya protects the liver

against steatosis and fibrosis. In contrast, available evidence was not sufficient to show a beneficial effect of papaya in decreasing the cytokines level among NAFLD.

The results of the study showed that papaya administration to high fat diet rats, improved their liver injury and hepatic steatosis, and attenuated the production of hepatic SREBP-1c and FAS expression. When on a high fat diet, FAS appears in hepatic lipid metabolism by stimulating the de novo lipogenic activity in the liver. The high fat diet causes excessive fat accumulation, which increases hepatic de novo lipogenesis and impaired triglyceride (Bae et al., 2018). Our result verified that hepatic TC levels and fat droplets in high fat diet rats were reduced by being treated with papaya, which may down-regulated SREBP-1c and its downstream FAS (Fu, Cui, & Zhang, 2018). Our previous study suggested that abnormal accumulation of triglyceride and cholesterol is associated with disorders in the lipid transporters (Mayes, 1969). The histopathological observation of oil red O and H&E staining on the liver, further confirmed that papaya could improve the hepatic steatosis in rats that was caused by their high fat diet.

Papaya contain several antioxidants, including carotenoids (β-carotene, α-carotene, β-cryptoxanthin, α- cryptoxanthin, lutein, 9-cis-β-carotene), phenolic compounds (ferulic acid, caffeic acid, p-coumaric acid, rutin, quercetin, kaempferol) and glucosinolates (benzyl glucosinolate, benzyl isothiocyanate) (Nguyen TTetal.,2013), that might have effective antioxidant effects. This study showed that papaya has active ingredients, carotenoids which include lutein and zeaxanthin. Carotenoids have the ability to reduce the severity of free radicals and to isolated oxygen radicals. Carotenoids therefore have antioxidant activity (Hamed Safafar, 2015).

Adipocyte differentiation and fat deposition are correlated to the emergence and development of obesity (Gesta, Tseng, & Kahn, 2007). In this study, the effect of papaya in the 3T3-L1 preadipocyte model was examined, with the results showing that it suppressed lipid accumulation in a dose-dependent manner, and inhibited triglyceride accumulation when treated with a high dose of papaya compared to the control group ($p<0.01$) without any toxicity at high concentrations. Obesity is usually associated with increased mitochondrial ROS production, causing oxidized lipids, synthesis of faulty proteins, and mtDNA mutations. Moreover, it has been established that high ROS production leads to a dysregulation of adipokine secretion and contributes to the production of pro-inflammatory cytokines such as the tumor necrosis factor- α (TNF-α)

and interleukin-6 (IL-6) (Ouchi, Parker, Lugus, & Walsh, 2011). In these results, we found that papaya decreased the intracellular ROS levels induced by hydrogen peroxide and decreased the mRNA gene levels of TNF-α and IL-6 at high concentrations. Similarly, mature adipocytes treated with papaya decreased TNF-α secretion. Moreover, papaya can improve antioxidant gene expression such as superoxide dismutase (SOD) and catalase (CAT) in the 3T3-L1 adipocytes model. The result showed that in the treatment group papaya increased SOD and CAT expression when compared with the 100 μM H_2O_2 group.

Conclusion

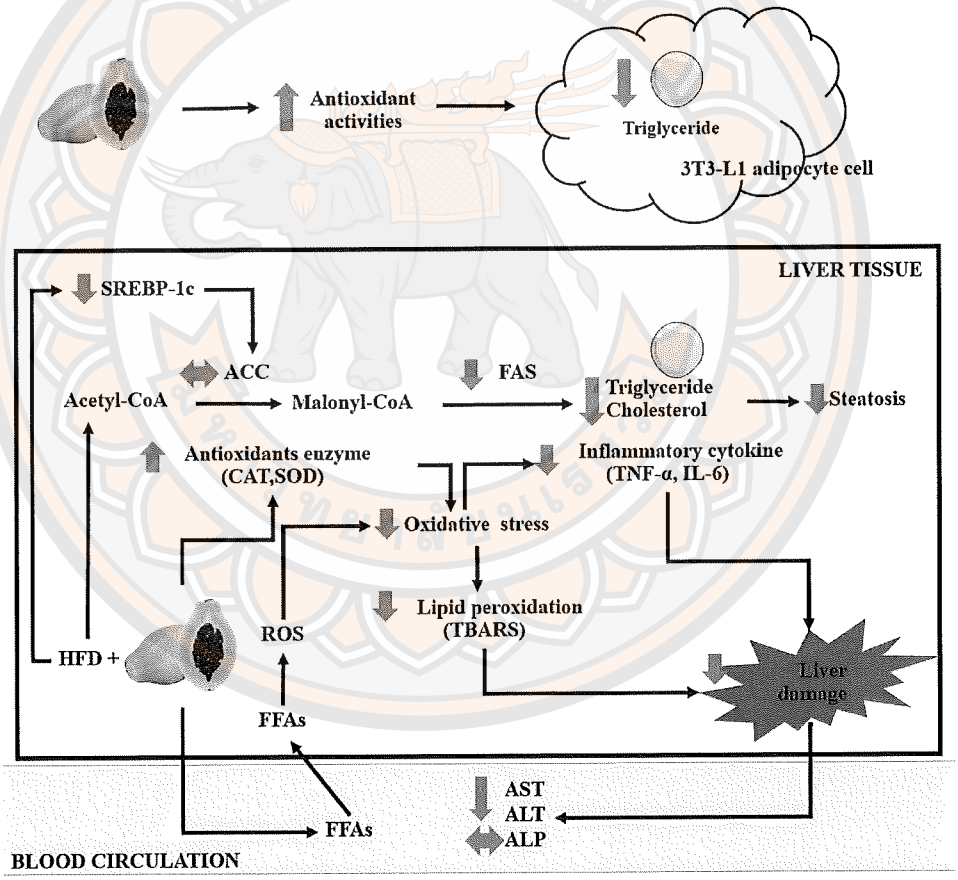
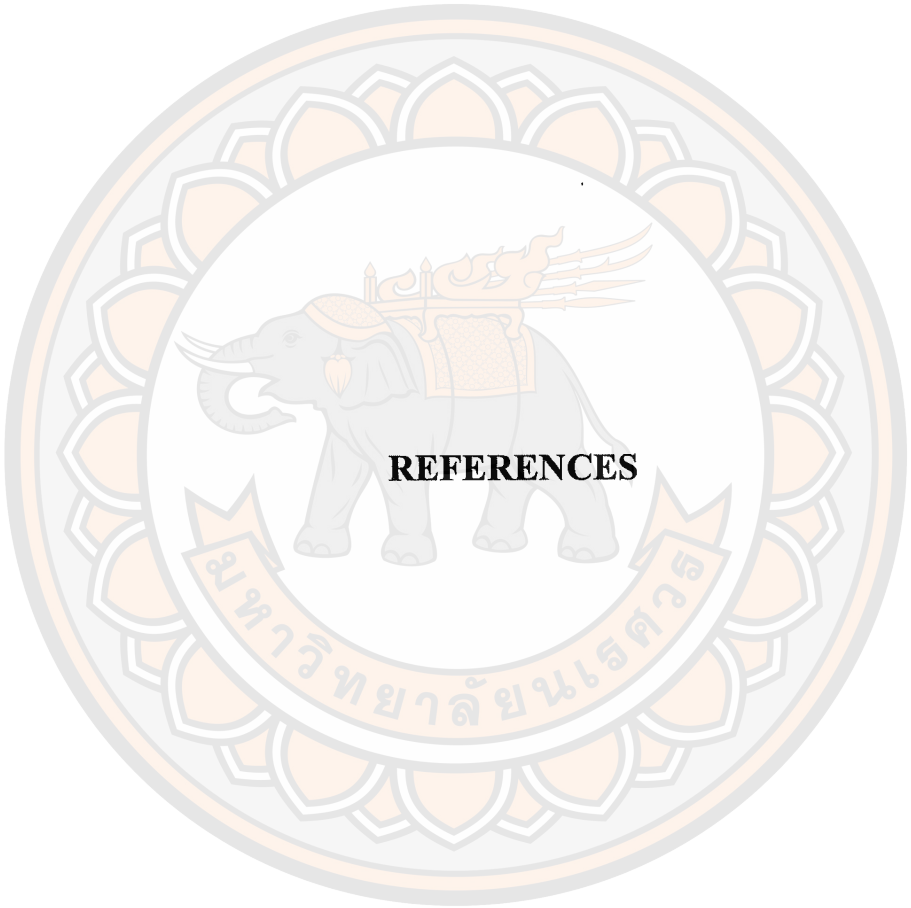


Figure 38 The proposed mechanism of NAFLD induction by high fat diet in obese rats and H_2O_2 in 3T3-L1 adipocytes

Papaya is a source of natural dietary antioxidants. This study was to explore the antioxidative effects of papaya in 3T3-L1 adipocytes, as considered as a promising

strategy to fight against obesity. Antioxidant papaya effect was evaluated by determining the antioxidant assay using ABTS, DPPH and total phenolic content assay. Induction of oxidative stress in 3T3-L1 adipocytes were measure by DCFH-DA assay. Oil red O staining and cellular levels of triglyceride (TG) were used to evaluate the in vitro anti-obesity effects of papaya in 3T3-L1 cells. The expression levels of antioxidant and inflammation genes using reverse transcriptase polymerase chain reaction. Papaya which have antioxidant effect significantly attenuated triglyceride level and decrease expression of antioxidant gene (SOD, and CAT) and inflammation gene (IL-6, and TNF-α). Papaya also reduced the production and release of reactive oxygen species. The current data suggest possible roles for papaya in 3T3-L1 adipocytes. Thus, papaya may be explored as a potentially promising therapeutic agent for the prevention of obesity. The beneficial effect of papaya against hepatic steatosis in obese rats may occur through the inhibition of lipogenic pathways by reducing *SREBP-1c* and *FAS* gene expression causing the reduction of hepatic fat accumulation. Papaya can improve enzymatic antioxidants (CAT and SOD) and decrease lipid peroxidation in the liver. The administration of papaya significantly decreased proinflammatory cytokines such as TNF-α and IL-6 to modulate liver damage. Papaya is therefore able to reduce the activities of AST and ALT in serum. Overall, this study provides evidence for the beneficial effects of papaya to reverse the progression of NAFLD in obese rats.



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APPENDIX

APPENDIX A PREPARATION OF 1X PBS

Table 5 Preparation of 1X PBS (1 litre)

Reagent	Amount (g)
NaCl	8
KCl	0.2
Na_2HOP_4	1.44
KH_2PO_4	0.24

Recipe for 1 litre of 1X phosphate buffered saline (PBS) solution starts with 800 ml of distilled water. Dissolve the solution as described above, and then adjust pH stock is will be approximately 7.4. If necessary, pH can be adjusted using hydrochloric acid or sodium hydroxide. Dispense the solution into aliquots and sterilize by autoclaving (15 min, 121°C, liquid cycle).

APPENDIX B PROCESS OF OIL RED O STAINING

1. Prefix Frozen Section by wash tissue with PBS or Normal saline 10 minutes 3 times
2. If embedding in OCT, need sucrose cryoprotection by

    Fix in 15% sucrose for 3 hours

    Fix in 30% sucrose for 10 hours

    Embed in OCT and freeze in -20 ºC
3. Cut 5 µm thick cryostat sections for liver and mount on silane coat slides.
4. Store slides at -20 ºC until needed. The slides can be store at -20 ºC for short term storage (within a few weeks).
4. Before staining, warm slides at room temperature for 30-60 minutes
5. Wash in distilled water 2 minutes and proceed to standard staining procedure.

<u>Oil Red O staining for frozen section</u>

1. Dip slide in distilled water for 1 minute
2. Soak slide in 60% isopropanol for 2 minutes at room temperature
3. Stain with Oil Red O working solution for 15 minutes
4. Soak slide in 60% isopropanol for 2 minutes at room temperature
5. Stain nucleus by glycerin jelly
6. Observe under the microscope

APPENDIX C PROCESS OF H&E STAINING

Table 6 Process of Haematoxylin &Eosin staining

	Solution	Time(s)
1. Deparafinization	Xylene	10
	Xylene	10
2. Rehydration	Isopropyl alcohol	10
	Isopropyl alcohol	10
	95% ethanol	10
	95% ethanol	10
	Tap water	60
3. H&E Staining	Haematoxylin	420
	Tap water	60
	Lithium carbonate	10
	Tap water	60
	95% ethanol	10
	Eosin	25
4. Dehydration	95% ethanol	10
	95% ethanol	10
	Isopropyl alcohol	10
	Isopropyl alcohol	10
	Isopropyl alcohol	10
5. Clearing	Xylene	10
	Xylene	10
	Xylene	10
6. Dehydration	95% ethanol	10
	95% ethanol	10
	Isopropyl alcohol	10
	Isopropyl alcohol	10
	Isopropyl alcohol	10

Table 6 (CONT.)

7. Clearing	Xylene	10
	Xylene	10
	Xylene	10
8. Deparafinization	Xylene	10
	Xylene	10
9. Rehydration	Isopropyl alcohol	10
	Isopropyl alcohol	10
	95% ethanol	10
	95% ethanol	10
	Tap water	60
10. H&E Staining	Haematoxylin	420
	Tap water	60
	Lithium carbonate	10
	Tap water	60
	95% ethanol	10
	Eosin	25
11. Dehydration	95% ethanol	10
	95% ethanol	10
	Isopropyl alcohol	10
	Isopropyl alcohol	10
	Isopropyl alcohol	10
12. Clearing	Xylene	10
	Xylene	10
	Xylene	10
13. Dehydration	95% ethanol	10
	95% ethanol	10
	Isopropyl alcohol	10
	Isopropyl alcohol	10
	Isopropyl alcohol	10

Table 6 (CONT.)

14. Clearing	Xylene	10
	Xylene	10
	Xylene	10

APPENDIX D PREPARATION ABTS RADICAL

Table 7 Preparation Trolox

Substances	Blank	1 (control)	2	3	4	5	6	Sample
Trolox (µl)	-	-	10 0.5 mM	10 1 mM	10 1.5 mM	10 2 mM	10 2.5 mM	-
Final Conc. (mM)	-	-	0.02	0.04	0.06	0.08	0.1	-
100% Methanol (µl)	250	10	-	-	-	-	-	-
Papaya extract (µl)	-	-	-	-	-	-	-	10
ABST radical (µl)	-	240	240	240	240	240	240	240
Total	250	250	250	250	250	250	250	250

APPENDIX E PREPARATION DPPH

Table 8 Preparation Vitamin C

Substances	Blank	C	1	2	3	4	5	6	sample
Final conc. (mg/ml)	-	-	-	4	10	20	30	40	-
Vitamin C (mg/ml)	- -	- -	- 0	10 100	10 250	10 500	10 750	10 1000	-
DPPH (µl)	-	250	140	140	140	140	140	140	140
100% MtOH (µl)	250	-	110	100	100	100	100	140	100
Papaya extract (µl)	-	-	-	-	-	-	-	-	10
Total	250	250	250	250	250	250	250	250	250

APPENDIX F PREPARATION FOLIN REAGENT (10 ml)

Table 9 Preparation of gallic acid

Substance	Blank	C	1	2	3	4	5	sample
Gallic acid (µl)	-	-	10	10	10	10	10	-
			0.2 mg/ml	0.4 mg/ml	0.6 mg/ml	0.8 mg/ml	1 mg/ml	
Final Concentration (mg/ml)	-	-	0.008	0.016	0.024	0.032	0.04	-
DW (µl)	225	225	215	215	215	215	215	215
Papaya extract (µl)	-	-	-	-	-	-	-	10
Folin working reagent (µl)	-	25	25	25	25	25	25	25
Total	250	250	250	250	250	250	250	250

APPENDIX G PRIMER FOR GENE EXPRESSION

Table 10 Primer for gene expression

Gene	Primer Sequence(5'→3')	PCR product (bp)	Reference
FAS	Forward primer: 5'GGACATGGTCACAGACGATGAC3' Reverse primer: 5'GTCGAACTTGGACAGATCCTTCA3'	94	(Xu et al., 2017)
SREBP -1c	Forward primer: 5' TGGATTGCACATTTGAAGACAT 3' Reverse primer: 5' GCTCCTCTTTGATTCCAGGC 3'	300	Lab design
ACC	Forward primer: 5' GCCTCTTCCTGACAAACGAG 3' Reverse primer: 5' TCCATACGCCTGAAACATGA 3'	101	(Xu et al., 2017)
SOD	Forward primer: 5' AGCGTGACTTTGGGTCTTTT3' Reverse primer: 5' CAGCAATCTGTAAGCGACCT3'	123	Design from AH0054432.2
CAT	Forward primer: 5'ACTTCTGGAGCCTACGTCCT 3' Reverse primer: 5'AAGTCTCGCCGCATCTTCAA 3'	209	Design from NM_001752.3
TNF-α	Forward primer: 5' AGCGTGACTTTGGGTCTTTT 3' Reverse primer: 5' CAGCAATCTGTAAGCGACCT 3'	129	Lab design

Table 10 (CONT.)

IL-6	Forward primer: 5' ACTTCTGGAGCCTACGTCCT 3' Reverse primer: 5' AAGTCTCGCCGCATCTTCAA 3'	130	Lab design
B-actin	Forward primer: 5'TGTCCACCTTCCAGCAGATGT 3' Reverse primer: 5' AGCTCAGTAACAGTCGCGCTAGA3'	101	Ho et.al.,2012

APPENDIX H PREPARATION REVERSE TRANSCRIPTION REAGENT

Table 11 Preparation reverse transcription reagent

Reagents	Volume(µl)
5X Buffer	4
dNTP(10 mM each)	2
RNase inhibitor (100 U/µl)	1
Random primer (25 ρmol/µl)	1
Reverta Ace (Reverse transcriptase)	1
RNase Free water	8
RNA sample	3
Total volume	20

APPENDIX I PREPARATION PCR REAGENT

Table 12 Preparation Polymerase chain reaction (PCR) reagent

Reagents	**Volume(µl)**
Master mix	2
Forward primer	0.25
Reverse primer	0.25
cDNA	2
Water	7.5
Total volume	12