THE EFFECTS OF LAURIC ACID ON PI3K AND NFκB SIGNALING PATHWAYS IN ALCOHOL-INDUCED HEPG2 CELLS

By

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ABSTRACT

THE EFFECTS OF LAURIC ACID ON PI3K AND NFκB SIGNALING PATHWAYS IN ALCOHOL-INDUCED HEPG2 CELLS

LUA YING HUAN

The adverse consequences of alcoholism have drawn scientific attention to investigate the underlying mechanism in alcohol metabolism. The metabolism of alcohol involves cytochrome P450 2E1 (CYP2E1)-induced oxidative stress, with the association of phosphatidylinositol-3-kinases (PI3K) and nuclear factor kappa B (NFκB) pathways. These pathways are important in regulating cellular survival, proliferation and inflammation. Lauric acid, a major fatty acid in palm kernel oil, has been shown as a potential antioxidant. This leads to the main objectives of this study which were to investigate the possible involvement of PI3K and NFκB pathways in acute ethanol-induced HepG2 cells and the potential effect of lauric acid on these pathways. In this study, HepG2 cells were treated with 2% (v/v) of ethanol and different concentrations of lauric acid (5 µM, 10 µM and 20 µM) for 24 hours. Western blot analysis was performed to quantify protein expression against phosphorylated- PI3K, total- PI3K and NFκB antibodies and housekeeping β-actin antibody. The normalized densitometry analysis showed that the phosphorylated PI3K p85 (Tyr458) protein was significantly elevated in

ethanol-treated HepG2 cells, whereas co-treatment with lauric acid and ethanol showed opposite results. This suggests that ethanol activated PI3K pathway via phosphorylation on PI3K p85 (Tyr458) in HepG2 cells, but the effect was suppressed by lauric acid in a dose-dependent manner. However, there was no significant difference in NFκB pathway in which the normalized NFκB p105 (Ser933) phosphorylation remained constant in any treatment conditions in this study. In short, lauric acid antagonized the ethanol-activated PI3K pathway, but it has no effect on NFκB p105 (Ser933) activation in acute ethanol-induced HepG2 cells. The suppression of PI3K pathway in the co-treatment of lauric acid and ethanol indicates the therapeutic potential of lauric acid in alcoholism.

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DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

LUA YING HUAN

APPROVAL SHEET

This project report entitled "**THE EFFECTS OF LAURIC ACID ON PI3K AND NFκB SIGNALING PATHWAYS IN ALCOHOL-INDUCED HEPG2 CELLS**" was prepared by LUA YING HUAN and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that **LUA YING HUAN** (ID No: 15ADB06989) has completed this final year project entitled "THE EFFECTS OF LAURIC ACID ON PI3K AND NFκB SIGNALING PATHWAYS IN ALCOHOL-INDUCED HEPG2 CELLS" under the supervision of Dr. Chew Choy Hoong (Supervisor) from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(LUA YING HUAN)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
ALD	Alcoholic liver disease
ALDH	Acetaldehyde dehydrogenase
APS	Ammonium persulfate
BCP	1-bromo-3-chloropropane
BSA	Bovine serum albumin
CDK	Cyclin-dependent kinase
CO_2	carbon dioxide
COX	Cyclooxygenase
CYP2E1	Cytochrome P450 2E1
DNA	Deoxyribonucleic acid
EG	Ethanol : Glycerol
FBS	Fetal bovine serum
FOXO	Forkhead box class O
g	Acceleration of gravity (approximately 9.8 m/s^2)
GEG	Guanidine hydrochloride : Ethanol : Glycerol
GSK3α/β	Glycogen synthase kinase 3 alpha/beta
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
HRP	Horseradish peroxidase
IKK	IκB kinase
IL	Interleukin

IκB	Inhibitor of κB
LCFA	Long chain fatty acid
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MCFA	Medium chain fatty acid
MDM 2	Mouse double minute 2 homolog
MEM	Minimum essential medium
mTOR	Mammalian target of rapamycin
NFκB	Nuclear Factor kappa B
Nrf 2	Nuclear factor erythroid 2-related factor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered Saline
PDK 1	Phosphatidylinositol-dependent kinase 1
PH	Pleckstrin homology
PI3K	Phosphatidylinositol-3-kinases
PIP 2	Phosphatidylinositol 4,5-diphosphate
PIP 3	Phosphatidylinositol 3,4,5-triphosphate
PTEN	Phosphatase and tensin homologue
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
Ser	Serine
SPSS	Statistical Package for the Social Sciences
SREBP	Sterol regulatory element binding protein

STAT 3	Signal transducer and activator of transcription 3
TAD	Transactivation domain
TBS	Tris-Buffered Saline
TEMED	N,N,N'N'-Tetraethylmethylenediamine
TGF β	Transforming growth factors β
TLR	Toll like receptor
TNFα	Tumor necrosis factor alpha
Tyr	Tyrosine

CHAPTER 1

INTRODUCTION

Alcohol can be a common addictive drug as approximately 40% of the world population drink alcohol in the year 2010. However, there are 3.3 million of deaths worldwide every year due to alcoholism (World Health Organization, 2014). Despite the beneficial roles of moderate alcohol consumption, excessive alcohol intake is detrimental to health and it can lead to various health problems especially alcoholic liver disease (ALD) (World Health Organization, 2014). The spectrum of liver diseases encompassed by ALD are steatosis, hepatitis and cirrhosis (Walsh and Alexander, 2000).

Alcohol metabolism involves cytochrome P450 2E1 (CYP2E1) enzyme and this enzyme generates acetaldehyde as well as reactive oxygen species (ROS) which lead to ALD (Koop, 2006). Numerous studies have proven the increment of CYP2E1 mRNA and protein expressions upon ethanol exposure (Jin et al., 2013; Ong, 2017). The rise in CYP2E1 expression is hypothesized to be associated with activation of phosphatidylinositol-3-kinases (PI3K) and nuclear factor kappa B (NFκB) pathways as these pathways are involved in alcohol induction (Mandrekar and Szabo, 2009; Zeng et al., 2012).

Besides alcohol abstinence, antioxidant therapy is recommended as alternative ALD treatment (Menon et al., 2001). Lauric acid is proven to have antioxidative and anti-inflammatory properties (Henry et al., 2002; Hamsi et al., 2015) and it would be interesting to investigate its application in ameliorating ALD. A study by Ong (2017) proved that lauric acid successfully reduced the ethanol-induced ROS formation and CYP2E1 expression in HepG2 cells. However, to our knowledge, the impact of lauric acid on PI3K and NFκB signaling pathways in acute ethanol-induced HepG2 cells is yet to be elucidated. Here, it is hypothesized that lauric acid could suppress the PI3K and NFκB signaling pathways in this study. Understanding on the underlying signaling pathways utilized by lauric acid would definitely contribute to its future role as a therapeutic agent for treating the effects of alcoholism.

Therefore, the objectives of this study were:

1. To study the impact of alcohol on PI3K and NFκB signaling pathways in HepG2 cells.
2. To determine the co-treatment effect of lauric acid on PI3K and NFκB signaling pathways in ethanol-induced HepG2 cells.
3. To link the possible connection of PI3K, NFκB signaling and lauric acid to CYP2E1 in ethanol-induced HepG2 cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Alcoholism

According to the report from World Health Organization (2014), the worldwide alcohol consumption per person with aged 15 years and above was 6.2 liters in the year 2010. The mortality rate was recorded at 5.9% due to alcohol consumption. In Malaysia, it is reported that one out of two current drinkers aged 13 years and above is involved in binge drinking (Hatta et al., 2013). These findings had raised public concern on the alcohol consumption.

The effects of alcohol consumption depend on the volume, patterns and quality as well as the influence of societal and demographic factors (Rehm et al., 2010). Moderate consumption of alcohol is able to reduce the risk cardiovascular diseases especially ischemic vascular disease but higher amount of consumption places higher risk for hemorrhagic stroke and mortality. In addition to that, lower risks for diabetes and dementia are associated with low to moderate consumption of alcohol (Li et al., 2016; Xu et al., 2017).

On the other hand, alcoholism has given rise to many adverse impacts. Binge drinking of alcohol during adolescence affects the key genes expression within the periaqueductal gray which is responsible for fear, pain and anxiety processing (McClintick et al., 2016). According to Sesso et al. (2008) and Fernández-Solà (2015), myocardial contractility was reduced while arrhythmias and hypertension were induced after alcohol consumption.

Liver diseases are the second highest alcohol-attributable fraction after fetal alcohol syndrome (World Health Organization, 2014). Alcohol abuse leads to a link between alcohol and cancer. The risks of breast, esophagus, pharynx and mouth cancers are elevated in female even among the individuals who only drink lightly (Bagnardi et al., 2012). It was well-documented that most liver diseases are associated with alcohol consumption, known as ALD such as fatty liver, hepatitis, cirrhosis and hepatocellular carcinoma (El-Serag, 2001).

2.2 Role of Cytochrome P450 (CYP2E1) in the Pathogenesis of ALD

Alcohol is metabolized in the hepatocytes via three major pathways. The first pathway takes place in cytosol involving alcohol dehydrogenase (ADH) to catalyze the oxidation of ethanol to acetaldehyde followed by oxidation to acetate via the enzyme acetaldehyde dehydrogenase (ALDH) and finally converted into carbon dioxide and water (Edenberg, 2007). Catalase not only catalyzes the elimination of hydrogen peroxide (H_2O_2) but also alcohol

oxidation. However, its role in oxidizing alcohol in the liver is less remarkable because the pathway is restricted by low H_2O_2 production rate under physiological condition (Cederbaum, 2012). The other pathway to metabolize alcohol is through the enzyme CYP2E1. This pathway is activated under heavy alcohol consumption (Gaviria et al., 2016).

The pathogenesis of ALD is mainly due to the oxidative stress and studies found that CYP2E1 is the key enzyme for ROS generation. Bardag-Gorce and her colleagues (2000) showed that ethanol administration increased CYP2E1 in wild-type mouse by five-fold but not in CYP2E1 knockout mouse. Wu et al. (2012) suggested that in CYP2E1 knockin mice, ethanol administration led to the accumulation of lipid and steatosis as compared to CYP2E1 knockout mice. In a previous study by senior in the lab Ong (2017), 24-hours of 2% (v/v) ethanol administration also increased the mRNA expression of CYP2E1 in HepG2 cells.

CYP2E1 mRNA level corresponds to its protein expression level in many studies (Novak and Woodcroft, 2000; Ho et al., 2004). Jin et al. (2013) proved that both CYP2E1 mRNA and protein were elevated at 6-hours of ethanol exposure in their studies. Ethanol is found to inhibit proteasomal degradation of CYP2E1 (Roberts et al., 1995; Osna and Donohue, 2007). Therefore, the stability and protein level of CYP2E1 are increased upon alcohol administration.

The decrement of NADPH and oxygen as a result of CYP2E1 activity generates abundant amount of superoxide anion radical and H_2O_2 (García-Suástegui et al., 2017). ROS is necessary for transduction of signal, cellular physiology and several metabolic pathways but it is detrimental when present in high concentration (Leung and Nieto, 2013). ROS integrates with a number of profibrotic factors to initiate fibrosis (Cichoż-Lach and Michalak, 2014). Diesen and Kuo (2011) showed that oxidative radicals stimulated transforming growth factors β (TGFβ) expression, which in turn up-regulated collagen gene expression and mediated fibrosis formation in hepatic stellate cells. Besides, ROS alters the mitochondrial membrane permeability leading to its destruction and initiates proapototic factors to activate the caspase cascade, and finally results in cell death (McVicker et al., 2007).

CYP2E1 is able to stimulate lipid peroxidation and cause alcoholic liver diseases (Leclercq et al., 2000; Lieber, 2004). Electrophiles such as malondyaldehyde or 4-hydroxynonenal which are produced from lipid peroxidation can modify some essential proteins. As a consequence, the function of the essential proteins and cell homeostasis are lost (Galicia-Moreno and Gutiérrez-Reyes, 2014).

Liangpunsakul et al. (2017) showed that serum lipopolysaccharide (LPS) was increased in excessive alcohol drinkers. LPS aggravates the alcoholic liver injury by inducing the activation of Kupffer cell. Activated Kupffer cells release cytokines, such as tumor necrosis factor α (TNFα) which played a role

in ALD (Mandrekar and Szabo, 2009). Similarly, Zeng et al. (2016) indicated that hepatocytes sensitivity to injury was increased by elevating LPS-induced TNFα production. Liu et al. (2002) showed that the hepatocyte TNFα response was shifted from proliferation to apoptotic cell death when CYP2E1 was overexpressed.

Several studies have showed that alcohol-induced cells could be mediated by a few potential pathways including PI3K and NFκB pathways (Hill et al., 2000; Mandrekar and Szabo, 2009; Zeng et al., 2012; Zeng et al., 2018). Thus, it is warranted to understand how the pathways regulate CYP2E1 in hepatotoxic or hepatoprotective way.

2.3 Phosphatidylinositol-3-kinases (PI3K) Signaling Pathway

2.3.1 Key Components and Overview of PI3K Signaling Pathway

PI3K signaling pathway regulates a diverse cellular functions and properties such as cell growth, proliferation, metabolism, protein synthesis, survival, motility and morphology (Engelman et al., 2006; Bader et al., 2015). The key proteins involved in PI3K pathway are PI3K, Akt and mammalian target of rapamycin (mTOR).

PI3Ks are categorized into three classes: class I, II and III. Class I PI3Ks mainly convert phosphatidylinositol 4,5-diphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3). It can be further divided into class I A and I B based on the receptors that they couple to. Class I A of PI3K is a heterodimer composed of a p110 catalytic subunit and a p85 regulatory subunit. A common p110-binding domain (known as inter-SH2 domain) presents in all of the class I A p85 isoforms. Meanwhile, class I B PI3K consists of heterodimer of p110γ catalytic subunit and a p101 regulatory subunit, which is distinct from p85 protein (Engelman et al., 2006). There is only little information about class II and III PI3K that have been discovered so far. However, Engelman et al. (2006) suggested that class II PI3K consisted of a p110-like catalytic subunit only whereas class III PI3K consisted only one member known as vacuolar protein-sorting defective 34.

The downstream effector of PI3K is Akt, also known as protein kinase B, is a Serine/Threonine kinase. There are three isoforms of Akt: Akt 1, Akt 2 and Akt 3. Three of these have C-terminal regulatory phobic motif, central catalytic domain and N-terminal pleckstrin homology (PH) domain which interacts with PIP3 (Bellacosa et al., 2005). mTOR is another Serine/Threonine kinase that is governed by mTOR-associated proteins and functions as core component of mTOR complex 1 and mTOR complex 2 (Jason and Cui, 2016).

It is vital to control this pathway as any dysregulation may result in a variety of diseases including heart diseases, diabetes, multiple hamartoma syndromes and so on (Danielsen, 2015). Many tumors are associated with high activated Akt level (Vivanco and Sawyers, 2002).

2.3.2 Activation and Inhibition of PI3K Signaling Pathway

There are four major types of sensors to activate PI3K signaling pathway, namely receptor tyrosine kinases, cytokines receptors, G protein-coupled receptors, and integrins. After the binding of these sensors with cofactors, downstream kinases in the PI3K family are activated to phosphorylate PIP2 and forming PIP3 (Danielsen, 2015). A modification in the intracellular membrane composition is induced and eventually proteins with PIP3 binding domains involving phosphatidylinositol-dependent kinase 1 (PDK 1) and Akt are recruited. PDK 1 activates Akt through phosphorylation of Thr308 while mTOR activates Akt through phosphorylation of Ser473. Dual phosphorylation is required for full activation of Akt to activate downstream targets (Sarbassov et al., 2005; Danielsen, 2015).

A negative regulator of PI3K/Akt signaling pathway is phosphatase and tensin homologue (PTEN). PTEN converts PIP3 back to PIP2 by removing phosphate (Phin et al., 2013). A group of inhibitors had been identified to target components of the PI3K pathway such as class I PI3K and mTOR dual

inhibitor, ATP-competitive inhibitors and PI3K isoform-specific inhibitors (Dienstmann et al., 2014).

2.3.3 Downstream Effects of PI3K Signaling Pathway

Literature showed that there are more than 100 non-redundant Akt substrates, such as forkhead box class O (FOXO), glycogen synthase kinase 3 alpha/beta (GSK3α/β), mouse double minute 2 homolog (MDM2) and IκB kinase alpha (IKKα) (Manning and Cantley, 2007). Phosphorylation of the specific site(s) contributes to specific effects. Akt may promote cell survival through blocking of a component of the cell death machinery. According to Datta et al. (1997), Akt phosphorylates BAD at Ser136 *in vitro* and *in vivo* to prevent BAD-mediated cell death. Besides, Akt inhibits MDM2, an E3 ubiquitin ligase which degrades p53 (Manning and Cantley, 2007). Similarly, Ozes et al. (1999) supported that PI3K/Akt pathway was necessary to trigger NFκB survival signaling.

Cellular proliferation and growth are related to the biological consequences of Akt activation as well. Cell cycle regulation is maintained by the actions of cyclin-cyclin-dependent kinase (CDK) and CDK inhibitors. Akt phosphorylates GSK-3β to inhibit its kinase activity. Inhibition of GSK-3β prevents cyclin D1 from degradation (Vivanco and Sawyers, 2002). Ouwens et al. (1999) identified that growth factors activated PI3K and Akt, which

subsequently activated mTOR. mTOR mediates synthesis of protein and emerges as a cell growth regulator (Vivanco and Sawyers, 2002). Thus, cell growth is promoted through activation of mTOR via PI3K/Akt pathway.

Manning and Cantley (2007) claimed that lipid metabolism is regulated by Akt signaling through GSK3 inhibition. GSK3 is found to be able to degrade sterol regulatory element binding proteins (SREBPs), which are transcription factors in regulating biosynthesis of cholesterol and fatty acid (Sundqvist et al., 2005). Hence, Akt pathway stimulates lipid production via inhibition of GSK3 to stabilize SREBP.

Konstandi et al. (2013) emphasized that inactivation of insulin/PI3K/Akt/FOXO 1 pathway could lead to the upregulation of progesterone-induced CYP2E1. In contrast to the study, Sidhu et al. (2001) indicated that the activation of PI3K-dependent pathway had no direct association with the downregulation of insulin-induced CYP2E1 mRNA expression.

PI3K/Akt pathway is shown to negatively regulate NFκB and inflammatory genes expression in macrophages (Guha and Mackman, 2002). However, Thomas et al. (2002) argued that activation of NFκB by respiratory syncytial virus or interleukin-1 (IL-1) stimulation was positively regulated by PI3K-dependent pathway activation. The difference outcomes may be explained by

different type of stimulants and/or different cell types being used (Zhao et al., 2008). Figure 2.1 represents the overview of PI3K/Akt pathway.

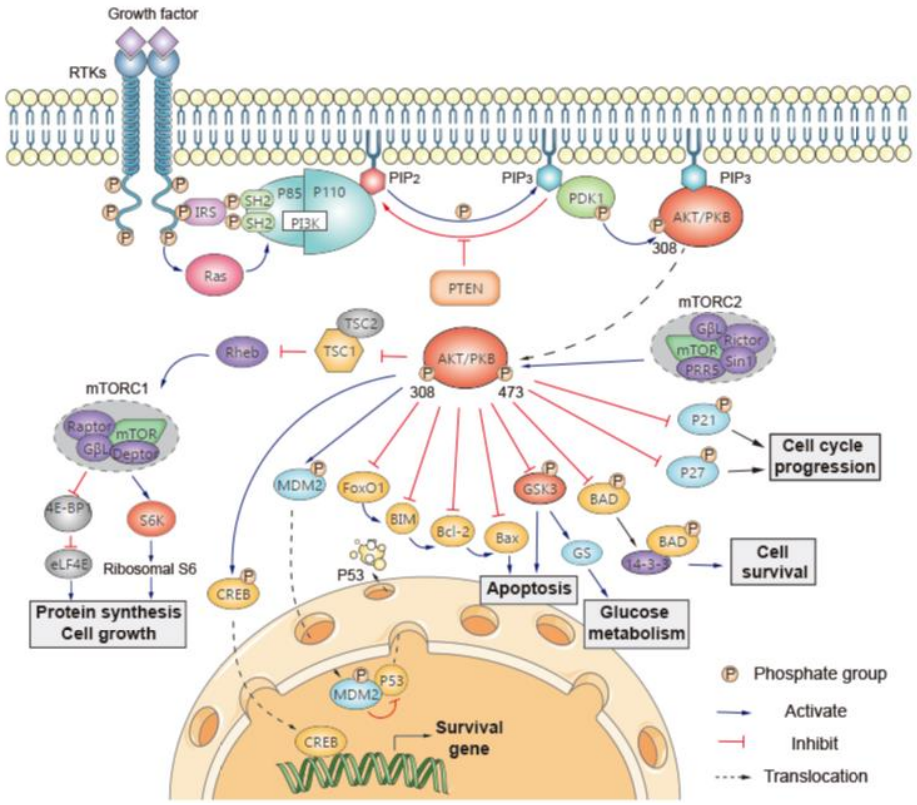

Figure 2.1: Schematic representation of PI3K signaling pathway with the impacts on cell cycle progression, cell survival, glucose metabolism, apoptosis and protein synthesis. After activation of PI3K by RTK or Ras, PI3K catalyzes the formation of PIP3. PTEN inhibits the conversion of PIP2 to PIP3. PIP3 recruits PDK 1 and Akt. A fully activated Akt mediates downstream effects by phosphorylating a variety of substrates (Adopted from Creative Diagnostics, 2018).

2.3.4 Association of CYP2E1 and PI3K Signaling Pathway in Alcohol-induced Cells

A recent study from Zeng et al. (2018) revealed that CYP2E1 activation may be associated with chronic ethanol-induced Akt suppression. Activation of PI3K/Akt pathway was denoted by the phosphorylation of the proteins. Hence, in the study by Zeng et al (2012), western blotting was used to detect the

protein levels of phosphorylated Akt and total Akt to determine whether Akt was activated after ethanol treatment.

Previous studies showed that there might be other mechanisms (other than PI3K) which contribute for the Akt suppression under chronic ethanol state as the protein level of phosphorylated Akt at Thr308 was reduced but PI3K protein level were not affected after four week of ethanol exposure in male Kun-Ming mice (Zeng et al., 2018). However, the result was in contrast during acute ethanol stage. Zeng et al. (2012) proved that the protein level of PI3K p85 was decreased significantly with the increment in SREBP-1c protein after three hours of ethanol exposure. This result showed that activation of PI3K/Akt pathway with accumulation of SREBP-1c could be mediated by PI3K p85 in acute-ethanol toxicity stage.

SREBP-1, a downstream effector of PI3K/Akt pathway (Luu et al., 2012; Xiao and Song, 2013) was significantly upregulated in ethanol treatment (Yin et al., 2007; Wang et al., 2013; Yang et al., 2013). The rise in the amount of mature SREBP-1 might due to the inhibition of proteasomal degradation by ethanol (You et al., 2002).

mTOR, a downstream effector of PI3K/Akt pathway is found to be positively activate signal transducer and activator of transcription 3 (STAT3) (Yokogami et al., 2000; Zhou et al., 2007). The activated STAT3 then induces CYP2E1

transcription by binding to the CYP2E1 promoter region after translocating into the nucleus (Patel et al., 2014).

2.4 Nuclear Factor kappa B (NFκB) Signaling Pathway

2.4.1 Key Components and Overview of NFκB Signaling Pathway

NFκB plays a role in regulation of inflammation and development of immune system (Hayden and Ghosh, 2012). Thus, liver injury, fibrosis and hepatocellular carcinoma are found to be associated with NFκB which possesses the role of inflammation regulator (Luedde and Schwabe, 2011). NFκB activation results in transcription of genes involved in cell proliferation, survival, differentiation, and metastasis (Gupta et al., 2010). NFκB signaling pathway consists of NFκB transcription factor, inhibitor of κB (IκB) and IκB kinase (IKK).

There are five members of NFκB including RelA (p65), RelB, cRel, NFκB1 (p50) and NFκB2 (p52). They remain in the cytoplasm as dimer in a complex with IκB proteins in resting cells. All of the members have a common N-terminal Rel homology domain, that is necessary for dimerization, association with IκB, nuclear translocation and DNA binding (Xiao and Ghosh, 2005; Uwe, 2008). C-terminal transactivation domain (TAD) which is necessary for gene transcription is found in p65, RelB and cRel while active DNA-binding proteins of p50 and p52 are formed only after the cleavage of the precursors of

p105 and p100 respectively. p50 and p52 are lacking of TAD and thus act as transcriptional repressors when forming homodimer but are transcriptional active if heterodimerizing with members of Rel subfamily (Gilmore, 2006).

IκB-α and IκB-β control the biological activity of NFκB by restricting NFκB from binding to DNA. IκB-α exerts a stronger inhibition effect on NFκB as compared to IκB-β *in vivo* (Tran et al., 1997). IKK is composed of two kinases responsible for phosphorylation (IKKα and IKKβ) and a regulatory subunit (IKKγ/ NEMO) (Israël, 2009).

Interestingly, NFκB regulates both antiapoptotic and proinflammatory responses (Hoesel and Schmid, 2013). Therefore, a precise balance of activation is vital to avoid cell death due to insufficient protection or increased inflammation or cancer (Moynagh, 2005; Luedde and Schwabe, 2011).

2.4.2 Activation and Inhibition of NFκB Signaling Pathway

There are two major pathways to activate NFκB, canonical pathway and non-canonical pathway. Canonical pathway is activated by cytokines such as TNF, IL-1 or toll like receptor (TLR) agonists while non-canonical pathway is essential in B cells (Senftleben et al., 2001; Dejardin, 2006).

Activation of canonical pathway is based on the inducible degradation of IκB in the cytoplasm. NFκB proteins are kept inactive by binding with inhibitory molecules of IκB (Israël, 2009) to mask the nuclear translocation sites and sequester it in the cytoplasm. When a cell is stimulated by inflammatory cytokines, ROS, bacterial or viral products, signal transduction pathway is activated which will lead to IKK activation (Brasier, 2006; Ghosh and Karin, 2002). Phosphorylation of IκB by IKK results in polyubiquitination and proteasomal degradation of IκB (Israël, 2009). Upon degradation of IκB, translocation site of NFκB is exposed, allowing nuclear translocation and DNA binding to activate transcription (Ghosh and Karin, 2002).

Non-canonical pathway is activated by limited TNF family members, CD40 ligand, RANK ligand, and lymphotoxin α, β heterodimers (Sun and Karin, 2008). Only IKKα is responsible for non-canonical pathway but not IKKβ and IKKγ. IKKα mediates p100 precursor processing, increases certain NFκB-target gene expression and for B cell maturation (Senftleben et al., 2001). However, canonical pathway is the main survival function regulated by NFκB.

There are many small molecules that can suppress NFκB activation pathway such as antioxidants, small RNA/DNA and peptides. Examples of antioxidants with anti-NFκB effect are isoflavones, curcumin, resveratrol, and cyaniding-3-glucoside (C3G) (Sarkar et al., 2008; Wang et al., 2015). Inhibition of pathway activation happens through several ways including blocking of IKK

activation, inhibiting proteasomal degradation of IκB and blocking the binding of NFκB to DNA (Gupta et al., 2010).

2.4.3 Downstream Effects of NFκB Signaling Pathway

NFκB activation induces cytokines (TNFα, IL1, IL6, IL8) and adhesion molecules, resulting in leukocytes recruitment to the inflammatory sites (Hoesel and Schmid, 2013). Studies from Yamamoto and Gaynor (2001) and Sarkar et al. (2008) found that cytokines produced by NFκB can exert positive autoregulation loop to directly activate NFκB pathway, thus the inflammatory response and its duration are amplified. Besides, NFκB stimulates inducible form of nitric oxide synthase expression which subsequently generates cyclooxygenase (COX) and nitric oxide that are involved in inflammation (Yamamoto and Gaynor, 2001).

Activation of NFκB signaling pathway is associated with development of B cells and T cells, especially regulatory T cell and natural killer T cells (Siebenlist et al., 2005). NFκB pathway is also highly activated in the development of cancers such as pancreatic cancer and prostate cancer (Fujioka et al., 2003; Shukla et al., 2004). It is activated through constitutive IKK activation and subsequently IκB degradation in cancer cells (Sarkar et al., 2008).

NFκB induces few Bcl-2 family members, especially the antiapoptotic molecules of Bcl-X_L and A1/Bfl-1 (Luo et al., 2005). Schwabe and Brenner (2006) suggested that NFκB activation increased the transcription of superoxide dismutase, an antioxidant enzyme to prevent TNF-induced cell death. However, ROS may regulate NFκB negatively by direct interference with NFκB dimers DNA-binding activity (Bubici et al., 2006).

NFκB signaling pathway controls cellular processes as well, such as cell proliferation. Cyclin D1 activity is reduced when activation of NFκB is inhibited. The decrease in retinoblastoma protein phosphorylation as a result of a decrease in cyclin D1, seems to delay the cell cycle progression (Guttridge et al., 1999). This result shows that NFκB is required in the activation cyclin D1 expression. Figure 2.2 illustrates the overview of NFκB signaling pathway.

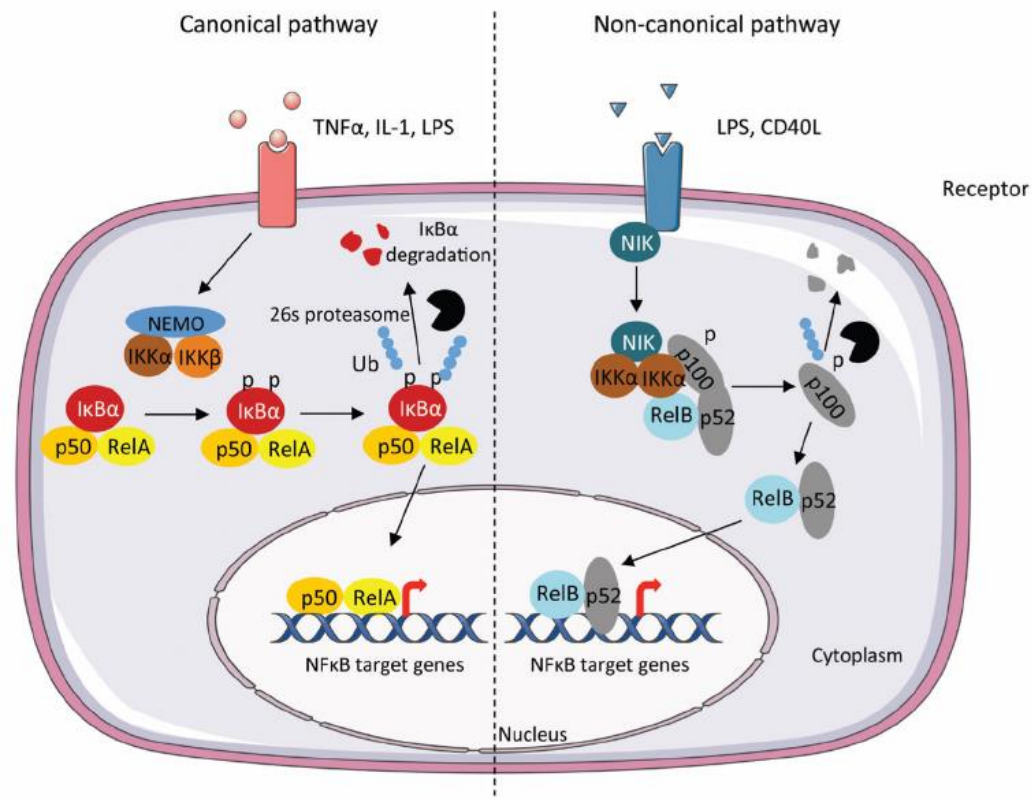

Figure 2.2: Schematic representation of canonical and non-canonical pathway leading to NFκB activation. Canonical pathway involves degradation of IκB-α by 26S proteasome and Rel A/p50 complex enters the nucleus freely for gene transcription. Non-canonical pathway involves NFκB-inducing kinase (NIK) to activate IKKα. The NIK-IKKα-P100 complex leads to phosphorylation of p100 subunit. This produces p52 from p100 subunit leading gene transcription by p52-Rel B complex (Adopted from Viennois, 2013).

2.4.4 Association of CYP2E1 and NFκB Signaling Pathway in Alcohol-induced Cells

Study from Wang et al. (2015) showed that chronic moderate alcohol consumption activated NFκB signaling pathway in HepG2 cells, but the effect upon acute alcohol is yet to be investigated. The activation was proven through western blotting experiment in which ethanol enhanced nuclear translocation of NFκB p65, increased IκB-α phosphorylation and subsequently reduced IκB-α level (Wang et al., 2015). On the other hand, Mandrekar et al.

(1999) demonstrated that activation of LPS-induced NFκB was inhibited by acute alcohol exposure in human monocytes as there was lesser binding of p65/p50 heterodimer to DNA.

The association of NFκB to CYP2E1 was shown by Zordoky and El-Kadi (2009), in which NFκB was found to be a transcription factor of CYP2E1. Activation occurs during binding of NFκB on CYP2E1 gene which promoter region contains a NFκB binding site.

2.5 Lauric Acid

2.5.1 Overview of Lauric Acid

Palm oil is one of the major exports in Malaysia as it possesses profound uses. It is then revealed that approximately half of the fatty acid composition in palm kernel oil is lauric acid. Fife (2013) showed that the amount of lauric acid in coconut oil was similar to that of palm kernel oil as well.

Lauric acid is also known as dodecanoic acid as it consists of twelve carbon atoms. It is a saturated medium chain fatty acid (MCFA) that possesses different properties compared to long chain fatty acid (LCFA) which is more common in human diet. Although lauric acid is a saturated fatty acid, it is less obesogenic as compared to LCFA intakes (McCarty and DiNicolantonio,

2016). The differences in digestive and metabolic pathway also make LCFA and MCFA distinguishable. LCFA passes through the stomach and gastrointestinal tract for digestion with the aid of digestive enzymes and bile. However, digestion of MCFA does not require digestive enzyme or bile. They are absorbed into portal vein and directed to liver to be used as a fuel for energy production instead of packaging into lipoprotein to transport cholesterol in artery walls (DebMandal and Mandal, 2011; Fife, 2013). Study from Dayrit (2015) reported that lauric acid was metabolized rapidly although part of the ingested lauric acid entered blood stream.

2.5.2 Applications of Lauric Acid

Antioxidant and anti-inflammatory properties of lauric acid are well studied (Henry et al., 2002; Hamsi et al., 2015). From the study by Alves et al. (2017), lauric acid reduced the production of superoxide in spontaneously hypertensive rats. Previous studies also proved that virgin coconut oil, which is rich in lauric acid, reduced the oxidative stress, increased catalase and SOD activities (Nevin and Rajamohan, 2006; Alves et al., 2015; Kamisah et al., 2015). These results are in line with result from Ong (2017) who indicated that lauric acid decreased the production of alcohol-induced ROS. Moreover, the formation of alcohol-induced H_2O_2 and mRNA expression of CYP2E1 were reduced in the presence of lauric acid in that study.

The application of lauric acid is able to reduce the number of *Propionibacterium acnes* and thus relieving *P. acnes*-induced inflammation without triggering apoptosis of human skin cells (Nakatsuji et al., 2009). Lauric acid would lower down the COX-1 and COX-2 activities (Henry et al., 2002). In contrast to Henry and his colleagues' study, Lee et al. (2001) proved that NFκB was activated and inflammatory markers such as COX-2 were increased by saturated fatty acids but not unsaturated fatty acids.

There are many studies published regarding that lauric acid possesses the antimicrobial activity. Palm kernel oil without steeping contains the highest percentage of lauric acid content and exhibits the greatest inhibitory effect on *Staphylococcus aureus* and *Streptococcus sp* compared to palm kernel oil with steeping (Ubgogu et al., 2006). In addition, Sun et al. (2003) and Anzaku et al. (2017) also proved the effectiveness of lauric acid against other gram positive bacteria such as *Mycobacterium tuberculosis* and it has a much lower inhibitory effect on gram negative bacteria, such as *Escherichia coli, Salmonella* and *Helicobacter pylori*. The mechanism of antimicrobial effect on *Clostridium perfringens* is observed through transmission electron microscope in which the inner and outer membranes are separated as well as the disorganization of the cytoplasma in cells treated with lauric acid (Skřivanová et al., 2005). Although the inhibitory effect on microorganism is quite low, microorganism does not become resistant to MCFA over time (Kabara, 2000) and this reason contributes to the wide spread use of MCFA.

In the past, saturated fat was thought to associate with cardiovascular diseases by increasing low density lipoprotein (LDL) and high density lipoprotein (HDL) but with no alteration in total cholesterol to HDL ratio (Siri-Tarino et al., 2010). However, previous studies showed that lauric acid increased TC and greater increased on HDL as compared to palmitic acid and oleic acid (German and Dillard, 2004; Siri-Tarino et al., 2010). Thus, the overall effect was a drop in the ratio of TC to HDL which is actually beneficial in cardiovascular outcomes (German and Dillard, 2004). Although study from McCarty and DiNicolantonio (2016) suggested that LDL was increased in diets rich in lauric acid, the proportional impact on HDL was still greater and beneficial. With these properties, it is hypothesized that lauric acid could interfere with the cell signaling pathways, focusing on PI3K and NFκB pathways, in this study.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials and Equipment

All of the materials utilized in the study were purchased from the suppliers as shown in Table 3.1.

Table 3.1: Materials and equipment used with their respective suppliers.

Materials	Suppliers
100X MEM non-essential amino acid solution, Penicillin (5000 units/ mL), Streptomycin (5000 µg/ mL), Phosphate buffered saline, Absolute ethanol, Polyvinylidene fluoride (PVDF) membrane, SNAP i.d.® 2.0 Protein Detection System (MultiBlot Frame), Immobilon™ Western chemiluminescene HRP substrate	Merck Milipore, USA
2-mercaptoethanol	GE Healthcare Bio-Sciences Lab, Sweden
75 cm^2 Tissue culture flask, 25 cm^2 Tissue culture flask	Techno Plastic Products (TPP), Switzerland
95% Ethanol	EMPARTA®, Germany

Table 3.1 continued: Materials and equipment used with their respective suppliers.

96-well plate	Grenier bio-one, Germany
Acetic acid , Methanol	Synerlab, French
Acetone, Butanol, Sodium bicarbonate	Quality Reagent Chemical (QRëC), New Zealand
Ammonium persulfate (APS)	Amersham Biosciences, Sweden
Cell scraper	NEST Biotechnology Co., LTD, China
Fetal bovine serum (FBS), Minimum essential medium (MEM), Sodium pyruvate, Trypan blue stain	Gibco, USA
Filter paper	GE Healthcare, UK
Glycerol, Glycine	Systerm®, Malaysia
Guanidine hydrochloride	Fluka BioChemika, Switzerland
Hemocytometer	Assistant, Germany
HepG2 cell line	American Type Culture Collection (ATCC), USA
Hydrochloric acid	Fisher Scientific (M) Sdn Bhd, Malaysia
Lauric acid, 40% Acrylamide/ bisacrylamide	Sigma Aldrich Inc, USA
Microcentrifuge	Thermo Scientific, USA

Table 3.1 continued: Materials and equipment used with their respective suppliers.

N,N,N'N'-Tetraethylmethylenediamine (TEMED), Coomassie brilliant blue	Bio Basic Canada Inc, Canada
NFκB p105/50 antibody, phospho-NFκB p105(Ser933) antibody, PI3K p85 antibody, phospho-PI3K p85 (Tyr458)/ p55(Tyr199) antibody, Goat-anti-rabbit horseradish peroxidase (HRP) conjugated antibody	Cell Signaling Technology, USA
Omega microplate reader	BMG lab tech, Germany
Mini-PROTEAN® Tetra Systems, Mini Trans-Blot® Electrophoretic Transfer Cell, Protein DC assay reagent kit	Bio-Rad, USA
SeeBlue™ Plus2 Pre-stained Protein Standard, Beta actin antibody	Thermo Fisher Scientific, USA
Resveratrol	ChromaDex, USA
SDS-PAGE loading dye	Life Technologies, USA
See-saw rocker	Stuart, UK
Serological pipette	Techno Plastic Products (TPP), Switzerland
Sodium dodecyl sulfate (SDS)	Fisher Scientific, UK
Sodium hydroxide, Sodium chloride	Merck KGaA, Germany
TRI Reagent® LS	Molecular Research Center (MRC), USA

Table 3.1 continued: Materials and equipment used with their respective suppliers.

Tris base	1st Base, Singapore
Trypsin, Bovine serum albumin (BSA)	Nacalai Tesque, Japan
Tween 20	Merck Schuchardt OHG, Germany
Voltmeter	Medigene Sdn Bhd, Malaysia
Vortex	Science Lab Asia Sdn Bhd, Malaysia
Weighing balance, Hot plate stirrer	Corpens Scientific (M) Sdn Bhd, Malaysia

3.2 Glassware and Plasticware Preparation

All of the glassware and plasticware such as Schott bottles, microcentrifuge tubes and pipette tips were autoclaved at 120ºC for 15 minutes with the pressure of 975 kPa. Besides, distilled water required for solution preparation and protein work was sent for autoclave under the same condition.

3.3 Solution Preparation

3.3.1 Cell Culture Medium

Minimum essential medium (MEM) was used for culturing HepG2 cells and prepared according to the composition as demonstrated in Table 3.2. The medium was filtered-sterilized using a 0.22 µm filter prior to use.

Table 3.2: Composition of a liter of MEM.

Solution	Composition
100X MEM non-essential amino acid solution	1X
MEM powder	9 g
Penicillin, 5000 units/mL	1%
Sodium bicarbonate	30 mM
Sodium pyruvate, 100 mM	1 mM
Streptomycin, 5000 µg/mL	1%
Sterile deionized water	Top up to 1L

3.3.2 Phosphate-buffered Saline (PBS)

One tablet of PBS buffer was dissolved in deionized water to make up a liter of PBS. The solution was sent for autoclaved at 121ºC for 15 minutes with the pressure of 975 kPa.

3.3.3 Stock Solutions

All the stock solutions were prepared as shown in the tables below.

Table 3.3: Solutions required for protein extraction.

Solution	Composition
Guanidine hydrochloride : Ethanol : Glycerol (GEG)	0.3 M Guanidine hydrochloride 2.5% (v/v) Glycerol Top up with 95% ethanol
Ethanol : Glycerol (EG)	2.5% (v/v) Glycerol Top up with 95% ethanol

Table 3.4: Solutions required for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Solution	Composition
SDS-PAGE Upper Gel Buffer, pH 6.8	0.5 M Tris-base 10% (w/v) SDS
SDS-PAGE Lower Gel Buffer, pH 8.8	1.5 M Tris-base 10% (w/v) SDS
10X SDS-PAGE Running Buffer	0.25 M Tris-base 1.92 M Glycine 1% (w/v) SDS

Table 3.5: Solution required for Coomassie blue staining.

Solution	Composition
Coomassie blue staining solution	0.1% (w/v) Coomassie blue 40% (v/v) Methanol 10% (v/v) Acetic acid

Table 3.6: Solutions required for western blot assay.

Solution	Composition
10X Towbin Transfer Buffer	0.25 M Tris-base 1.92 M Glycine
10X Tris-Buffered Saline (TBS), pH 7.4	0.5 M Tris-base 1.5 M Sodium chloride
Blocking Buffer	1% Bovine Serum Albumin (BSA) Top up with 1X TBST
Stripping Solution, pH 2.2	0.4 M Glycine 0.2% (w/v) SDS 2% Tween 20
Immobilon Western Chemiluminescent HRP substrate	350 µL of HRP substrate peroxidase 350 µL of HRP substrate luminol

3.4 Cell Culture Techniques

3.4.1 Maintenance of Cells

A total volume of 10 mL and 3 mL of medium mixture were maintained in the 75 cm^2 and 25 cm^2 tissue culture flasks respectively. The HepG2 cells were grown in minimum essential medium (MEM) with the composition as listed in Table 3.2. Fetal bovine serum (FBS) with 10% (v/v) concentration was included to the flask as well. The cells were then incubated in an incubator with 5% (v/v) carbon dioxide (CO_2) at 37ºC. The medium was replaced every two to three days by discarding the initial medium from the flask and washing with phosphate buffered saline (PBS) twice before fresh medium was added. The cells were checked regularly under inverted phase contrast microscope for confluency and contamination, if any.

3.4.2 Subculture of Cells

Subculture of cells was carried out when 80% of cell confluence was achieved. The medium in the 75 cm^2 tissue culture flask was first discarded and cells were washed twice with 6 mL of PBS each time. A volume of 2 mL of trypsin was added to the flask and incubated at 37ºC for approximately 10 minutes so that the cells detached from the flask. The reaction was then stopped by adding 4 mL of medium which contained trypsin inhibitor. All of the cell suspension was transferred to a sterile 15 mL centrifuge tube for centrifugation at 800 x *g* for 10 minutes to pellet the cells. The supernatant was discarded and the pellet

was resuspended with appropriate volume of new medium gently for hundreds of time to ensure even distribution of cells in new flask. New tissue culture flask was filled with desired amount of cell suspension and fresh medium supplemented with 10% (v/v) of FBS. The cells were incubated in 5% (v/v) CO_2 incubator at 37ºC.

3.4.3 Counting and Seeding of Cells

Following trypsinization and centrifugation, the pellet was suspended with appropriate amount of medium. A volume of 10 µL of cell suspension was pipetted out and diluted with 90 µL of trypan blue on a parafilm. After thorough mixing, 10 µL of the mixture was loaded to the edge of a clean hemocytometer chamber and drawn into the chamber via capillary force. Viewing was done by using the inverted phase contrast microscope. The hemocytometer has nine squares and each can accommodate a volume of 0.0001 mL. Under the microscopy view, one of the four outer squares was focused for cell counting and counting was then repeated with another three squares. Only viable cells were counted whereas dead cells that were stained blue were excluded from counting. The concentration of cells after ten times dilution was calculated as: (total number of cells/ 4) x 10^4 cells/ mL. A number of 2.0 x 10^6 of cells was seeded into a new 25 cm^2 tissue culture flask with fresh medium and 10% (v/v) FBS topped up to 3 mL. The flask was incubated in incubator with 5% (v/v) CO_2 at 37ºC.

3.4.4 Cell Treatment for Ethanol and Lauric Acid or Resveratrol

HepG2 cells were treated with 2% (v/v) ethanol and different concentration of lauric acid. The initial medium was discarded and washed with 3 mL of PBS twice. A total of five 25 cm^2 tissue culture flasks were treated with the 2% ethanol in addition to different concentration of lauric acid (5 µM, 10 µM and 20 µM) with reference to Cheah et al. (2014). In the presence of ethanol treatment, positive control was performed with the addition of 20 µM of resveratrol while negative control was performed with solely ethanol. Two more flasks were treated with solely 20 µM of lauric acid or 20 µM of resveratrol respectively in the absence of ethanol. An eighth flask was used as a vehicle control in which ethanol (diluent for lauric acid) was added. Fresh medium was then added up to approximately 3 mL after the addition of the reagent and 10% (v/v) FBS. All of the eight flasks were placed in 5% (v/v) CO_2 incubator at 37ºC for 24 hours prior to the protein isolation as described in Section 3.5.

3.5 Preparation of Protein Samples

3.5.1 Phase Separation

The protein extraction was performed using TRI Reagent® LS following the protocol stated. The initial medium in 25 cm^2 flask was discarded and washed twice with 3 mL of PBS following by the addition of 750 µL of TRI Reagent® LS. The cells were scrapped off from the base of the flask and the suspension

was pipetted up and down several times to allow for homogenization. The homogenates from different treatments were transferred to 1.5 mL microcentrifuge tubes with respective labeling.

For phase separation, a volume of 100 µL of 1-bromo-3-chloropropane (BCP) was added into the microcentrifuge tube per 750 µL of TRI Reagent® LS. The sample was vortexed for 20-30 seconds until the color turned milky pink and it was allowed to stand at room temperature for 10 minutes. After 10 minutes, centrifugation was carried out at 12 000 x *g* for 15 minutes at 4ºC. Three phases could be observed and the lower pink organic phase contained the protein. The upper aqueous phase was removed.

3.5.2 Protein Isolation

Firstly, a volume of 300 µL of 95% ethanol was added to 750 µL of TRI Reagent® LS to precipitate DNA in the interphase layer. The sample was mixed by inversion several times and centrifuged at 2 000 x *g* for 5 minutes at 4ºC. For protein precipitation, the supernatant was drawn out to a new microcentrifuge tube and mixed vigorously with acetone at a ratio of 1:3. The sample was allowed to stand at room temperature for 10 minutes before centrifugation at 12 000 x *g* for 10 minutes at 4ºC.

The pellet was then washed with 500 µL of guanidine hydrochloride: ethanol: glycerol (GEG). A sterile paper clip was used to help in solubilizing the pellet in the solution. Another 500 µL of GEG was added to the sample. During the wash, the sample was kept in GEG for 10 minutes at room temperature. Centrifugation was carried out at 8000 x *g* for 5 minutes at 4ºC. The washing step was repeated twice with GEG followed by ethanol: glycerol (EG).

Protein solubilization was performed after washing with EG. The pellet was air dried for 7-10 minutes. Then, the pellet was dissolved in appropriate volume of 1% (w/v) sodium dodecyl sulfate (SDS) and incubated at 37ºC overnight or longer until complete solubilization. After solubilization, the protein sample was stored for future use in -20ºC refrigerator.

3.5.3 Bio-Rad DC Protein Assay

The concentration of extracted protein was determined using Bio-Rad DC Protein Assay reagent kit. The principle of the reaction is close to Lowry assay (Lowry et al., 1951). Protein standard curve was prepared every time before the protein assay was carried out. A series of protein standard dilutions containing from zero to 1.46 mg/mL of bovine serum albumin (BSA) were prepared by using 1% (w/v) SDS as diluent. Meanwhile, protein samples were also diluted with 1% (w/v) SDS in a dilution factor of 5, to avoid extrapolation from the standard curve.

According to the instruction from manufacturer, reagent A' was prepared by adding 20 µL of reagent S to 1000 µL of reagent A (alkaline copper tartrate solution). A volume of 5 µL of standards and also diluted samples were pipetted into a dry and clean 96 well microtiter plate. A volume of 25 µL of reagent A' was added into each well followed by the gentle addition of 200 µL of reagent B (Folin reagent). The plate was covered with aluminium foil and incubated for 15 minutes at room temperature before the absorbances were read at 750 nm using a microplate reader. The samples and standards were repeated thrice in order to get an average values. Finally, a standard curve was made and the protein sample concentrations were determined using Microsoft Excel 2010.

3.6 Western Blot Assay

3.6.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Resolving gel with 10% (w/v) of acrylamide and staking gel with 5% (w/v) acrylamide were prepared according to Table 3.7 using the buffer stock solutions from Table 3.4. TEMED and APS were added to the solution and mixed well just prior to pouring.

Table 3.7: Composition of resolving and stacking gel of SDS-PAGE.

Components of gel	**10% (w/v) Resolving gel**	**5% (w/v) Stacking gel**
SDS-PAGE upper gel buffer (mL)	-	0.63
SDS-PAGE lower gel buffer (mL)	1.25	-
40% Acrylamide/bisacrylamide (mL)	1.25	0.31
10% (w/v) SDS (µL)	50	25
10% (w/v) Ammonium persulfate (APS) (µL)	50	25
N,N,N'N'-Tetraethylmethylenediamine (TEMED) (µL)	5	2.5
Distilled water (mL)	2.45	1.54
Total volume (mL)	**5.00**	**2.50**

SDS-PAGE was conducted using Mini-PROTEAN® Tetra Systems and the apparatus were assembled according to the manufacturer's instruction. Resolving gel was prepared, mixed thoroughly and then poured in between the glass plates until 2.5 cm away from the upper edge of short plate. The resolving gel solution was overlaid with butanol to exclude bubbles and produce a smooth level surface on top of the resolving gel. The gel was allowed to polymerize for 30 minutes. After that, filter paper was used to absorb excess butanol before pouring in stacking gel solution. A ten-well comb was inserted immediately. After one hour of polymerization, the comb was removed carefully from the gel. The gel together with the glass plates were then placed into an electrophoresis tank filled with 1X SDS-PAGE running buffer that was diluted from the stock solution in Table 3.4. The inner

chamber must be fully filled with 1X SDS-PAGE running buffer throughout the electrophoresis whereas outer chamber was filled up to the indicator mark according to the number of gels.

While waiting for polymerization, protein samples were prepared. Firstly, SDS-PAGE gel loading buffer was made by mixing loading dye with 2-mercaptoethanol in a ratio of 20:1 in a fume hood. An amount of 25 µg of protein samples were mixed with the loading buffer in a ratio of 3:1. The mixtures were mixed using vortex before they were incubated at 70ºC in water bath for 10 minutes.

The samples were loaded into respective wells and eight microliter of SeeBlue™ Plus 2 Pre-stained Protein Standard was loaded into one of the wells. Electrophoresis was performed at 100 V for 70 minutes or until the bromophenol blue tracking dye was approximately 0.5 cm from the bottom of the gel.

3.6.2 Western Blotting

After SDS-PAGE electrophoresis, wet transfer was carried out to transfer the proteins from the gel to a polyvinylidene fluoride (PVDF) membrane using Mini Trans-Blot® Electrophoretic Transfer Cell. 1X Towbin transfer buffer

was firstly prepared from the stock solution in Table 3.6 with the addition of 10% (v/v) methanol and chilled at 4ºC. The gel was dismantled from the glass plates and trimmed to remove stacking gel. PVDF membrane was marked using a pencil to show the protein side-up surface.

Next, it was soaked in methanol for two minutes for activation followed by two more minutes in distilled water. The PVDF membrane, filter papers, foam pads and gel were equilibrated in 1X Towbin transfer buffer for 15 minutes. Then, the PVDF membrane was placed on the gel with protein side-up surface facing to the gel. These were sandwiched in between filter papers and foam pads on each site to provide tight contact between PVDF membrane and the gel (foam pad> filter papers>PVDF membrane> gel> filter papers> foam pad). Any air bubbles were removed. The sandwich was clamped tightly in a gel holder cassette and this cassette was submerged vertically into the tank filled fully with chilled 1X Towbin transfer buffer. Transfer was carried out in cold environment for 90 minutes at 100 V. After transferring, the cassette was disassembled and the membrane was inactivated by soaking in methanol for two minutes and air dried. The membrane was kept at -20ºC until immunodetection was carried out as described in Section 3.6.4 while the gel was stained as described in Section 3.6.3.

3.6.3 Coomassie Blue Staining of SDS-PAGE gel

The gel was soaked in Coomassie blue staining solution (Table 3.5) for 15 minutes. The gel was destained using distilled water overnight to remove the excess stain so that the protein bands can be visualized. The image of the gel was captured using ChemiDoc™ XRS+ System under white light conversion screen (Bio-Rad, USA).

3.6.4 Probing of PVDF Membrane

SNAP i.d.® 2.0 Protein Detection System (MultiBlot Frame) was used for immunoblotting following the procedures by manufacturer. Before starting, PVDF membrane was cut into an appropriate size that can fit into the blot holder, activated in methanol for two minutes and then soaked in distilled water for another two minutes. The PVDF membrane was placed in the center of the blot holder with protein side-down. The blot was rolled gently after membrane was placed on it to remove air bubbles. The blot holder was then placed into blot holder frame in the correct orientation with protein side-up on the PVDF membrane.

In order to block non-specific protein sites, 15 mL of blocking buffer was loaded and incubated for 15 minutes. After that, the frame was pressed down and vacuum was applied to remove the blocking buffer. A volume of 2 mL of primary antibody was diluted in a ratio of 1:1000 with blocking buffer and

added onto the surface of blot holder and incubated for 15 minutes. The primary antibodies used were NFκB p105/50 antibody, phospho-NFκB p105 (Ser933) antibody, PI3K p85 antibody, phospho-PI3K p85 (Tyr458)/p55 (Tyr199) antibody, and β-actin antibody. All of the primary antibodies were diluted in 1:1000 except β-actin antibody which was diluted in a ratio of 1:1500.

After incubation, excess primary antibody was removed by turning the vacuum on. Approximately 10 mL of 1X TBST was used to wash the membrane and the washing step was repeated three more times with incubation of a minute each. A volume of 2 mL of secondary antibody, goat anti-rabbit HRP conjugated (in a ratio of 1:3000 with blocking buffer) was added and incubated for 10 minutes. Following incubation, vacuum was applied to remove the excess antibody and the blot was washed with 1X TBST for four times as described above.

3.6.5 Chemiluminescence Detection

Immobilon Western Chemiluminescent HRP substrate was prepared according to Table 3.6 and incubated in dark for 10 minutes in room temperature. Chemiluminescence detection was done using ChemiDoc^{TM} XRS+ System (Bio-Rad, USA). The HRP substrate was added to the membrane. The image was captured and saved for further analysis.

3.6.6 Stripping of Membrane

Any antibody on the membrane was stripped off by immersing the membrane in stripping solution for 3 minutes at room temperature in a see-saw rocker. The stripping solution was replaced with fresh stripping solution and rocked for another 5 minutes, followed by two washes of 1X TBST for 15 minutes each. Finally, the membrane was rinsed with distilled water and soaked in methanol for 2 minutes, air dried and stored in -20ºC for next usage.

3.6.7 Densitometry Analysis of Western Blot Assay

Image Lab^{TM} version 6.0 software (Bio-Rad, USA) was used for the quantification of the immunodetected protein bands. The expression of each proteins were quantified and normalized against the protein expression of the housekeeping gene, β-actin.

3.7 Statistical Analysis

Statistical Package for the Social Sciences (SPSS) version 22 software was used in this study to calculate the independent-samples T test. Statistically significant result was indicated with $p<0.05$ while $p<0.01$ indicated statistically very significant.

CHAPTER 4

RESULTS

4.1 HepG2 Cell Line

HepG2 is an epithelial cell line which was derived from a 15-year old Caucasian male suffered from liver hepatocellular carcinoma. HepG2 cells are adherent cells (American Type Culture Collection, 2016). According to a study from Bouma et al. (1989), HepG2 cells were able to synthesize most of the hepatic proteins and the cellular features of healthy human hepatocytes. This allows hepatoblastoma cell line to be used extensively as an *in vitro* model system for metabolic activities in many researches. Thus, it is justifiable to use HepG2 cell line in this study to investigate the effect of lauric acid on PI3K and NFκB pathways in alcohol-induced cells. The microscopic morphology of attached HepG2 cells under an inverted phase contrast microscope is displayed in Figure 4.1.

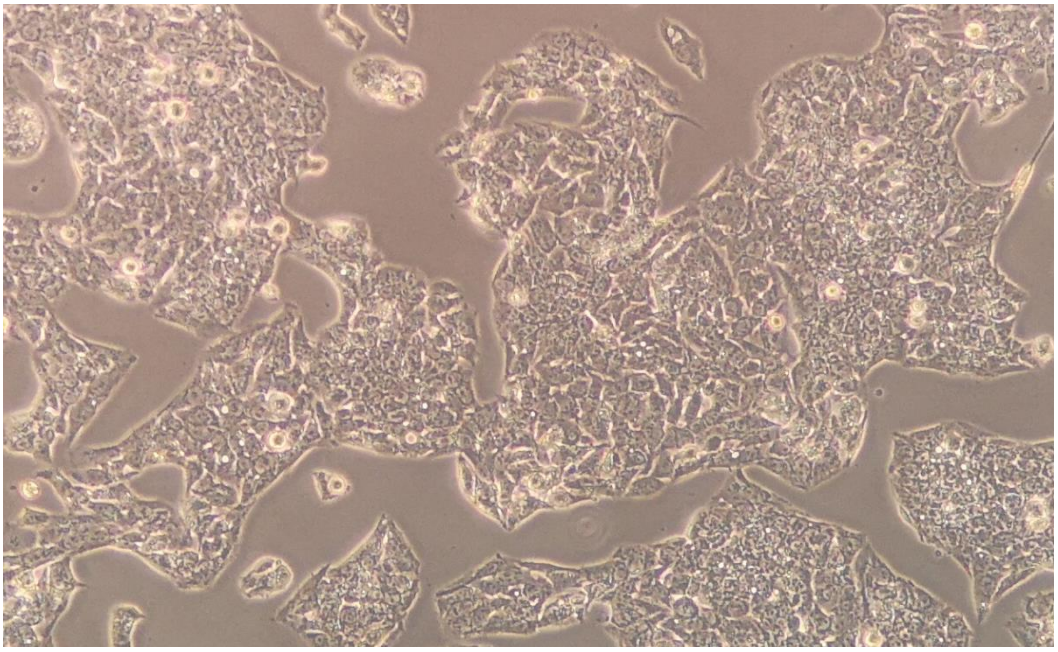

Figure 4.1: Human hepatocellular carcinoma cells, HepG2 (Magnification: 200X). HepG2 cells have epithelial-like appearances.

4.2 Total Cellular Protein Concentration of Cells Co-treated with Ethanol and Lauric Acid or Resveratrol

Co-treatment experiment was performed according to Section 3.4.5 once the cells reached 60% confluency. After the extraction of total cellular protein, the concentrations of the extracted protein were measured using Bio-Rad DC Protein Assay reagent kit with the procedure as described in Section 3.5.3. A standard curve was constructed using Microsoft Excel 2010 and the curve is shown in Appendix A. With that, the concentrations of protein samples were calculated from the equation and tabulated in Table 4.1.

Table 4.1: Concentration of total cellular protein extracted from HepG2 cells under different treatments. The extraction was done using TRI Reagent® LS while concentrations were determined using Bio-Rad DC Protein Assay reagent kit.

Condition of treatment	Protein Concentration, mg/ mL
Vehicle control	0.208
20 µM of Lauric acid only	0.134
20 µM of Resveratrol only	0.254
2% (v/v) of Ethanol only	0.093
2% (v/v) of Ethanol + 5 µM of Lauric acid	0.176
2% (v/v) of Ethanol + 10 µM of Lauric acid	0.158
2% (v/v) of Ethanol + 20 µM of Lauric acid	0.161
2% (v/v) of Ethanol + 20 µM of Resveratrol	0.111

4.3 Western Blot Analysis for the Expression of Phosphorylated PI3K p85 (Tyr458) Proteins under the Effect of Lauric Acid or Resveratrol in Alcohol-induced HepG2 Cells

The protein bands were transferred from the gel to the PVDF membrane as described in Section 3.6.4. The bands were blotted with PI3K p85 antibodies and chemiluminesently detected using Immobilon Western Chemiluminescent HRP substrate. The effects of lauric acid and resveratrol on PI3K signaling pathway in alcohol-treated HepG2 cells were investigated from the expression of phosphorylated and total proteins.

Based on Figure 4.2 (a), treatment with 2% (v/v) of ethanol (Lane 4) and co-treatment of ethanol with lauric acid (Lane 5 to 7) demonstrated denser bands among the various treatments. On the other hand, faded bands were observed in the treatment with solely 20 µM of lauric acid (Lane 2) and solely 20 µM of resveratrol (Lane 3).

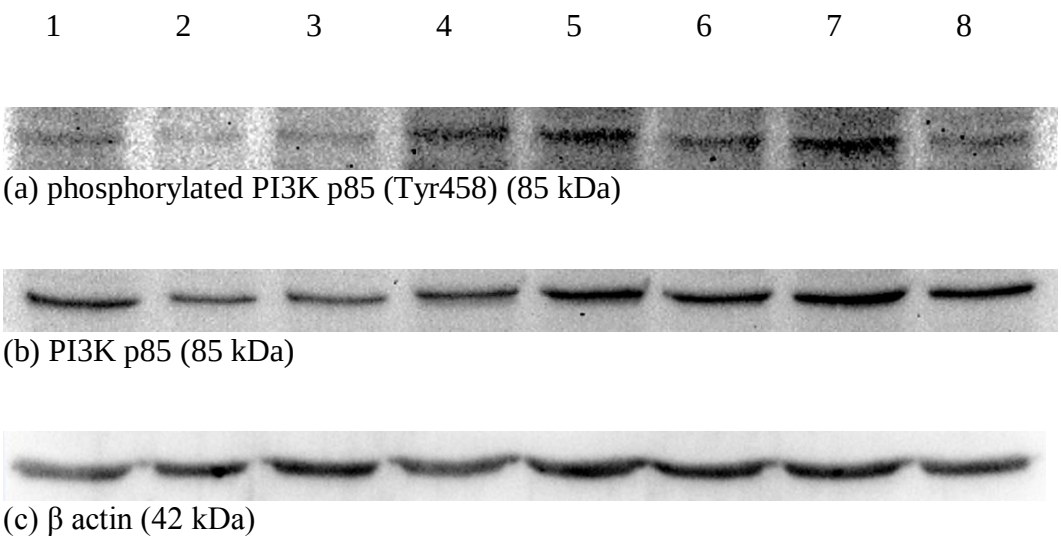

Figure 4.2: (a) Phosphorylated PI3K p85 (Tyr458), (b) PI3K p85 and (c) β actin protein bands on blotted PVDF membrane under the chemiluminescent detection for PI3K signaling pathway in alcohol-induced HepG2 cells. Lane 1 to 8 were loaded with 25 µg of protein extracted from the cells under different treatments. Lane 1: Vehicle control. Lane 2: Cells treated with 20 µM lauric acid only. Lane 3: Cells treated with 20 µM resveratrol only. Lane 4: Cells treated with 2% (v/v) of ethanol only. Lane 5: Cells co-treated with 2% (v/v) of ethanol and 5 µM of lauric acid. Lane 6: Cells co-treated with 2% (v/v) of ethanol and 10 µM of lauric acid. Lane 7: Cells co-treated with 2% (v/v) of ethanol and 20 µM of lauric acid. Lane 8: Cells co-treated with 2% (v/v) of ethanol and 20 µM of resveratrol.

The β actin protein intensities remained fairly constant (Figure 4.2 (c)) in each treatment. Densitometry analysis was conducted in order to quantify the protein expression accurately. The PI3K p85 protein bands were normalized to

respective β actin bands. HepG2 cells that were treated with 0.001% (v/v) of ethanol (0.3 µL) alone acted as a vehicle control with its protein fold expression fixed at 1.00.

To investigate the activation of PI3K pathway, the phosphorylated PI3K p85 expression was analyzed in this study. Densitometry analysis was carried out by normalizing phosphorylated protein against the total PI3K p85 and also β actin. According to Figure 4.3, the 2% (v/v) of ethanol treatment elevated the expression of phosphorylated PI3K p85 at position Tyr458 significantly to 1.42-fold. With the addition of lauric acid in alcohol-induced HepG2 cells, the phosphorylation of PI3K p85 was reduced to 0.98-fold, 0.93-fold and 0.83-fold as the lauric acid concentration was increased from 5 µM, 10 µM and 20 µM. A significant reduction to 0.85-fold in the phosphorylated protein expression was observed in cells co-treated with 2% (v/v) of ethanol and 20 µM of resveratrol as compared to cells treated with ethanol only (Bar D). Although there was not much alteration in the phosphorylated PI3K p85 (Tyr458) protein expressions in HepG2 cells treated with sole lauric acid or resveratrol, the expressions were reduced in dose response manner when the alcohol-induced cells were co-treated with the additives. This demonstrates that lauric acid repressed the phosphorylation and activation of PI3K p85 at position Tyr458.

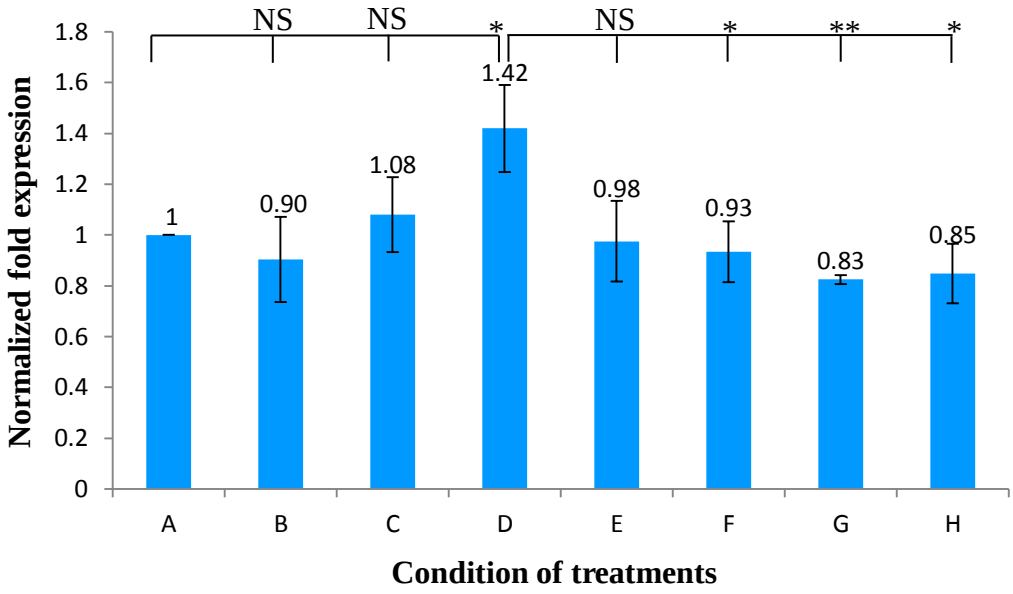

Figure 4.3: Graphical representation of the quantitative protein expression of phosphorylated PI3K p85 (Tyr458) in HepG2 cells. Y axis represents the normalized fold protein expression of phosphorylated PI3K p85 (Tyr458) whereas X axis represents the different condition of treatments. The number listed on top of each bar represents the fold value of phosphorylated PI3K p85 (Tyr458) protein expression with normalization to total PI3K p85 and β actin, relative to vehicle control which is assigned as 1.00-fold. A: Vehicle control. B: Cells treated with 20 µM lauric acid only. C: Cells treated with 20 µM resveratrol only. D: Cells treated with 2% (v/v) of ethanol only. E: Cells co-treated with 2% (v/v) of ethanol and 5 µM of lauric acid. F: Cells co-treated with 2% (v/v) of ethanol and 10 µM of lauric acid. G: Cells co-treated with 2% (v/v) of ethanol and 20 µM of lauric acid. H: Cells co-treated with 2% (v/v) of ethanol and 20 µM of resveratrol. Error bars are expressed as standard deviation and the data represent mean± SD; n= 3. *p<0.05 and **p<0.01 represent the statistically significant changes in the expression. NS represents non-significance.

4.4 Western Blot Analysis for the Expression of Phosphorylated NFκB p105 (Ser933) Proteins under the Effect of Lauric Acid or Resveratrol in Alcohol-induced HepG2 Cells

According to Figure 4.4 (a), the protein expression of phosphorylated NFκB p105 (Ser933) extracted from 2% (v/v) of ethanol treated cells (Lane 4) showed the lowest intensity as compared to the other treatments. In contrast, the cells co-treated with ethanol and lauric acid or resveratrol (Lane 5 to 8) exhibited higher protein expressions, whereas the protein expressions of cells treated solely with lauric acid or resveratrol (Lane 2 and 3) were similar to that of the vehicle control.

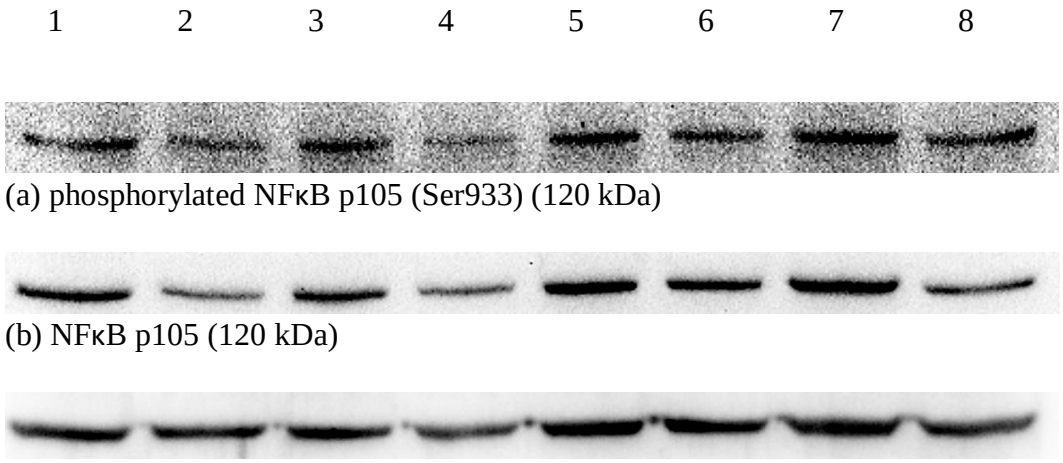

Figure 4.4: (a) Phosphorylated NFκB p105 (Ser933), (b) NFκB p105 and (c) β actin protein bands on blotted PVDF membrane under the chemiluminescent detection for NFκB signaling pathway in alcohol-induced HepG2 cells. Lane 1 to 8 were loaded with 25 µg of protein extracted from the cells under different treatments. Lane 1: Vehicle control. Lane 2: Cells treated with 20 µM lauric acid only. Lane 3: Cells treated with 20 µM resveratrol only. Lane 4: Cells treated with 2% (v/v) of ethanol only. Lane 5: Cells co-treated with 2% (v/v) of ethanol and 5 µM of lauric acid. Lane 6: Cells co-treated with 2% (v/v) of ethanol and 10 µM of lauric acid. Lane 7: Cells co-treated with 2% (v/v) of ethanol and 20 µM of lauric acid. Lane 8: Cells co-treated with 2% (v/v) of ethanol and 20 µM of resveratrol.

The graphical representation of densitometry analysis of phosphorylated NFκB p105 (Ser933) protein expression under different treatments in HepG2 cells is illustrated in Figure 4.5. The phosphorylated NFκB p105 (Ser933) protein bands were normalized to respective NFκB p105 and β actin bands with the normalized protein fold expression of vehicle control assigned as 1.00. Based on Figure 4.5, cells treated solely with 20 μM of lauric acid and resveratrol showed unchanged expression level of phosphorylated NFκB p105 (Ser933) to the basal level. The phosphorylated protein band from cells with 2% (v/v) of ethanol treatment was lower but the densitometry normalization result showed a 1.19-fold to vehicle control. This is because its total NFκB p105 was reduced as well (Figure 4.4 (b)). Even though the intensities of protein bands from co-treated HepG2 cells with ethanol and lauric acid were higher, the normalized phosphorylation were lower as compared to cell treatment with solely 2% (v/v) of ethanol. In cells co-treated with ethanol and resveratrol, the normalized densitometry was increased to 1.40-fold. However, all the normalized fold changes were statistically insignificant. This indicates that the activation of NFκB signalling pathway at position Ser933 may not be involved in mediating lauric acid action in ethanol-induced HepG2 cells.

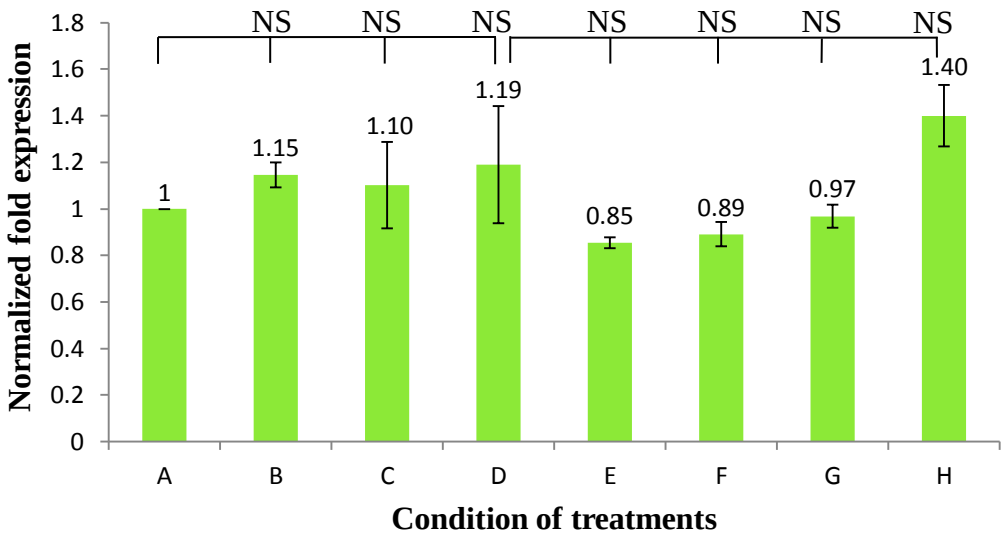

Figure 4.5: Graphical representation of the quantitative protein expression of phosphorylated NFκB p105 (Ser933) in HepG2 cells. Y axis represents the normalized fold protein expression of phosphorylated NFκB p105 (Ser 933) whereas X axis represents the different condition of treatments. The number listed on top of each bar represents the fold value of phosphorylated NFκB p105 (Ser 933) protein expression with normalization to total NFκB p105 and β actin, relative to vehicle control which is assigned as 1.00-fold. A: Vehicle control. B: Cells treated with 20 µM lauric acid only. C: Cells treated with 20 µM resveratrol only. D: Cells treated with 2% (v/v) of ethanol only. E: Cells co-treated with 2% (v/v) of ethanol and 5 µM of lauric acid. F: Cells co-treated with 2% (v/v) of ethanol and 10 µM of lauric acid. G: Cells co-treated with 2% (v/v) of ethanol and 20 µM of lauric acid. H: Cells co-treated with 2% (v/v) of ethanol and 20 µM of resveratrol. Error bars are expressed as standard deviation and the data represent mean± SD; n= 3. All experiments were statistically non-significant (NS) from untreated to ethanol-treated cells.

CHAPTER 5

DISCUSSION

5.1 Review

One of the aims in this study is to identify the putative signaling pathway that is involved in ethanol-induced HepG2 cells. Previous study by Ong (2017) reported that ethanol treatment induced the expression of CYP2E1 mRNA expression in HepG2 cells but lauric acid reduced the induction. Numerous studies from other researchers coincided with the result that alcoholism is associated with the induction of CYP2E1 (French et al., 1993; Maza et al., 2000). The induction seems to involve via translation, post-translation and transcriptional mechanisms of CYP2E1. The activity and post-translational stabilization of CYP2E1 protein is enhanced in the presence of alcohol (Novak and Woodcroft, 2000). Experiments from Raucy et al. (2004) and Jin et al. (2013) also supported that ethanol increased the CYP2E1 protein expression level when compared to the control.

In this study, PI3K and NFκB pathways were hypothesized to be involved in the lauric acid-ethanol regulation of CYP2E1 expression in HepG2 cells. Thus, the expression of proteins and their respective phosphorylation sites were analyzed mainly through western blot assay. These two pathways were postulated by Ong (2017) with numerous evidences of their involvements (Hill

et al., 2000; Mandrekar and Szabo, 2009; Zeng et al., 2012; Zeng et al., 2018), so similar experimental condition was conducted in this study to further investigate their roles in lauric acid-ethanol regulation. To mimic the condition, the concentration of ethanol used in this study (2%) was chosen based on the result from the dose response effect on CYP2E1 in the previous study (Ong, 2017). The mentioned study showed that 2% (v/v) of ethanol was the optimum concentration to induce CYP2E1 expression to the highest fold of expression (Ong, 2017). Thus, western blot analysis was conducted to observe the changes of protein expression levels. The protein bands were quantified using densitometry and data was analyzed using independent-samples T test program. Each experiment was repeated three times.

5.2 HepG2 Cells as a Model System

Liver is the main organ that is responsible for the metabolism of alcohol. It is comprised of multiple distinctive cell types and the chief liver cell is hepatocyte. Hepatocytes are responsible for various activities including intermediary metabolism regulation, detoxification, protein synthesis and bile-acid-dependent bile flow generation (Malhi et al., 2010). ALD were found to be associated with the specific transcription factors, intracellular signaling pathways, and chemokines interaction (Gao and Bataller, 2011). Therefore, in order to investigate the signaling pathways involved in ALD in the liver, HepG2 cell line was chosen.

HepG2 cells are commonly utilized in many hepatotoxicity studies and drug metabolism (Donato et al., 2013). They are known to share the functional similarities with normal human hepatocytes such as the production and secretion of plasma proteins and bile acids, mRNA expression for interferon γ, TNFα, TGFβ and interleukins (Stonans et al., 1999; Gonzales et al., 2015). Bouma et al. (1989) pointed out that HepG2 cells express most of the proteins including albumin, alpha-fetoprotein, apolipoproteins and so on. Apart from that, HepG2 cells have stable phenotype and high proliferation rate over the passage of cells, which make them hassleless while handling (Donato et al., 2013). Due to the close morphological and cellular properties, HepG2 cells are widely used as *in vitro* cell line instead of primary human hepatocytes (Donato et al., 2013) which are phenotypically unstable and come with high functional variability from batch-to-batch.

5.3 Extraction of Total Cellular Protein

As described in Section 3.5, the total cellular protein was isolated using Tri-Reagent® LS. It is a convenient reagent for fast and easy isolation of RNA, DNA and protein simultaneously from liquid samples. This isolation is achieved in a single-step via the liquid phase separation. This method is based on the combination of phenol and guanidine thiocyanate in monophase solution (Chomczynski, 1993).

BCP was added into a homogenized biological sample to separate the mixtures into three phases, namely, aqueous phase consisting RNA, interphase consisting DNA, and an organic phase consisting proteins. According to Zumbo (2014), when the pH of phenol is low, most proteins and small DNA fragments are separated into organic phase while larger DNA fragments remain at interphase and RNA are retained in the aqueous phase. The hydrophobic cores of the protein interact with phenol and precipitated out of the aqueous phase. The protein samples from the organic phase were precipitated, washed and solubilized according to the protocol.

5.4 Analysis on PI3K Signaling Pathway

5.4.1 Effect of Ethanol on PI3K Signaling Pathway

A precise molecular balance between the catalytic and regulatory subunits is a determinant for optimum PI3K signaling pathway (Ueki et al., 2002). Engelman et al. (2006) supported that a drop in PI3K p85 enhanced the PI3K signaling pathway. Based on the proteins expressions and densitometry, the phosphorylated PI3K p85 (Tyr458) protein band was denser in ethanol treatment as compared to vehicle control (Figure 4.2 (a) and Figure 4.3). This proved that PI3K signaling pathway in ethanol-treated HepG2 cells was highly activated at Tyr458 phosphorylation site. This result was in agreement with study from Umoh et al. (2014) that PI3K signaling pathway mediated effect of alcohol. Early works by Yu et al. (1998a) and Yu et al. (1998b) have demonstrated that activation of PI3K pathway happens via phosphorylation of

tyrosine residues in the SH2 domain of p85. The tyrosine residue identified from this study was at the position 458. Likewise, the activation of this pathway might be due to the improved stoichiometry between p110 and p85 subunits when the PI3K p85 subunit is reduced (Zeng et al., 2012), which was correlated with this study.

Activation of PI3K pathway might due to formation of ROS as a consequence of alcoholism (Zhang et al., 2016). During alcohol metabolism, acetaldehyde has been shown to increase the amount of mature SREBP by decreasing the sterol concentration (You et al., 2002; Wada et al., 2008). The regulation of SREBP activity in hepatic lipogenesis which may contribute to alcoholic fatty liver development is controlled by PI3K/Akt pathway (Porstmann et al., 2008). Hence, PI3K pathway might be activated in this study in order to regulate SREBP which was induced in response to ethanol exposure.

Besides, the activation may be promoted by somatic mutation in *PIK3R1* and *PIK3CA* genes, which encodes for p85α and p110α (Mizoguchi et al., 2004; Luo and Cantley, 2005). The presence of acetaldehyde from the metabolism of alcohol by CYP2E1 may be the cause of the mutation as it is a very toxic carcinogen and mutagen (Pöschl and Seitz, 2004). In addition, the inhibitory action of PI3K p85 subunit was diminished with the deletion on either part of the inhibitory domain (LED or C-terminal SH2) found in the inter-SH2 region (Chan et al., 2002). With such deletion, it is expected to produce an incompetent truncated protein without the inhibitory domain and eventually

the protein can activate PI3K activity (Mizoguchi et al., 2004). As a result of hyperactivation of this pathway, the cells were shown to grow extensively and form tumor in alcohol-induced cells (Luo and Cantley, 2005).

Ma (2013) concluded that alcohol consumption led to the activation of nuclear factor erythroid 2-related factor (Nrf2) and PI3K was found to be responsible to regulate Nrf2 (Wang et al., 2008). Walker et al. (2013) proposed that any oxidative burst such as alcohol exposure activated PI3K/Akt pathway and a complex was formed between Keap1 and Nrf2. mTOR cleaved the complex to allow the entry of Nrf2 into the nucleus. Subsequently, expression of antioxidant machinery is induced when Nrf2 interacts with the antioxidant response element in the nucleus (Walker et al., 2013).

5.4.2 Effect of Resveratrol/ Lauric Acid on PI3K Signaling Pathway

Based on Figure 4.3, the densitometry analysis of phosphorylated PI3K p85 protein was not heavily affected when resveratrol was administered alone (Bar C) but it is worth to note that its expression was repressed when HepG2 cells were co-treated with ethanol and resveratrol (Bar H). Resveratrol was used as a positive control in this study because there are many evidences supporting resveratrol as a potent antioxidant (Lastra and Villegas, 2007; Iuga et al., 2012) to inhibit the activities of CYP2E1 (Piver et al., 2001; Wu et al., 2013). Its hepatoprotective effect with antioxidant properties had been documented as

well (Bujanda et al., 2006; Kasdallah-Grissa et al., 2007; Ko et al., 2017). Studies showed that resveratrol reduced the phosphorylation of Akt, which is a downstream molecule of PI3K, and subsequently inhibited PI3K signaling pathway in different cell lines with association in reduced cell proliferation (Jiang et al., 2009; Parekh et al., 2011). With these abundant evidences, resveratrol was chosen to be a positive control in this study.

This study proved that ethanol treatment activated the PI3K pathway (Figure 4.3), but co-treatment with lauric acid suppressed the pathway. As mentioned earlier, activity of SREBP can be activated by PI3K/Akt signaling pathway (Krycer et al., 2010; Yamauchi et al., 2011). Its activity can also be activated by oxidative stress and it causes lipid accumulation in HepG2 cells (Sekiya et al., 2008). Here, we showed that lauric acid possess antioxidant properties which counterbalance the oxidative stress produced from the alcohol metabolism by decreasing the activation of the pathway. In this study, lauric acid inactivated the pathway by blocking on the same phosphorylated site as the protein expressions of phosphorylated Tyr458 were attenuated in a dose dependent manner. Thus, with lesser SREBP-1 protein and accumulation of fat, the risk of alcohol-induced fatty liver could be reduced. Indeed, lauric acid was shown to exert a similar magnitude of effect on PI3K pathway as resveratrol in similar concentrations. In addition, this study proved that lauric acid may attenuate the ethanol induction of CYP2E1 by inactivating PI3K pathway.

5.5 Analysis on NFκB Signaling Pathway

5.5.1 Effect of Ethanol on NFκB Signaling Pathway

Based on Figure 4.5, the phosphorylation of NFκB seemed increased upon ethanol exposure but the increment was shown to be insignificant which may be due to the large standard deviation among experimental repeats. Technical errors during transferring or blotting might have caused the inconsistency of the band intensities. In literature, alcohol is supposed to activate the NFκB signaling pathway. The insignificant result obtained here is in discrepancy with previous studies which proved that chronic ethanol induced accumulation of ROS and activated NFκB signaling pathway to trigger inflammation (Vallés et al., 2004; Wang et al., 2015). This showed that duration of ethanol exposure might play a role in the activation of NFκB signaling pathway in HepG2 cells.

Acute ethanol treatment has been found to reduce the NFκB phosphorylation at the site of NFκB p65 Ser536 in human monocytes (Mandrekar et al., 2007). However, Blanco et al. (2005) showed that acute ethanol exposure activated NFκB in astrocytes. These studies explained that the activation of NFκB pathway could be cell line specific which could explain the non-significant effect observed in HepG2 cells in this study.

Thus, it can be deduced from Figure 4.5 that the activation of NFκB pathway in the presence of acute alcoholism did not occur at the p105 Ser933 phosphorylation site in HepG2 cells. This incidence has been observed by

many researchers suggested that the alteration NFκB-mediated responses in acute and chronic alcoholism in different cell lines such as hepatocytes and macrophages (Hill et al., 2000; Uesugi et al., 2001).

5.5.2 Effect of Resveratrol/ Lauric Acid on NFκB Signaling Pathway

Once again, treatment with resveratrol or its co-treatment with ethanol did not exert any statistically significant result on the activation of NFκB pathway (Figure 4.5). This finding was in opposition to previous studies that resveratrol is a very potent antioxidant and able to suppress TNFα mediated NFκB expression (Yu et al., 2008). However, resveratrol did not exert changes on NFκB p105 (Ser933) protein in this current study because Lastra and Villegas (2005) proved that inhibition of NFκB pathway activation was through the degradation of IκB.

NFκB p105 is phosphorylated when the cells are stimulated with proinflammatory cytokines and undergoes proteasomal processing into p50 (Lang et al., 2003). NFκB p50 is preferentially to heterodimer with p65 rather than forming homodimer (Chen et al., 1998), to activate transcription of genes responsible in regulation of cell survival and inflammation (Luedde and Schwabe, 2011). This implies that lauric acid might able to counterbalance with proinflammatory cytokines and block the conversion of p105 to p50.

However, there was no statistically prominent effect on NFκB pathway in the co-treatment of ethanol and lauric acid (Figure 4.5). Christian et al. (2016) showed that limited proteasomal processing of p105 to generate p50 involve site-specific phosphorylation on p105 but the precise mechanisms remain unclear. The insignificant effect of lauric acid on NFκB pathway could be explained as Ser933 may not be the site for NFκB p105 phosphorylation in HepG2 cells and thus no p50 is generated to heterodimer with p65 in order to activate the pathway. The non-involvement of phosphorylated of NFκB p105 (Ser933) protein in lauric acid-ethanol regulation in HepG2 cells can be supported by Gupta et al. (2010) that antioxidant can act at various steps in NFκB pathway, in different cell lines.

5.6 Summary of the Investigation on Interaction between the Signaling Pathways and CYP2E1 Expression under the Effect of Lauric acid

Hayat (2014) concluded that elevation of CYP2E1 and oxidative stress as a result of acute alcoholism has caused a gain in lipogenic SREBP, which consequently lead to steatosis. As mentioned before, SREBP-1 activity can be enhanced via PI3K/Akt signaling pathway. Thus, the antioxidant property of lauric acid is possibly able to reduce the activity of SREBP through suppression of PI3K signaling pathway. Suppression of PI3K pathway eventually reduces the activity of mTOR and STAT3, so does the transcription of CYP2E1 (Patel et al., 2014). Here, we proved that lauric acid abolished the phosphorylation of PI3K p85 at Tyr458 by ethanol, and this could most likely

be the mechanism behind the reduction of CYP2E1 expression in HepG2 cells shown by Ong (2017).

Although NFκB is the transcription factor to activate CYP2E1, the activation of NFκB could be cell line specific and dependent on alcohol exposure duration. This could explain the discrepancy of the result in this study. Therefore, it can be concluded that the increase in CYP2E1 mRNA expression in HepG2 cells shown by Ong (2017) did not occur at the Ser933 phosphorylation site of NFκB p105. Both ethanol treatment and subsequent co-treatment with the antioxidants did not produce any significant changes to the activation site.

5.7 Future Studies

From this experiment, there was a large variation of the phosphorylated NFκB p105 (Ser933) protein bands expression as illustrated from the standard deviation error bars from three separate experiments (Figure 4.5). This would call for the experiment to be repeated a few more times to verify the results. Besides, other phosphorylation sites on the PI3K and NFκB, such as Akt and NFκB p65, can be included in future studies to have a deeper understanding on the activation of the signaling pathways upon the effect of ethanol and lauric acid. It will be beneficial knowledge to identify if lauric acid could target any other phosphorylation sites on the pathway to counter the effects of alcohol. In

this study, sole treatment with lauric acid and resveratrol produced insignificant results on both PI3K and NFκB pathways in HepG2 cells. It would be interesting to further evaluate if these antioxidants might exert a different mechanism due to the amount of ROS or acetaldehyde in HepG2 cells, if their actions are affected by ROS or acetaldehyde.

The connection between alcohol or lauric acid and PI3K or NFκB signaling pathway were established in this study. However, the relationship of CYP2E1 protein expression to the pathways was elucidated from previous study (Ong, 2017). The direct confirmation of the role of these pathways on CYP2E1 could be further confirmed by performing small interference RNA (siRNA) experiment in which the PI3K pathway is knockdown and CYP2E1 expression is analyzed.

Histological examination, hepatic triglyceride level, hepatic enzymes level including aspartate transaminase and alanine transaminase can be performed to observe the differences in cell morphology and biochemical status under different treatments. Chronic alcohol exposure in HepG2 cells can be conducted to compare its effect with this study, in which the exposure time to alcohol was shorter. Nuclear translocation study on NFκB could be carried out to investigate if there is any change in the nuclear and cytoplasmic extracts.

CHAPTER 6

CONCLUSION

This study was conducted with all the objectives accomplished successfully. The protein expression of phosphorylated PI3K p85 (Tyr458) was increased in 2% (v/v) of ethanol treatment. By using specific antibodies against phosphorylated and total protein, the PI3K pathway was shown to be activated at position Tyr458 in ethanol-induced HepG2 cells. HepG2 cells under the co-treatment with ethanol and different concentrations of lauric acid exhibited a decrement in the level of phosphorylated PI3K p85 in a dose dependent manner. In this study, lauric acid was proven to attenuate the activation of PI3K pathway at Tyr458 in a dose response manner. Nonetheless, there was no significant change observed in NFκB p105 (Ser933) protein expression in lauric acid-ethanol regulation in HepG2 cells. Hence, it can be concluded that position Ser933 of NFκB p105 may not the site of activation for NFκB pathway activation by ethanol in this study. Therefore, other phosphorylation sites in PI3K and NFκB pathways should be performed to have a more thorough understanding of the mechanism. The direct role of these pathways on CYP2E1 can be further confirmed through siRNA experiment.

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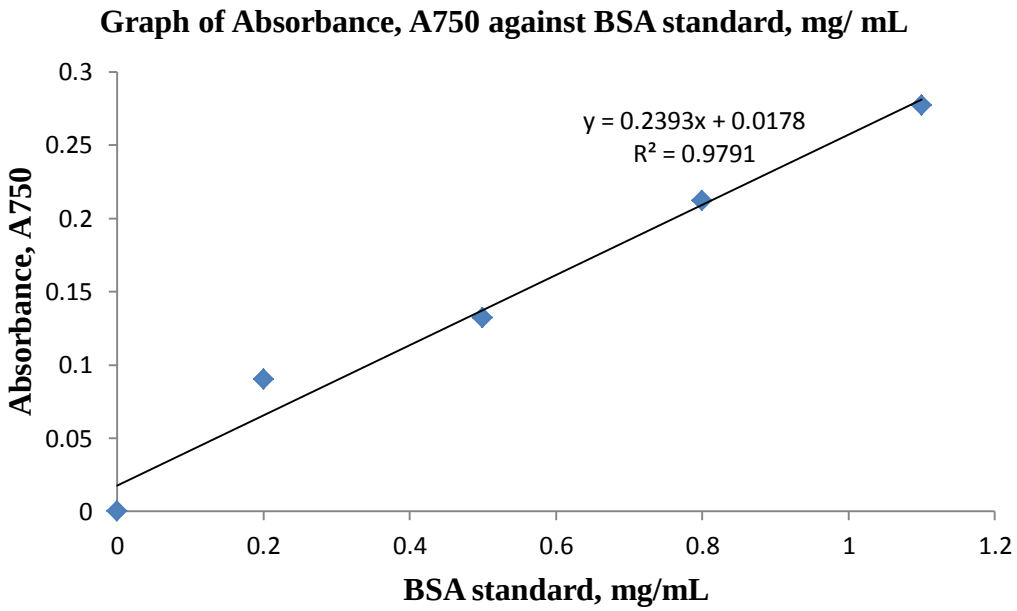
APPENDIX

Appendix A

The BSA standard calibration curve was plotted with the concentration of 0 mg/ mL (blank), 0.2 mg/ mL, 0.5 mg/ mL, 0.8 mg/ mL and 1.1 mg/ mL. The concentrations of extracted total cellular protein sample from various treatment of ethanol with lauric acid or resveratrol were calculated with respect to the absorbance values.



Appendix 1-4

Universiti Tunku Abdul Rahman			
Form Title : Supervisor's Comments on Originality Report Generated by Turnitin for Submission of Final Year Project Report (for Undergraduate Programmes)			
Form Number: FM-IAD-005	Rev No.: 0	Effective Date: xx/xx/xxxx	Page No.: 12 of 1

FACULTY OF SCIENCE

Full Name(s) of Candidate(s)	LUA YING HUAN
ID Number(s)	15ADB06989
Programme / Course	BACHELOR OF SCIENCE (HONS) BIOMEDICAL SCIENCE
Title of Final Year Project	THE EFFECTS OF LAURIC ACID ON PI3K AND NFκB SIGNALING PATHWAYS IN ALCOHOL-IINDUCED HEPG2 CELLS

Similarity	**Supervisor's Comments** **(Compulsory if parameters of originality exceeds the limits approved by UTAR)**
Overall similarity index: __12____ % **Similarity by source** Internet Sources: ___7___ % Publications: ___8___ % Student Papers: ___4___ %	
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Note: Parameters (i) – (ii) shall exclude quotes, bibliography and text matches which are less than 8 words.

Note Supervisor/Candidate(s) is/are required to provide softcopy of full set of the originality report to Faculty/Institute

Based on the above results, I hereby declare that I am satisfied with the originality of the Final Year Project Report submitted by my student(s) as named above.

_____ _____

Signature of Supervisor Signature of Co-Supervisor

Name: _____ Name: _____

Date: _____ Date: _____