INVESTIGATION OF THE EFFECT OF LAURIC ACID ON LIVER X RECEPTOR α (LXRA) mRNA EXPRESSION LEVEL IN ALCOHOL-INDUCED HEPG2 CELLS

By

LEE SOKE SUN

A project report submitted to the Department of Biomedical Science

Faulty of Science

UniversitiTunku Abdul Rahman

in partial fulfilment of the requirements for the degree of

Bachelor of Science (Hons) Biomedical Science

September 2018

ABSTRACT

INVESTIGATION OF THE EFFECT OF LAURIC ACID ON LIVER X RECEPTOR α (LXRA) mRNA EXPRESSION LEVEL IN ALCOHOL-INDUCED HEPG2 CELLS

LEE SOKE SUN

Liver X Receptor α (LXRA) is a member of nuclear receptor superfamily. LXRA is a xenobiotic sensor that mainly helps in cholesterol and lipid metabolism. LXRA was suggested to regulate the cytochrome P450 enzyme, CYP3A4. CYP3A4 plays an important role in metabolising ethanol and other xenobiotics. Ethanol metabolism by CYP3A4 will cause oxidative stress which helps in development of alcoholic liver disease (ALD). Lauric acid is a medium length fatty acid chain which is a potential antioxidant that may help in treating ALD. In this study, the effect of ethanol on LXRA mRNA expression level in HepG2 cells was investigated. Lauric acid was also used in this study to investigate its role in the regulation of LXRA in alcohol-induced HepG2 cells. The study was conducted in two phase. In Phase 1, HepG2 cells was treated with 1%, 2% and 5% (v/v) ethanol while in Phase 2 HepG2 cells was treated with 2% (v/v) ethanol and different concentration of lauric acid (5 µM, 10 µM and 20 µM) for 24 hours. By using quantitative reverse transcription polymerase chain reaction (qRT-PCR), an increase in LXRA

mRNA expression level was observed in alcohol-induced HepG2 cells while a reduction was observed in co-treated HepG2 cells with ethanol and lauric acid. The most significant reduction of LXRA mRNA expression level was shown at 20 μM lauric acid. This study showed that lauric acid has significant effect in decreasing LXRA mRNA expression level, and this may in turn, reduced CYP3A4 expression.

ACKNOWLEDGEMENT

It is an honour to be given the chance to express my gratitude to those people that helped me during my final year project. First of all, I would like to express my deepest gratitude to my beloved supervisor, Dr, Chew Choy Hoong for her guidance, advices and patience throughout my project period. My final year project and thesis wouldn't be done without her assistance and guidance.

After that, my sincere gratitude goes to my senior postgraduates students, Mr. Wong Hong Kin, Mr. Tham Yong Yao, Ms. Cheong Hui Ting and Ms. Esther Thou MunHuieh for guiding and helping me whenever I had met difficulties during my project time. Besides, I would also like to thank all the lab officers, Mr. Tie Shin Wei, Mr. Saravanan A/L Sivasangaran and Mr. Gee Siew Meng for their assistance and tolerance when I encountered problem in my project.

Then, I would like to show my gratefulness to my bench mates, Ms. Lua Ying Huan and Mr. Yong Wee Shen for helping me during the whole project period. I would also thank my friends and course mates for giving me emotional and mentally support throughout my final year project. Lastly, I would like to thank my parents and siblings for their encouragement and support that have provided me strength to complete my final year project.

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.

_____

(LEE SOKE SUN)

APPROVAL SHEET

This project report entitled "**INVESTIGATION OF THE EFFECT OF LAURIC ACID ON LIVER X RECEPTOR α (LXRA) mRNA EXPRESSION LEVEL IN ALCOHOL-INDUCED HEPG2 CELLS**" was prepared by LEE SOKE SUN and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at UniversitiTunku Abdul Rahman.

Approved by:

_____

(Assoc. Prof. Dr. CHEW CHOY HOONG) Date:……..

Supervisor

Department of Biomedical Science

Faculty of Science

UniversitiTunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: 23 August 2018

PERMISSION SHEET

It is hereby certified that **LEE SOKE SUN** (ID No: **15ADB07785**) has completed this final year project entitled "**INVESTIGATION OF THE EFFECT OF LAURIC ACID ON LIVER X RECEPTOR α (LXRA) mRNA EXPRESSION LEVEL IN ALCOHOL-INDUCED HEPG2 CELLS**" under the supervision of Associate Prof. 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,

_____

(LEE SOKE SUN)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
ABCA1	ATP binding cassette subfamily A member 1
ABCG5	ATP-binding cassette sub-family G member 5
ABCG8	ATP-binding cassette sub-family G member 8
ACTB	β-actin
ADH	Alcohol dehydrogenase
AF-1	N-terminal ligand-independent activation function domain
AF-2	C-terminal ligand-dependent transactivation sequence/ activation function-2
ALD	Alcoholic liver disease
ALDH2	Aldehyde dehydrogenase 2
ATP	Adenosine triphosphate
BCP	1-Bromo-3-Chloropropane
cDNA	Complementary deoxyribonucleic acid
CO2	Carbon dioxide
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CYP1A2	Cytochrome P450 Family 1 Subfamily A Member 2
CYP2E1	Cytochrome P450 Family 2 Subfamily E Member 1
CYP3A4	Cytochrome P450 Family 3 Subfamily A Member 4
CYP7A1	Cytochrome P450 Family 7 Subfamily A Member 1

-d(RFU)/dT	Rate of change of the relative fluorescence units with time
DBD	DNA-binding domain
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
Egr-1	Early growth response-1
ER	Oestrogen receptor
et al.	“et alia” (Italia word referring to ‘and other’)
FBS	Foetal bovine serum
FXR	Farsenoid X Receptor
g	Acceleration of gravity (~9.8 m/s2)
HCC	Hepatocellular carcinoma
HDL	High density lipoprotein
HepG2	Human hepatocellular carcinoma cell line
HSC	Hepatic stellates cells
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon-gamma
kb	kilobases
LBD	Ligand-binding domain
LDL	Low density lipoprotein
LXR	Liver X receptor
LXRA	Liver X receptor alpha
LXRB	Liver X receptor beta
LXRE	Liver X response elements
MEM	Minimum Essential Medium

mRNA	Messenger ribonucleic acid
NAD^+	Oxidized nicotinamide adenine di-nucleotide
NADH	Reduced nicotinamide adenine dinucleotide
$NADH/NAD^+$	Reduced nicotinamide adenine dinucleotide/oxidized nicotinamide adenine di-nucleotide ratios
NHRs	Nuclear hormone receptors
nM	Nanomolar
NRs	Nuclear receptors
OD	Optical density
PBS	Phosphate buffered saline
PXR	Pregnane X Receptor
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RCT	Reverse cholesterol transport RFU
RFU	Relative fluorescence units
RNA	Ribonucleic acid
ROS	Reactive oxidative species
rRNA	Ribosomal ribonucleic acid
SREBP	Sterol regulatory element-binding protein
SREBP-1	Sterol regulatory element-binding protein-1
SREBP-1a	Sterol regulatory element-binding protein-1a
SREBP-1c	Sterol regulatory element-binding protein-1c
SREBP-2	Sterol regulatory element-binding protein-2
TBE	Tris-Boric acid-EDTA
Tm	Melting temperature

TNFα	tumour necrosis factor alpha
v/v	Volume/volume
VCAM-1	vascular cell adhesion molecule-1
w/v	Weight/volume

CHAPTER 1

INTRODUCTION

Lauric acid is a saturated fatty acid that can be primarily found in palm oil and coconut oil (Fife, 2013; Dayrit, 2015). In this study, lauric acid's role as a potential antioxidant was investigated. Previous research by senior showed that lauric acid possessed potential in anti-inflammatory and anti-oxidative properties (Cheah et al., 2014). This was also supported by Henry et al. (2007) showed that lauric acid reduced pro-inflammatory cytokines. In addition, lauric acid causes decreased level of CYP3A4 and CYP2E1 mRNA expression and reduces the oxidative stress in alcohol-induced HepG2 cells (Lim, 2017; Ong, 2017). In this study, lauric acid was hypothesised to down regulate the mRNA expression level of LXRA in alcohol-induced HepG2 cells. Since LXRA is the regulator for CYP3A4, this effect on LXRA may in turn reduce the CYP3A4 expression level.

Alcohol is the most common xenobiotic that causes liver failure in the human population. Based on World Health Organization Report in 2014, there were about 534,600 deaths in the world due to alcohol attributed liver cirrhosis in 2012. It was also reported that there was 30.8% male and 28.2% female in Malaysia who has developed liver cirrhosis due to alcoholism (Rehm et al., 2013). Liver cirrhosis is an irreversible fibrosis stage of liver tissue due to long term liver damage. It is the end stage in the spectrum ranges of alcoholic liver

disease (ALD). The liver is considered as non-functional or failure at this stage (Walsh and Alexander, 2000).

The pathogenesis of ALD is still unclear, but it has been hypothesised to be caused by ethanol metabolism which will cause production of toxic metabolites, reactive oxygen species (ROS) and oxidative stress in the cells. Ethanol metabolism is associated with cytochrome P450 such as CYP2E1 and CYP3A4 (Osna et al., 2017). The role of CYP3A4 is less commonly known in ethanol metabolismas compared to CYP2E1 but previous studies had suggested that CYP3A4 might act similarly with CYP2E1 in metabolising ethanol. Michael (2017) showed that ethanol metabolism by CYP3A4 could cause liver damage as it increased the production of ROS and toxic metabolites.

Liver X receptor α (LXRA) is a nuclear hormone receptor superfamily of ligand activated transcription factor (Janowski et al., 1996). Many previous studies have shown that LXRA plays a vital role in cholesterol and carbohydrates metabolism. It is also an important transcription factor that regulates fatty acid metabolism and transport in liver cells (Kalaany and Mangelsdorf, 2006). LXRA control lipid metabolism by targeting the lipogenic gene expression such as CYP7A1, ABCA1, ABCG5, ABCG8, SREBP-1 and so on (Jump et al., 2013).

Based on Watanabe et al. (2013), LXRA was hypothesised to play dual role in the regulation of CYP3A4 gene expression level. CYP3A4 gene expression can be induced by ethanol and cholesterol consumption (Lim, 2017; Watanabe et al, 2013). Watanabe and his team showed that LXRA inhibited the PXR-mediated CYP3A4 gene expression and increased the CYP3A4 gene expression by binding on the CYP3A4 responsive elements. Thus, it can be hypothesised that the increase of CYP3A4 gene expression level in alcohol-induced HepG2 cells may be induced by LXRA.

Hence, the objectives of this study were:

1. To identify the dose impact of alcohol in LXRA mRNA expression level in HepG2 cells.
2. To determine the effect of lauric acid in mRNA expression level of LXRA in alcohol induced HepG2 cells.
3. To investigate the role of LXRAin the regulation of alcohol induction of CYP3A4in the presence of lauric acid.
4. To study the relationship of LXRA and CYP3A4 in alcohol induction with or without lauric acid treatment.

CHAPTER 2

LITERATURE REVIEW

2.1 Lauric Acid

Lauric acid, also known as dodecanoic acid, is a saturated fatty acid with 12 carbon atoms chain that is commonly found in laurel oil, coconut oil and palm kernel oil. It is also known as beneficial medium chain fatty acid (MCFA) that has antioxidants, anti-inflammation and also antimicrobial properties (Henry et al., 2002; Uday et al., 2014). Although lauric acid is a saturated fatty acid, it is stored as fat after ingestion but is directly transported into liver via portal vein and metabolised into energy (Dayrit, 2014). Energy production from lauric acid begins when lauric acid was metabolised into acetyl-CoA after β-oxidation in liver. Acetyl-CoA is one of the starting components in Krebs cycle which will eventually produce carbon dioxide and ATP (Dayrit, 2014; Dayrit, 2015).

Lauric acid had been proven that have a strong antioxidant and anti-inflammation properties. Based on Henry et al. (2002), they showed that lauric acid was able to control the inflammation in the cells by inhibit the production of cyclooxygenase isoform, COX-I and COX-II enzyme. They also suggested that the antioxidant effect of fatty acid was increased from C-8 to C-14 fatty acid. Besides that, lauric acid is further proven to be a potential anti-inflammatory agent by our seniors in the laboratory (Lim et al., 2015). In this

study, lauric acid was shown to inhibit the up-regulation of inflammatory cytokine and interferon-gamma (IFN-γ) on vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression in THP-1 differentiated human macrophages. The anti-inflammatory effect of lauric acid is suggested to give a huge contribution to treat atherosclerosis. The anti-oxidative properties of lauric acid are further supported by Nevin and Rajamohan (2004). Here, they showed that the lauric acid could reduce the lipid peroxidation process and increased the antioxidant enzyme in cells. Previous studies also proved that lauric could decrease the expression of CYP3A4 and CYP2E1 genes in alcoholic liver disease (Lim, 2017; Ong, 2017).

In addition, lauric acid has hypocholesterolemic effect as it increases high density lipoprotein (HDL) levels and decreases the total cholesterol/HDL ratio in blood (Mensink et al., 2003). This characteristic of lauric acid is further supported by De et al. (2001) who proved that lauric acid caused a huge increase in HDL-cholesterol in patients.

The derivative of lauric acid, monolaurin, comes with antimicrobial, antiviral and antiprotozoal effect (Murray et al., 2005). Conversion of lauric acid to monolaurin is carried out by esterification process (Fife, 2013). Monolaurin is able to kill the various gram-positive bacteria and protozoa by distorting the lipid bilayer of microorganism plasma membrane (Fife, 2013). Antiviral action of monolaurin is through the mechanism of solubilising the lipid

content in the viral envelope and this leads to the disintegration of viral outer membrane (Fife, 2013). There are several microorganism that were known killed effectively by lauric acid and these include *Streptococcus*, *Staphylococcus*, *Helicobacter pylori*, Herpes virus, Epstein-Barr virus and etc (Lieberman et al., 2006; Nakatsuji, et al., 2009).

2.2 Alcoholic Liver Disease (ALD) and Pathogenesis

Alcohol liver disease is common disease that leads to chronic liver damage or liver cirrhosis and fibrosis due to overconsumption of alcoholic drink for a long duration. ALD has spectrum ranges of phenotypes from alcoholic fatty liver (steatosis), alcoholic hepatitis, alcoholic liver cirrhosis to hepatocellular carcinoma (HCC) (Shrestha, 2011). Grant et al. (1988) stated that at least 80% of chronic alcoholic drinker will develop steatosis, 10% to 35% of them developed alcoholic liver inflammation and 10% eventually developed liver cirrhosis. The pathogenesis of each stage of ALD is not clearly studied yet but there are several hypotheses which have been made. The pathogenesis of ALD might be related to the intermediate metabolites produced during the ethanol metabolism process, production of acetaldehydes and also production of reactive oxygen species (ROS) (Ceni et al., 2014).

An overview of ethanol metabolism was shown in Figure 2.1. The major route of ethanol metabolism is oxidised by alcohol dehydrogenase (ADH) which will eventually oxidised ethanol into acetaldehydes, a toxic metabolites which

causes liver injury. The production of reduced nicotinamide adenine dinucleotide (NADH) during the ethanol metabolism is also highly toxic due to its ability to bind to the macromolecule in the cells and then disrupt the function and structure of the macromolecules (Mauch et al., 1986). Toxicity of acetaldehydes and NADH can be relieved when acetaldehyde is metabolised by aldehyde dehydrogenase 2 (ALDH2) into acetate. Another ethanol-oxidising pathway in hepatocytes is through the cytochrome P450 enzyme, CYP2E1. CYP2E1 is the major cytochrome P450 enzyme involved in ethanol metabolism. Other than, CYP2E1, CYP3A4 and CYP1A2 also function similarly to CYP2E1 in ethanol metabolism. Even though CYP2E1 will act slower than ADH enzyme, it has a 10-fold higher capacity for ethanol binding (Osna et al., 2017). The increase in CYP2E1 will increase the production of ROS and this would lead to oxidative stress and eventually causes the liver cell injury (Osna et al., 2017).

Most heavy drinker will develop the first stage of ALD which is steatosis. Steatosis is characterised by the lipid accumulation, mainly triglycerides in the hepatocytes. It can develop from the consumption of at least 80 g of alcohol per day (Teli et al., 1988; Walsh and Alexander, 2000). Formation of steatosis in early studies was shown related to the high level of reduced nicotinamide adenine dinucleotide/oxidized nicotinamide adenine di-nucleotide ratios ($NADH/NAD^+$) in hepatocytes caused by alcohol consumption. The increase in $NADH/NAD^+$ ratio had affected the β-oxidation process that happened in mitochondrial and lead to fatty liver formation (Baraona and Lieber, 1979). However, recent studies showed that alcohol consumption would regulate the

expression of lipogenic transcription factor which will stimulate the lipid synthesis and inhibit the β-oxidation process. Two main transcription factors that are induced by ethanol are early growth response-1 (Egr-1) andsterol regulatory element binding protein-1c (SREBP-1c). Acetaldehyde was stated by previous studies that it could increase the expression of SREBP-1c while decreasing the expression of peroxisome proliferator activated receptors (PPAR) (Orman et al., 2013). Egr-1functions to induce the expression of lipogenic cytokines, tumour necrosis factor alpha (TNFα) which will also activate the lipogenesis in liver (Donohue et al., 2012). On the other hand, Wei et al. (2013) suggested that alcohol consumption caused lipolysis in the adipose tissue and the free lipid molecules from the adipose tissue were up-taken by liver cells, leading to steatosis.

Accumulation of fat in liver can progress into alcoholic hepatitis which is liver inflammation due to excess alcohol consumption. Alcoholic hepatitis can be characterised by leukocytes infiltration and also presence of Mallory-Denk bodies in hepatocytes that underwent ballooning degenerations (Leftkowitch, 2005). The excessive alcohol intake will cause a huge number of toxic metabolites production. This induces the Kupffer cells or liver macrophages differentiate into M1 phenotypes (pro-inflammatory phenotypes). M1 phenotypes Kupffer cells will release numerous pro-inflammatory cytokines such as interleukins and chemokines. This will activate the immune system in the body and attract the inflammatory cells to the liver and thus lead to hepatitis. Besides that, induction of CYP2E1 will lead to formation of

oxidative stress and activate the release of pro-inflammatory cytokines from macrophages (Osna et al., 2017).

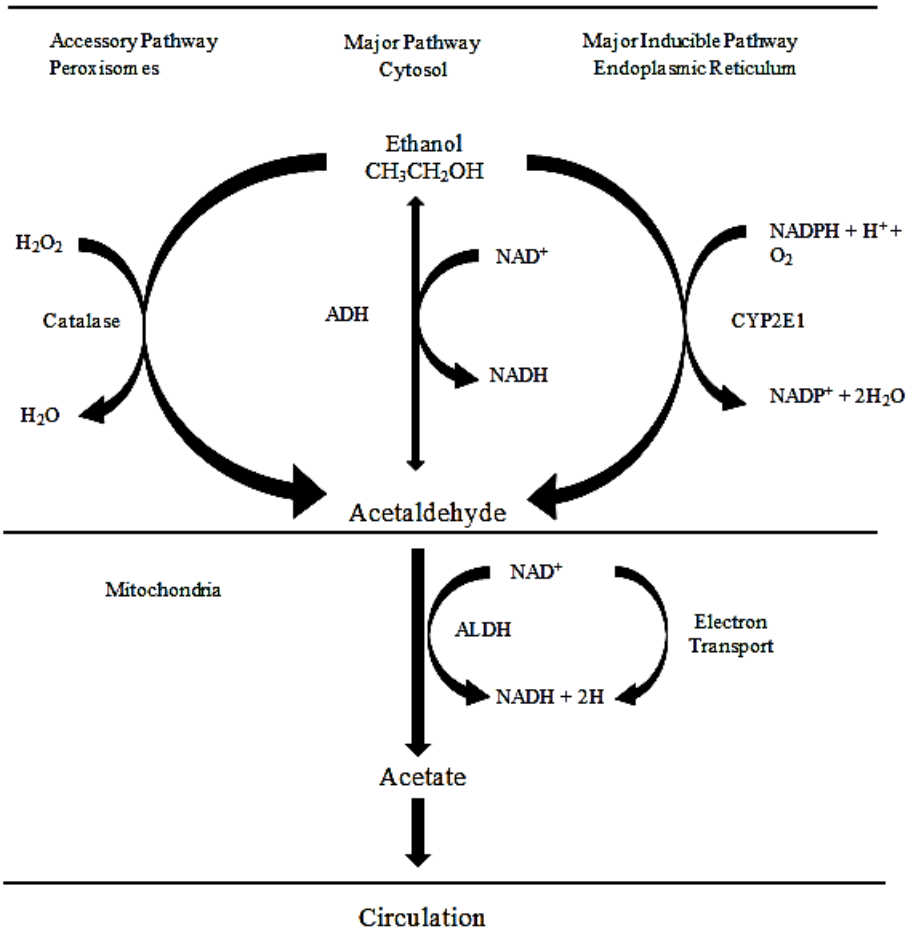


Figure 2.1: Major and minor route of ethanol metabolism (Adapted from Osna et al., 2017).

Liver cirrhosis is the last stage of ALD, it will be happened if continuous alcohol consumption is occurred. Liver cirrhosis is the accumulation of collagen and matrix in the extracellular region which result in fibrosis of liver. The main indication of liver cirrhosis is the surrounding of fibrous septa in the

hepatic parenchyma (Osna et al., 2017). Liver failure will happen when liver cirrhosis is developed due to the replacement of functional hepatocytes with scar tissue or fibrotic tissue (Walsh and Alexander, 2000). Hepatic stellates cells (HSC) plays and important role in liver fibrosis development. It was activated during the hepatic injury and then causes the deposition of collagen and extracellular-matrix protein in the liver cells. Hepatic stellates cells also activate the leukocytes to release cytokines that enhance the activation of HSC and exacerbating the fibrosis process (Friedman, 2008).

ALD is a main risk factor of hepatocellular carcinoma (HCC). Patient with alcoholic liver cirrhosis will have a high chance to develop HCC. The mechanism of ALD patient developing HCC includes the formation of acetaldehydes and the induction of CYP2E1 which help in metabolising pro-carcinogenic compound in alcoholic drink (McKillop and Schrum, 2009).

2.3 Nuclear Receptor Superfamily

Nuclear receptors (NRs) are transcriptional factors that made up by a central DNA-binding domain which specific to vary hormone response elements (Laudet and Gronemeyer, 2002). Nuclear receptor superfamily is categorised into nuclear hormone receptors (NHRs) and orphan nuclear receptors. Nuclear hormone receptors are those transcription regulators with identified hormonal ligands whereas orphan nuclear receptors are receptors with unknown ligands

bind on it. One specific characteristic of NRs is they can bind to targeted small hydrophobic molecules which cannot be done by other transcription factors (Germain et al., 2003).

Then the nuclear superfamily can be further sub-categorised into four classes: Class I, II, III and IV. The categorisation of nuclear receptors superfamily is based on the dimerization and DNA-binding properties of the receptors. Class I receptors are ligand-induced homodimers which bind to inverted repeats of DNA half-sites. Class II receptors will form heterodimer with Retinoid X Receptor (RXR) and will bind to direct repeats of DNA. Class III is similar to Class I receptors which will form homodimer but bind to the direct repeat of targeted DNA. Class IV receptors acts as monomers and bind to the extended core sites of DNA molecules. Class III and IV are mostly orphan receptors (Mangelsdorf et al., 1995).

Nuclear hormone receptors play an important role in most physiological function including homeostasis, cell differentiation, metabolism process and also development of embryo. Since nuclear hormone receptors related to mostly physiology function in human body, it is often studied as therapeutic target for certain diseases. Nuclear receptors also help in regulating the proteins expression by acting as co-activators or co-repressors (Olefsky, 2001). The modes of action of nuclear receptors basically act in three steps which are repression, derepression and transcription activation (Laudet and Gronemeyer, 2002). For examples, oestrogen receptor (ER) with different ligand binding on

it will lead to downregulation or upregulation of transcription factor such as c-fos and c-jun and lead to vary biological effects in human body (Hall et al., 2001). Another example is Liver X Receptor (LXR) and Farsenoid X Receptor (FXR) which play vital function in regulating cholesterol level in human body (Lu et al., 2001).

2.4 Liver X Receptor (LXR)

LXRs are NR subfamily 1 group H member which was first discovered in 1995 (Oosterveer et al., 2010). LXRs are divided into two isoforms which are LXRA (NR1H3) and LXRB (NR1H2). LXRA can be found on chromosome 11p11.2 while LXRB can be found on chromosome 19q13.3. (Zhao and Dahlman-Wright, 2010). Both isoforms of LXRs are identical in almost 80% in amino acid sequences and have same DNA and ligand binding domain. (Zhao and Dahlman-Wright, 2010). Compared to LXRB that expressed high level in brain, LXRA can be seen in predominantly active tissue such as liver, kidney and so on (Fan et al., 2008).

LXRs are grouped under adopted orphan receptors that will activated by cholesterol or oxysterol. The oxysterol that commonly detected by LXRs are 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol and 24(S), 25-epoxycholesterol (Makishima, 2006). Thus, LXRs are known as cholesterol sensors that help in cholesterol and carbohydrates metabolism (Lu et al., 2001).

Cholesterol metabolism by LXR includes 4 major pathways which are regulation of bile acid synthesis, reverse cholesterol transport (RCT), biosynthesis of cholesterol and absorption or excretion of cholesterol in the intestine (Zhao and Dahlman-Wright, 2010). This helps LXRs to induce the gene transcription and protect the cells from excessive cholesterol level.

Besides that, LXRs is involved in the lipogenesis process by regulating the lipogenic gene such as ABCA1, fatty acid synthase and so on (Repa et al., 2000; Tall et al., 2000). This suggests that the increase of LXRs might lead to the liver steatosis and atherosclerosis. In addition, research has showed that LXRA could modulate the inflammatory response in macrophages. It helps to inhibit the pro-inflammatory cytokines induction by SUMOylation-dependent pathway (Joseph et al., 2003; Ghisletti et al., 2007).

2.4.1 LXR Structure

Structure of LXR consists of four domains that help in transcription process (Wójcicka et al., 2007). The first domain is an N-terminal ligand-independent activation function domain (AF-1). It can stimulate transcription process without the presence of a ligand. Second domain is known as DNA-binding domain (DBD) which is formed by two zinc fingers with an approximately 76 amino acids. Third domain is a hydrophobic ligand-binding domain (LBD). It is vital in ligand binding and help in receptor dimerization process. The last

domain is a C-terminal ligand-dependent transactivation sequence, or also known as activation function-2 (AF-2). It helps in activating transcription process once the ligand is binding to the third domain (Wójcicka et al., 2007). Figure 2.2 showed the structure of an hLXRA molecule.

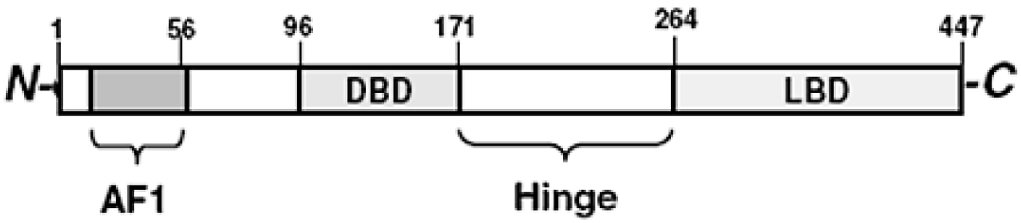


Figure 2.2: Schematic representation of hLXRA(Adapted from Chen et al., 2006).

Activation of LXRs transcription starts with LXRs forming a heterodimer with RXR. A co-repressor will bind to the LXR/RXR heterodimer and form a complex. The complex will bind on the LXR response elements (LXRE) with repression of target gene (Zelcer and Tontonoz, 2006). The LXRE sequence is a direct repeat-4 DNA sequences with two AGGTCA hexameric half sites (Wójcicka et al., 2007). The co-repressor will be exchanged into co-activator complexes once the oxysterol binds to the LXR/RXR heterodimer. Then, the target gene will be regulated. The activation process is shown in Figure 2.3.

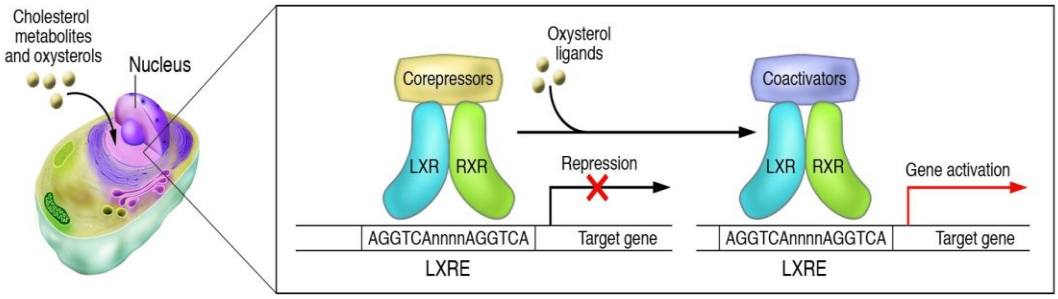


Figure 2.3: Schematic representation of LXR activation process. (Adapted from Zelcer and Tontonoz, 2006).

2.4.2 LXRA and Alcoholic Liver Disease

LXRA does not play any direct role in the ethanol metabolism pathway as it functions in the lipid metabolism after induction by alcohol in hepatocytes. As mentioned above, chronic consumption of alcohol will lead to the accumulation of fat in the hepatocytes. One of the reasons for fat accumulation is due to lipogenesis in the liver that activated by SREBP, which is one of the target gene of LXRA.

SREBP is a transcription factor that enters the nucleus and activates gene that functions in synthesis of cholesterol and fatty acid. There are three types of SREBP which are SREBP-1a, SREBP-1c and SREBP-2. Research has shown that SREBP-1c is the key factor that functions in regulation of lipogenic enzyme and induce lipogenesis. According to You et al. (2002), SREBP-1c was increased in the mice after ethanol feeding. Besides that, histological

slides also proved that fatty liver was developed the mice after ethanol feeding. This showed that alcoholic fatty liver was due to the increased of SREBP-1c.

Numerous studies have proven that LXRA is able to regulate or activate the SRECP-1c gene expression. Thus, the increase of SREBP-1c due to the huge amount of LXRA will lead to the activation of SREBP-1c-regulated lipogenic enzyme (Cha and Repa, 2006). LXRA activates SREBP-1c expression by forming a heterodimer with RXR and then bind on the LXRE before located in the SREBP-1c gene (Yoshikawa et al., 2003). Therefore, with the presence of chronic alcohol consumption, LXRA will be increased and this leads to the activation SREBP-1c which in turn, leads to steatosis formation.

2.4.3 LXRA and CYP3A4

CYP3A4 is a cytochrome P450 enzyme that is highly expressed in liver and small intestine. It is the most abundant CYP enzyme in liver and it helps in metabolising almost 50% of the drugs present in the market. The metabolism of ethanol by CYP3A4 is still unclear but it is hypothesised that CYP3A4 acts similarly to CYP2E1, which metabolises alcohol through oxidation process with the production of some free radicals or ROS (Katzung et al., 2012; Michael, 2017). A study by senior in our laboratory showed that CYP3A4 was actually up-regulated in the presence of alcohol in HepG2 cells (Lim, 2017).

The up-regulation of CYP3A4 is mainly induced by the Pregnane X Receptor (PXR) (Faucette et al., 2006). In short, gene expression of CYP3A4 is activated by binding of xenobiotics to PXR in the cytoplasm. Then, PXR with its ligand will form a heterodimer with RXR and bind to the PXR-responsive element located at the promoter region of CYP3A4 gene in the nucleus. Binding of PXR thus activates the gene expression of CYP3A4 (Tompkins and Wallace, 2007). The production of CYP3A4 will metabolise the xenobiotics and release some toxic metabolites such as reactive oxygen species (ROS) (Tompkins and Wallace, 2007). However, CYP3A4 could also be regulated by LXRA in both positive and negative way (Figure 2.4) (Watanabe et al., 2013).

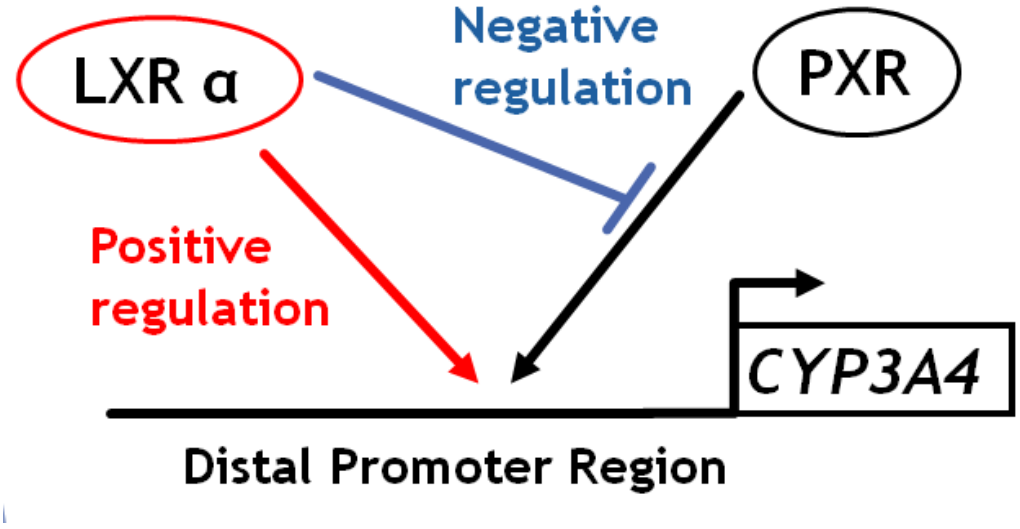


Figure 2.4: Graphical representation of dual roles of LXRA (Adapted from Watanabe, et al., 2013).

Based on Watanabe et al. (2013), they suggested that LXRA actually inhibited the activation of PXR-mediated CYP3A4 gene expression. This is due to the sharing of CYP3A4 responsive element between PXR and LXRA. This leads to the competition between PXR and LXRA. This will decrease CYP3A4 gene expression simultaneously. Wada et al. (2008) had suggested that there were also a co-activators competition between LXRA and PXR.

In contrast, LXRA is shown to increase the gene expression of CYP3A4 in another pathway. Watanabe et al. (2013) suggested that the association of LXRA and CYP3A4 gene might be due to CYP3A4 playing a vital function in cholesterol homeostasis. This suggestion is supported by Bodin et al. (2001) who showed that CYP3A4 actually catalyses the 4β-hydroxylation of cholesterol and produces metabolites, 4β-hydroxycholesterol which is an LXRA ligand. Besides that, Honda et al. (2011) also suggested that CYP3A4 actually metabolised the 25-hydrocholesterol which is an oxysterol that can activate LXRA gene expression. Up-regulation of CYP3A4 also can be induced by N- (2,2,2-trifluoroethyl)-N-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-benzenesulfonamide (T0901317) which is a synthesised agonist of LXRA (Shenoy et al., 2004). All these suggest that the increase in LXRA would also increase the CYP3A4 gene expression indirectly (Duniec-Dmuchowski et al., 2007)

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials and equipment

Table 3.1: Materials and their suppliers

Materials	Suppliers
100X MEM non-essential amino acid solution	Merck Millipore, USA
1-bromo-3-chloropropane (BCP)	Sigma Aldrich Inc., USA
75 cm^2 Tissue Culture Flask, 25 cm^2 Tissue Culture Flask	Techno Plastic Products (TPP), Switzerland
95% Ethanol	EMPARTA®, Germany
Absolute Ethanol (for cell treatment)	Merck Millipore, USA
Absolute Ethanol (for RNA extraction)	HmbG® Chemical, Germany
Agarose Powder	PhileKorea, South Korea
Beta-Actin Forward and Reverse Primers	Integrated DNA Technologies (IDT), Singapore
Bleach (4.25% of sodium hypochlorite)	Tesco, Malaysia
Boric acid	SYSTERM®, Malaysia
Cell Scraper	NEST Biotechnology Co., LTD, China

Table 3.1: Materials and their suppliers (continued)

Materials	Suppliers
CFX96™ Real-Time PCR Detection System, UV Transilluminator	Bio-Rad, USA
DEPC-Treated Water	Himedia Laboratories, India
DNA loading dye	Thermo Scientific, Malaysia
EtBr 'out' nucleic acid staining solution	Yeastern Biotech, Taiwan
Ethylenediaminetetraacetic acid (EDTA)	Promega, USA
Fetal Bovine Serum (FBS)	Gibco, USA
GeneRuler™ 100 bp DNA Ladder	Thermo Scientific, Malaysia
Haemocytometer	Assistant, Germany
HepG2 cells	American Type Culture Collection (ATCC), USA
Isopropanol	Bendosen, Malaysia
Lauric Acid	Sigma Aldrich Inc., USA
LXRA Forward and Reverse Primers	Integrated DNA Technologies (IDT), Singapore
Minimum Essential Medium (MEM)	Gibco, USA
MS® Micro Centrifuge Tubes	Membrane Solutions, USA
NanoDrop spectrophotometer	Nano Life Quest Sdn. Bhd., Malaysia
Penicillin (5000 units/ mL), Streptomycin (5000 µg/mL)	Merck Millipore, USA
Phosphate Buffered Saline (PBS)	Merck Millipore, USA

Table 3.1: Materials and their suppliers (continued)

Materials	Suppliers
Pipette Tips (10 µL, 200 µL, 1000 µL)	NEST Biotechnology Co., LTD, China
Resveratrol	ChromaDex, USA
Serological Pipette	Techno Plastic Products (TPP), Switzerland
Sodium bicarbonate	Quality Reagent Chemical (QRëC), New Zealand
Sodium pyruvate (100 mM)	Gibco, USA
TransScript® Green One-Step qRT-PCR SuperMix	TransGen Biotech Co., Ltd, China
TRI Reagent® LS	Molecular Research Center (MRC), USA
Tris base powder	1st Base Company, Singapore
Tryphan Blue Stain	Gibco, USA
Trypsin	NacalaiTesque, Japan

3.2 Preparation of stock solution

3.2.1 Minimum Essential Medium (MEM)

MEM was prepared for cell culture purpose using the components listed in Table 3.2. The medium was filter- sterilised.

Table 3.2: Composition of MEM (per litre)

Component	Final Composition
MEM powder	9 g
Sodium bicarbonate	30 mM
Sodium pyruvate(100 mM)	1 mM
100X MEM non-essential amino acid solution	1X
Penicillin (5000 units/ mL), Streptomycin (5000 µg/mL)	1 %
Sterile deionised water	Top up until 1L

3.2.2 TBE buffer

5X TBE buffer was prepared as shown in Table 3.3 for 1% RNA denaturing agarose gel electrophoresis.

Table 3.3: Composition of TBE buffer for RNA denaturing agarose gel electrophoresis.

Solution	Composition
5X TBE buffer	0.45 M Tris-Base, 0.44 M Boric Acid, 0.01 M EDTA, pH 8.0, sterile deionised distilled water was used to top up until 1 L.

3.3 Preparation of Glasswares and Plasticwares

Glasswares such as beakers and Schott bottles were washed and cleaned.They were autoclaved at 121 °C for 145 minutes at 975 kPa pressure. Plasticwares such as pipette tips, microcentrifuge tubes, qPCR tubes were autoclaved prior to use.

3.4 Cell Culture Techniques

3.4.1 Maintenance of cell culture

HepG2 cells (American Tissue Cell Culture, USA) were grown in Eagle's minimum essential medium (MEM) (Gibco, USA) prepared with 10% (v/v) of foetal bovine serum (Sigma-Alrich, USA). HepG2 cells were grown in 75 cm^2 and 25 cm^2 tissue culture flasks with a total volume of 10 mL and 3 mL respectively. Replacing of new cell medium was carried out every two to three days. Before fresh cell culture medium was added, the cells were washed twice using appropriate amount of PBS. The cells were then incubated at 37 °C and 5% (v/v) CO_2 supplied incubator. Observation of cells was done routinely to check the cells growing condition of the cells.

3.4.2 Sub-culturing of HepG2 cells

Sub-culturing of HepG2 cells were carried out once the cells achieved 80% to 90% confluence in 75 cm^2 tissue culture flask. The initial cell medium in the flask was discarded and the cells were washed twice using 6 mL of PBS. Then, 2 mL of 1X trypsin was added into 75 cm^2 tissue culture flask and it was incubated at 37 °C for 10 to 15 minutes to detach the monolayer of cells grown. After incubation, trypsin reaction was inhibited by adding 4 mL of MEM solution. All detached cells were re-suspended with the medium added and further transferred in a sterile 15 mL centrifuge tube. The tube was centrifuged at 800 x *g* for 10 minutes. After centrifugation, the supernatant with trypsin solution was discarded and an appropriate amount of MEM was added. The cell pellet was re-suspended gently to ensure the uniform cell distribution in MEM. The cells in MEM were added evenly into every new tissue flask. Fresh MEM and 10% (v/v) of foetal bovine serum were then added into each respective tissue culture flask until reach the final volume of 75 cm^2 and 25 cm^2 tissue culture flask. The tissue culture flasks were then placed into 5% (v/v) CO_2 incubator at 37 °C.

3.5 Treatment of HepG2 cells

3.5.1 Treatment with absolute ethanol for 24 hours

Treatment of HepG2 cells was carried out when the cells reached 60% to 70% confluence in 25 cm^2 tissue culture flask. The cells were treated with different concentrations of absolute ethanol which were 1% (v/v), 2% (v/v) and 5%

(v/v). Initial medium was discarded and then washed with 2 mL PBS for twice. Subsequently, fresh MEM with 10% (v/v) foetal bovine were added into each flask before treating the cells with different concentration of absolute ethanol. A set of control cells was done by adding fresh medium and 10% (v/v) foetal bovine serum only. All tissue culture flasks were incubated in 5% CO_2 at 37 °C for 24 hours.

3.5.2 Co-treatment with 2% (v/v) absolute ethanol and lauric acid for 24 hours

An optimum concentration of ethanol was obtained from Section 3.4.1 for co-stimulation of HepG2 cells with ethanol and lauric acid. Different concentrations of lauric acid (5 µM, 10 µM and 20 µM) were used to treat the cells with 2 % ethanol. Initial medium was discarded and washing step was performed twice using PBS. Then, fresh MEM with 10% (v/v) foetal bovine serum was added into 25 cm^2 tissue culture flask together with the different concentrations of lauric acid. A total of 20 µM of lauric acid was also added in one of the control cells to identify its sole effect on non-alcoholic induced cells. Another control which included the well- known antioxidant, resveratrol was included in this experiment. For this, one flask of cells were treated solely with 20 µM resveratrol, while another was treated 20 µM resveratrol with 2% (v/v) ethanol. A set of untreated cells with only fresh MEM and 10% (v/v) FBS added was acted as negative control in this experiment. All eight flasks of cells were then incubated in 5% CO_2 incubator at 37 °C for 24 hours.

3.6 RNA – Associated Techniques

3.6.1 Total Cellular RNA isolation using Tri-Reagent® LS

Total cellular RNA in HepG2 cells was isolated after 24 hours of treatment by using Tri-Reagent® LS according to the protocol provided from manufacturer. Based on manufacturer's instruction, 0.75 mL of Tri-Reagent® LS was needed for harvesting every 0.25 mL samples. Before adding Tri-Reagent® LS, cells were washed twice using PBS. Then, cell monolayer on growth surface was scraped using cell scraper after adding Tri-Reagent® LS. Cell suspension was re-suspended thoroughly and transferred into a sterile 1.5 mL microcentrifuge tube. The tubes were allowed to stand at room temperature for 5 minutes before adding 0.1 mL of 1-Bromo-3-Chloropropane (BCP) (Sigma-Alrich, USA). After adding BCP, the mixture was vortexed until it presented in milky pink colour and was incubated at room temperature for 10 minutes. Centrifugation process was then performed at 12,000 x *g* for 15 minutes at 4°C to obtain three layers in the solution. The first layer or aqueous phase which contained the RNA was transferred carefully to a new sterile 1.5 mL microcentrifuge tubes. The interphase and organic phase which containing DNA and protein respectively were left and kept at 4°C. Subsequently, 0.5 mL of isopropanol was added to the aqueous phase and incubated for 10 minutes for RNA precipitation. Centrifugation of the sample was performed again at 16,000 x *g* for 10 minutes at 4°C to obtain RNA pellet. Supernatant of the sample was discarded after centrifugation and 1 mL of 75% ethanol was added into the microcentrifuge tubes. Another centrifugation process was performed at 16,000 x *g* for 10 minutes to wash the RNA pellet obtained. After centrifugation, the supernatant was discarded and the RNA pellet was air-dried

for about 5 minutes. At last, the RNA pellet was re-suspended evenly by using 30 µL DEPC water. The solubilised RNA was then distributed evenly into three tubes to prevent RNA degradation due to frequent thawing. The RNA was then stored at -80 °C freezer.

3.6.2 1% RNA denaturing agarose bleach gel electrophoresis

RNA denaturing agarose bleach gel electrophoresis was carried out to identify the quality of RNA isolated in Section 3.5.1. One percent (w/v) of denaturing agarose gel was prepared using 0.2 g of agarose powder in 20 mL 1X TBE buffer and 400 µL of bleach solution (Tesco brand) was added into agarose to act as denaturing agent. Agarose with bleach solution was then heated in high temperature for complete dissolve of agarose powder in 1X TBE solution. The heated agarose solution was allowed to cool down to around 55 °C and 1 µL of pre-stain dye was added into the agarose solution before it was poured into a gel-casting tray and inserted with a comb. After the gel had solidified, the RNA samples were loaded into the wells with loading dye in the ratio of 1:5 (1 µL of 6X loading dye to 5 µL of RNA samples). Gel electrophoresis was then carried out in the gel tank filled with 1X TBE buffer for 40 minutes at 80 V. The gel was then viewed under UV transilluminator (Biorad Molecular Imager Chemi Doc ^{TM} XRS+, USA) (Aranda et al., 2012).

3.6.3 Spectrophotometric measurement of RNA

Concentration and purity of isolated total cellular RNA were measured by using nanospectrophotometer (Implen, USA). Purity of isolated RNA obtained from the optical densities at wavelength 230 nm, 260 nm and 280 nm. A_{260}/A_{280} and A_{260}/A_{230} were used to identify the quality of the isolated RNA and to check the phenol or ethanol contamination on the samples.

3.7 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

3.7.1 Relative Quantification of LXRA mRNA expression through qRT-PCR

Quantitative RT-PCR was performed by using TransScript® Green One-Step qRT-PCR SuperMix (TransGen Biotech Co., Ltd) to identify the amount of LXRA gene in alcoholic induce HepG2 cells after treatment. The primers sequences used in qRT-PCR for LXRA and ACTB are shown in Table 3.4. Master Mix was prepared on ice atccording to the components shown in Table 3.5 to the final volume of 10 µL. The samples were placed in CFX96TM real-time PCR Detection System (Biorad, USA). The parameters of amplification process were set up as shown as Table 3.6. Relative quantity of LXRA mRNA was normalised against ACTB mRNA which is a housekeeping gene that acts as internal control in this experiment. Amplification for each gene was done in triplicate for each experiment to ensure the accuracy of the results obtained.

Table 3.4: Nucleotide sequence of primers used in qRT-PCR

Primer	Primer sequence (5' to 3')	Expected product size
LXRA forward	CGGGCTTCCACTACAATGTT	213 bp
LXRA reverse	TCAGGCGGATCTGTTCTTCT	
ACTB forward	CGTACCACTGGCATCGTGAT	280 bp
ACTB reverse	CCATCTCTTGCTCGAAGTTC	

Note: Both LXRA primers sequence and ACTB primers sequence were adapted from Ng et al., 2011.

Table 3.5: Master Mix components used in qRT-PCR

Components	Final Concentration	Volume (µL)
2X TransScript® Green qRT-PCR SuperMix	1X	5.0
TransScript® One-Step RT/RI Enzyme Mix	0.2 µM	0.2
10 µM forward primer	0.2 µM	0.2
10 µM reverse primer	0.2 µM	0.2
RNA template (50 ng/µL)	50 ng/µL	2.5
RNase-free water	-	1.9
Total volume		10

Table 3.6: Protocol for the qRT-PCR Quantification of LXRA and ACTB mRNA Amplification

Step	Dwell Time	Set point (°C)	Activity	Cycle	Temperature change
1	10 min	45	Reverse Transcription	1	
2	30 sec	94	Denaturation	1	
3	5 sec	94	Denaturation	41	
	30 sec	60	Annealing		
			Extension		
4	1 min	95		1	
5	1 min	55		1	
6	5 min	65 to 95	Melt curve analysis	1	0.5 °C, increment every 5 sec

3.8 Statistical Analysis

Statistical Package for the Social Sciences (SPSS) was utilised to complete T-test for statistical analysis of data obtained from qRT-PCR*p*-value that less than 0.05 indicated as statistical significance.

CHAPTER 4

RESULTS

4.1 HepG2 cell culture

HepG2 cells were chosen as sample in this experiment for determination of LXRA mRNA expression level. The morphology of HepG2 cells is epithelial and can form small aggregate as shown in Figure 4.1. It is an adherent cell culture which can grow as a monolayer on the surface of tissue culture flask (American Type Culture Collection, 2018). Eagle's minimum essential medium and 10% (v/v) of FBS are needed for HepG2 cells culture as described in Section 3.4.1 (American Type Culture Collection, 2018).

4.2 Isolation of total cellular RNA from HepG2 cells

Total cellular RNA of each treated cell culture was extracted using Tri-Reagent® LS after 24 hours of stimulation. In phase one, different concentrations of ethanol was used for stimulation of HepG2 cells once the cells reached 60% to 70% confluence as mentioned at Section 3.5.1. In phase two, 2% (v/v) ethanol was selected as optimum concentration for HepG2 cell treatment and it was co-treated with different concentrations of lauric acid (5 µM, 10 µM and 20 µM) for 24 hours. The isolation of total cellular RNA was done as described at Section 3.5.1.

Then, the isolated total cellular RNA was electrophoresed on 1% (w/v) denaturing agarose gel electrophoresis to check the integrity of total cellular RNA isolated. One μL of 6XDNA loading dye and five μL of extracted total cellular RNA was loaded into each well for 1% (w/v) denaturing agarose gel electrophoresis. The results of 1% (w/v) denaturing agarose gel electrophoresis are shown in Figure 4.2. Two distinct bands were observed from the gel image, indicating the 28S ribosomal RNA (rRNA) and 18S rRNA bands, respectively. The ratio of 28S rRNA and 18S rRNA was approximate 2:1 are thus indicated the intact and minimal degradation of total cellular RNA isolated.

Besides that, nanospectrophotometric measurement was done to measure concentration and purity of the total cellular RNA extracted. Extracted RNA was measured spectrophotometrically at the wavelength of 260 nm and 280 nm using IMPLEN Nanospectrophotometer. The concentrations and A_{260}/A_{280} values obtained from nanospectrophotometric measurement are tabulated in Tables 4.1 and 4.2. The A_{260}/A_{280} values obtained fell between the range of 1.8 to 2.0 and it showed that there was no degradation and contamination of RNA.

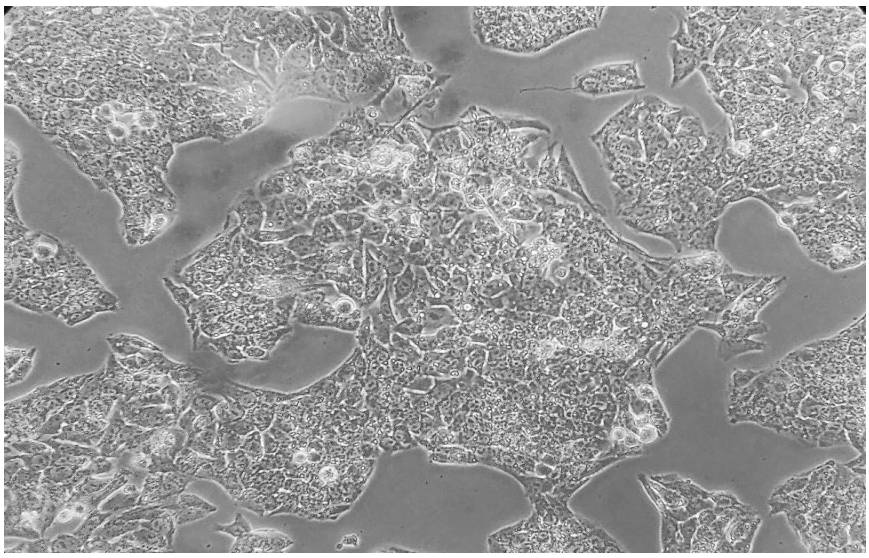


Figure 4.1: Human hepatocellular carcinoma cells, HepG2 cells (Magnification: 400 x)

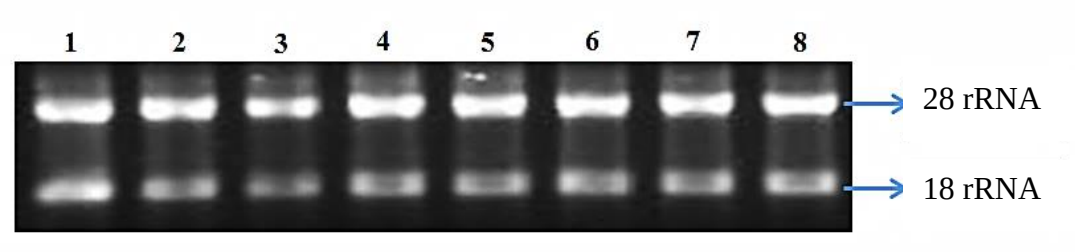


Figure 4.2: Gel image from 1% (w/v) RNA denaturing agarose gel electrophoresis of total cellular RNA isolated from HepG2 cells after treatment with 2 % (v/v) ethanol and different concentration of lauric acid for 24 hours.

Lane 1: RNA from untreated HepG2 cells.

Lane 2: RNA from HepG2 cells treated with 2% (v/v) of ethanol.

Lane 3: RNA from HepG2 cells treated with 2% (v/v) of ethanol and 5 μM of lauric acid.

Lane 4: RNA from HepG2 cells treated with 2% (v/v) of ethanol and 10 μM of lauric acid.

Lane 5: RNA from HepG2 cells treated with 2% (v/v) of ethanol and 20 μM of lauric acid.

Lane 6: RNA from HepG2 cells treated with 20 μM of lauric acid.

Lane 7: RNA from HepG2 cells treated with 2% (v/v) of ethanol and 20 μM of resveratrol.

Lane 8: RNA from HepG2 cells treated with 20 μM of resveratrol.

Table 4.1: Concentration and A_{260}/A_{280} ratios of total cellular RNA isolated from HepG2 cells treated with different concentrations of ethanol.

Ethanol % (v/v) Treated	Ratio of A_{260}/A_{280}	Concentration of RNA (ng/μL)
Untreated	1.929	918.0
1	1.920	860.0
2	1.923	746.0
5	1.875	390.0

Table 4.2: The Concentrations and A_{260}/A_{280} ratios of total cellular RNA isolated from HepG2 cells treated with 2% (v/v) ethanol and different concentrations of lauric acid.

Treatment	Ratio of A_{260}/A_{280}	Concentration of RNA (ng/μL)
Untreated	1.853	1564
2% (v/v) ethanol only	1.876	1328
2% (v/v) ethanol, 5 μM lauric acid	1.865	1738
2% (v/v) ethanol, 10 μM lauric acid	1.782	3454
2% (v/v) ethanol, 20 μM lauric acid	1.889	1402
20 μM lauric acid only	1.861	2498
2% (v/v) ethanol, 20 μM resveratrol	1.754	1442
20 μM resveratrol only	1.826	1976

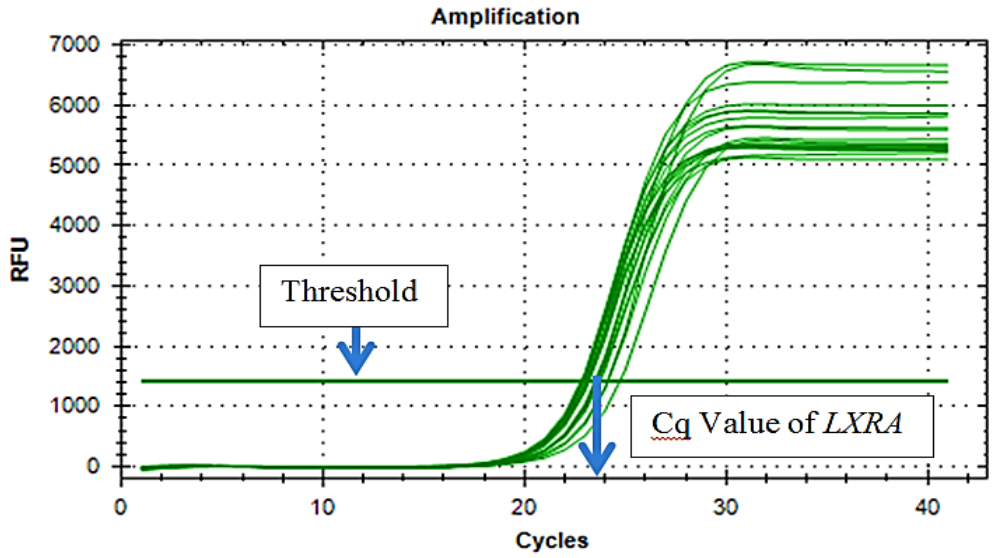
4.3 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

4.3.1 PCR amplification of LXRA and ACTB genes

Quantitative Reverse Transcription PCR was used to study the gene expression of target gene,LXRA after treatment with alcohol in the presence or absence of lauric acid for 24 hours. Reverse transcription of isolated RNA to cDNA was firstly performed in the reaction followed by amplification of cDNA forLXRA gene expression study. TransScript® Green One-Step qRT-PCR SuperMix (Section 3.6) was used for quantification of target gene, LXRA and housekeeping gene, ACTB expression study.

The amplification plots of both genes are shown in Figure 4.3. X-axis indicates the amplification cycle of PCR for each gene, while Y-axis indicates the relative normalised fluorescent unit (RFU). On the other hand, Cq values represent the number of cycle for each gene to reach the threshold level. Cq value of LXRA was around 23 cycles whereas Cq value of ACTB was approximately at 19 cycles. Gene expression of target gene, LXRA was relatively quantified automatically by using in-built software of CFX96TM real-time PCR Detection System (Biorad, USA).

(a)



(b)

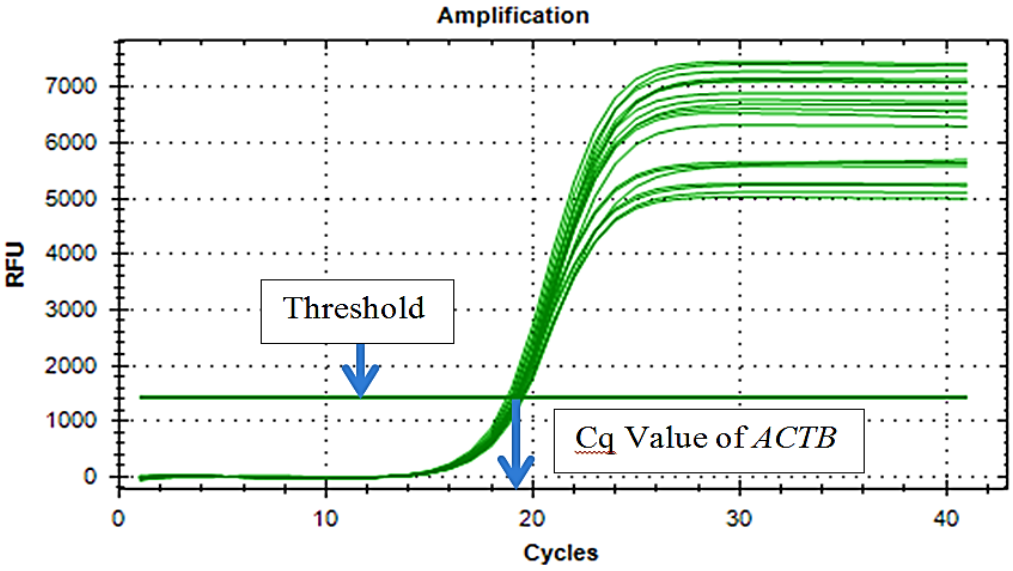


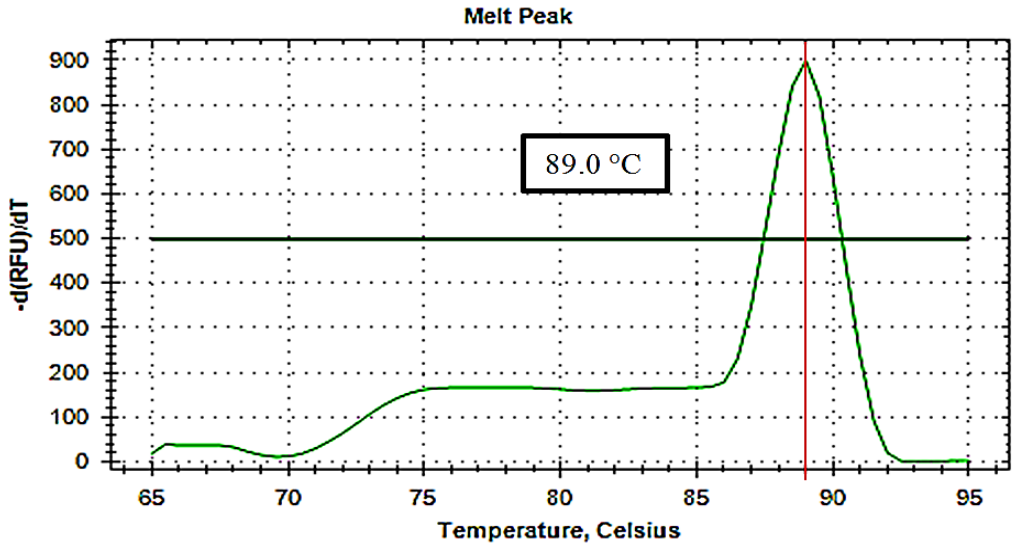
Figure 4.3: Graphical presentation of qRT-PCR amplification plot of (a)LXRA and (b) ACTB. X-axis represents the PCR amplification cycle numbers while Y-axis represents the relative normalised fluorescence unit (RFU). Cq values indicates the cycle number needed for each gene to reach the threshold level.

4.3.2 Melt curve analysis

Melting curve analysis was performed after final extension of target gene to determine the target specificity of primer used. The melting curve analysis was performed from 65 °C to 95 °C as described in Table 3.6. Changes of fluorescent intensity at every time point were detected and recorded by the software. Figure 4.4 showed the melting curve analysis of LXRA and ACTB respectively. The melting curve was plotted as the rate of RFU changes with time, -d(RFU)/dT (Y-axis) against the temperature, Celsius (X-axis).

The peak of melting curve shown in Figure 4.4 represents the melting temperature of target amplicons. Double-stranded amplicons will be melted into single stranded when the temperature reaches a specific temperature during the increment of temperature. Loss of double stranded structure at the specific time point will cause the sudden decrease of fluorescent intensity, and thus produce a sharp peak in the melting curve graph which indicates the melting temperature, T_m of the amplicons (Downey, 2014). Besides, a single and sharp peak in the melting curve represents the high specificity of primers used in the amplifications. Melting temperatures for both LXRA and ACTB are similar at 89.0 ˚C as shown in Figure 4.4.

(a)



(b)

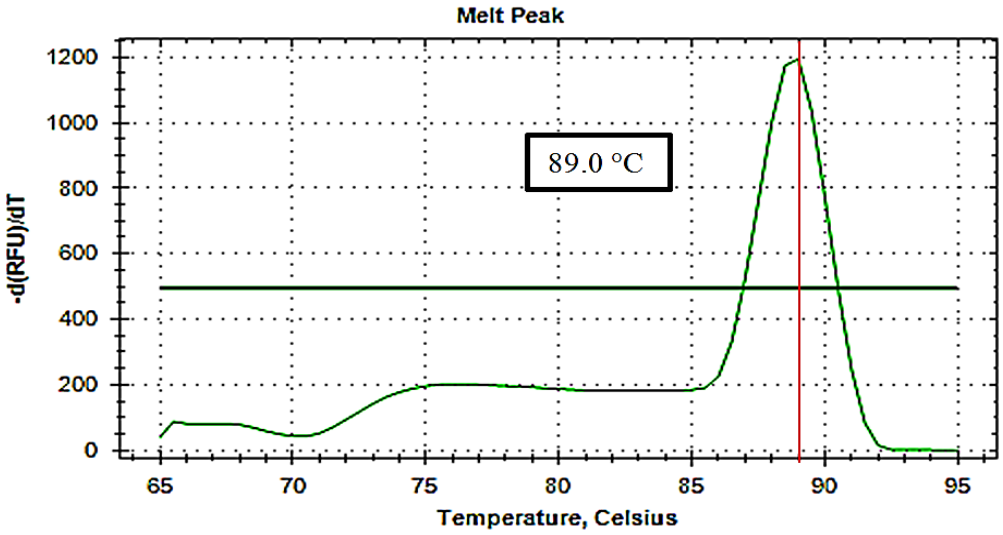


Figure 4.4: Melting curve analysis in qRT-PCR of (a) LXRA and (b) ACTB. X-axis of the melting curve plot indicates the temperature in Celsius while Y-axis of melting plot represents the rate of change of RFU with time, -d(RFU)/dT. Both of the LXRA and ACTB produced a single, sharp peak at the melting temperature, Tm of 89.0 ˚C.

4.4 The dose response effect of ethanol on LXRA mRNA expression level (Phase I)

HepG2 cells were stimulated with 1% (v/v), 2% (v/v) and 5% (v/v) absolute ethanol in phase I experiment (Section 3.5.1). Optimum concentration of absolute ethanol in inducing LXRA mRNA expression was determined in phase I experiment for next co-stimulation of lauric acid and resveratrol in HepG2 cells (Section 3.5.2). The normalised gene expression ofLXRA mRNA in every stimulated sample is as shown in Figure 4.5 and untreated sample and assigned with a gene expression value of 1.000. Based on Figure 4.5, LXRA mRNA expressionwas induced in HepG2 cells after ethanol treatment. The LXRA mRNA expression was increased to 1.228-fold, 2.512-fold and 1.424-fold respective in HepG2 cells stimulation with 1% (v/v), 2% (v/v) and 5% (v/v) of absolute ethanol. The decrease of LXRA mRNA expression in 5% (v/v) absolute ethanol might be due to the cell death that was caused by the high concentration of ethanol. Thus, 2% (v/v) absolute ethanolwas used in phase II experiment (Section 3.5.2).

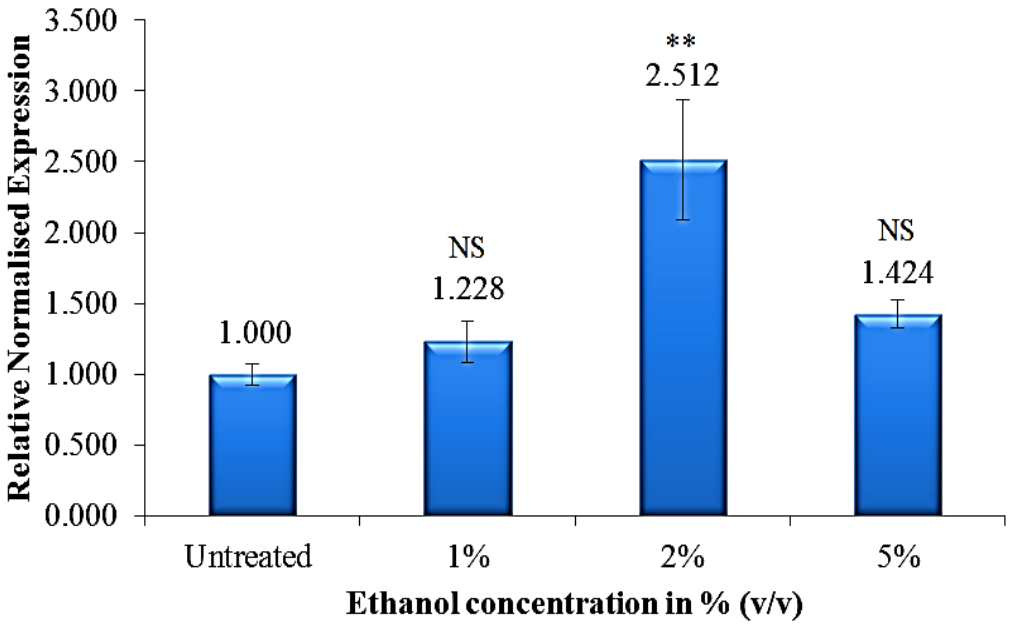


Figure 4.5: Graphical representation showing LXRA expression in HepG2 cells treated with 1% (v/v), 2% (v/v) and 5% (v/v) absolute ethanol.

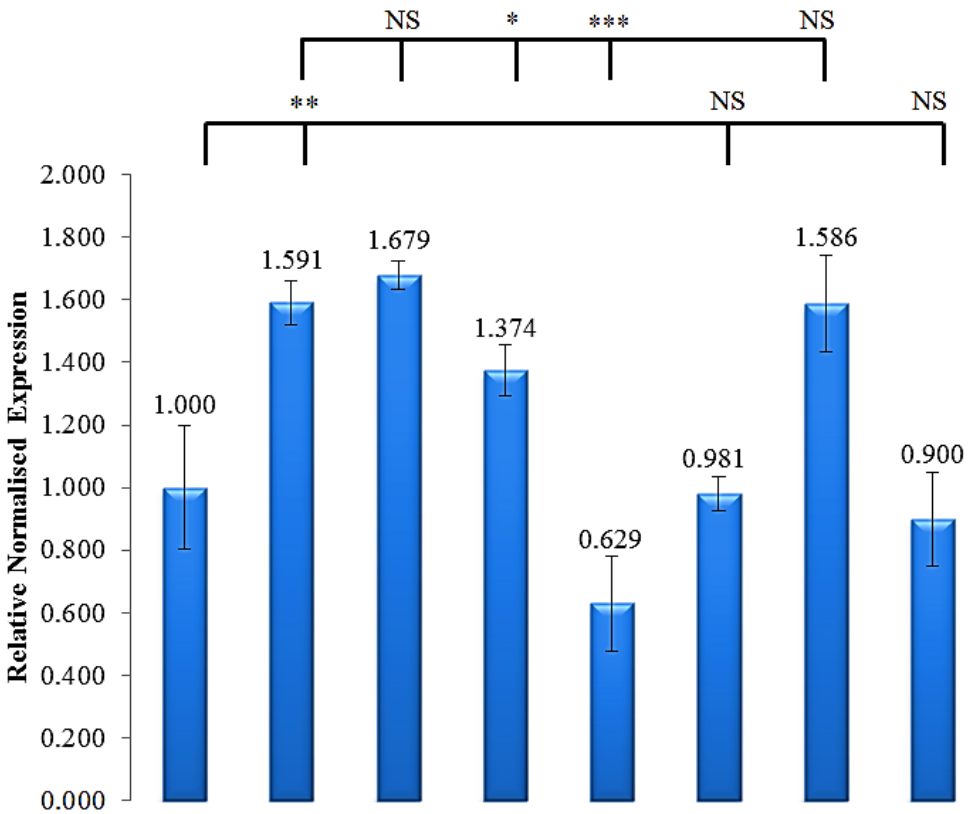
LXRA gene expression was analysed by the software of CFX96TM Real-Time PCR Detection System. The gene expression chart was plotted as relative normalised gene expression against the alcohol concentration in percentage, % (v/v) that was used in HepG2 cells stimulation. Each sample values of LXRA expression was normalised to ACTB housekeeping gene and relative to the negative control, untreated sample (1.00-fold). Error bar in each bars represent the standard error mean. NS indicates no statistical significance from the untreated samples; **p-value <0.01 indicates the statistical significance from the untreated samples

4.5 The dose response effect of 2% ethanol co-treatment with different concentrations of lauric acid on LXRA mRNA expression level (Phase II)

In phase II experiment (Section 3.5.2), HepG2 cells were stimulated by 2% (v/v) absolute ethanol and 5 µM, 10 µM, 20 µM lauric acid for determination of LXRA gene expression. A well-known antioxidant, resveratrol (20 µM) was also co-stimulated with 2% (v/v) absolute ethanol in phase II experiment. Resveratrol functions as a positive control in phase II experiment. In addition, LXRA gene expression was also determined by single treatment of 20 µM lauric acid and 20 µM resveratrol. Similar to Section 4.4, the gene expression ofLXRA mRNA in every stimulated sample was done by normalising to ACTB gene and then relative to untreated sample which was assigned with gene expression value of 1.000.

Figure 4.6 shows LXRA mRNA expressionin HepG2 cells stimulated with 2% (v/v) ethanol, different concentration of lauric acid and 20 µM resveratrol. The LXRA mRNA expression in 2% (v/v) ethanol treated HepG2 cells was increased 1.591-fold compared to untreated HepG2 cells. A gradual decrease in LXRA mRNA expression was observed in cells co-treated with 2% (v/v) absolute ethanol with 5 µM, 10 µM, 20 µM of lauric acid. The LXRA mRNA expression level was 1.679-fold, 1.374-fold and 0.629-fold, respectively in ethanol treated cells with 5 µM, 10 µM, 20 µM of lauric acid. This shows that the ethanol-induced LXRA mRNA expression level was down-regulated in the presence of lauric acid. However,LXRA mRNA expression level in ethanol treated cells with 5 µM was shown no significant different compared to 2% (v/v) absolute ethanol treated sample.

Under the treatment of 2% (v/v) absolute ethanol with 20 µM resveratrol, the LXRA mRNA expression level was increased to 1.586-fold but showed no significant different compared to 2% (v/v) absolute ethanol treated HepG2 cells. Besides, the LXRA mRNA expression level was decreased to 0.981 fold and 0.900-fold in healthy cells with sole 20 µM lauric acid and 20 µM resveratrol, respectively.



2% Ethanol (v/v)	-	+	+	+	+	-	+	-
Lauric acid (µM)	-	-	5	10	20	20	-	-
Resveratrol (µM)	-	-	-	-	-	-	20	20

Figure 4.6: Graphical representation showing LXRA expression in HepG2 cells treated with 2% (v/v) ethanol and 5 µM, 10 µM, 20 µM lauric acid and 20 µM of resveratrol.

LXRA gene expression was analysed by the software of CFX96TM Real-Time PCR Detection System. The gene expression chart was plotted as relative normalised gene expression against various treatments in HepG2 cells stimulation. Each sample values of LXRA expression was normalised to ACTB housekeeping gene and relative to the negative control, untreated sample (1.00-fold). Error bar in each bars represent the standard error mean. NS indicates there is no statistical significance different from the untreated samples; *p-value <0.05, **p-value <0.01, ***p-value <0.001 indicate the statistical significance different from the untreated samples.

CHAPTER 5

DICUSSIONS

5.1 HepG2 cells as the model system

HepG2 cells were utilised in this experiment for determination of LXRA mRNA expression level. HepG2 cell is an immortal cell line that was invented by Knowles and Aden in 1980. This cell is derived from human hepatoblastoma cells of a 15-year old Caucasian male from Argentina (Knowles and Aden, 1980). HepG2 cells are known as the gold standard *in vitro* model for the study of human biotransformation and cytotoxicity of xenobiotic (Wikening et al., 2003; Guillouzo et al., 2006). By using HepG2 cells in pharmaceutical and toxicological studies, this has helped to decrease the use of live animals for xenobiotic cytotoxicity assay. Based on Sassa et al. (1987), they stated that HepG2 cells were highly divided and differentiated continuous cells with many genotypic features of normal human liver cells. This statement is further supported by Wikening et al. (2003), who showed that the gene regulation in HepG2 cells was similar to the primary hepatocytes. Besides, HepG2 cells are easy to handle and high availability for experimental usage (Wikening et al., 2003). LXRA was also shown highly expressed in hepatocytes and this solidifies the usage of HepG2 cells for this experiment (Zhao and Dahlman-Wright, 2010).

5.2 Total cellular RNA isolation

5.2.1 Total cellular RNA isolation using Tri-Reagent® LS

RNA is a long, single stranded polymer that is formed by ribonucleoside monophosphate moieties. Compared to DNA, RNA is more labile and easier to undergo degradation due to its natural single strand structure (Farrell, 1993). Thus, the process of RNA isolation needs to be rapidly and carefully carried out to prevent the degradation of RNA by any contamination of RNase in the environment. RNA isolation is very important in study of gene expression in cell metabolism and function (Farrell, 1993). The quality of isolated RNA is important to ensure the reliability of the molecular-based analysis experiments. For instance, qRT-PCR needs a high quality of RNA to ensure the accuracy of target gene expression in the cells

In this experiment, Tri-Reagent® LS was used for isolation of RNA from the HepG2 cells. It is a fast, cheap and convenient method that has the advantages of inactivating the RNase activity present in the sample and can lyse the cells samples in a short time (Farrell, 1993; Chomczynski and Sacchi, 2006). The process of RNA isolation using Tri-Reagent® LS was developed by Chomczynski and Sacchi in 1987 by modifing tedious RNA isolation methods. Tri-Reagent® LS is a monophosphate solution with phenol and guanidine thiocyanate compound (Molecular Research Centre, 2007). Upon phase separation, The RNA molecules in the cells will remain in the upper aqueous phase under at low pH with the aid of chloroform or bromochloropropane. The interphase and organic phase of the lysate contains DNA and protein,

respectively. RNA molecules that are present in the aqueous phase are then precipitated with the action of isopropanol and the RNA pellet was collected by centrifugation (Molecular Research Centre, 2007).

5.2.2 RNA integrity and purity

The integrity of RNA isolated was validated by using 1% denaturing bleach agarose gel electrophoresis. This is a cheap, reliable and easy alternative of checking RNA integrity with the absence of expensive facilities such as microfluidic electrophoretic devices. Besides that, instead of formaldehyde or formamide compound, bleach solution in the denaturing gel electrophoresis could denature the secondary structure of RNA (Aranda et al., 2012). The denaturation of RNA secondary structure allows the proper analysis of the RNA integrity (Aranda et al., 2012). RNase is the major problem that affects the integrity of isolated RNA since it can easily contaminate the laboratory apparatus and samples, and degrading the RNA within a short time (Farrell, 1993). It can also resist a huge number of chemical components, low pH and extreme temperature. However, the sodium hypochlorite present in the bleach solution can damage and oxidise the protein including RNase (Aranda et al., 2012).

A small amount of bleach solution added into agarose gel is sufficient to fractionated RNA into mRNA, tRNA and rRNA based on their molecular weight (Aranda et al., 2012). However, only two visible bands that indicate 28S rRNA and 18S rRNA were observed after gel staining process. The mRNA and tRNA are not shown due to the low percentages in the total RNA and they are not readily detectable even using the most sensitive techniques (Ream and Field, 1999; Palmer and Prediger, 2018). Therefore, the integrity of mRNA can be identified indirectly by identifying the integrity of rRNA. The mRNA might be also visible between the two rRNA bands as a smear during the gel electrophoresis (Tirabassi, 2017).

Theoretically, the intensity ratio of 28S rRNA to18S rRNA should be approximately 2.7 :1 due to the molecular weight of 28S rRNA and 18S rRNA are 5 kb and 2 kb respectively in mammalian cells (Palmer and Prediger, 2018). However, a 2:1 intensity ratio of 28S rRNA to18S rRNA is considered as the benchmark for an intact RNA. If the rRNA bands have similar intensity, this might indicate the presence of RNA degradation. Contamination of DNA can be seen when there is a higher molecular weight band appears in the agarose gel (Tirabassi, 2017). As shown in Figure 4.2, the ratio of 2:1 was observed in cell samples.

Nanospectrophotometric measurement was done to determine the purity and concentration of extracted RNA by using IMPLEN nanospectrophotometer. This method is reliable and only consumes a small amount of RNA samples for detection. The optical densities of extracted RNA were measured at the wavelength of 230 nm, 260 nm and 280 nm. Optical density in 230 nm is specific for background absorption and possible contaminants while 260 nm and 280 nm are specific for nucleic acid and protein absorption respectively (Fleige and Pfaffl, 2006). The purity of extracted RNA can be determined by using A_{260}/A_{280}. RNA is considered as good quality and pure when its A_{260}/A_{280} value falls between the ranges of 1.8 to 2.0 (Sambrook et al., 1989). A_{260}/A_{230} value ratio is used to ensure the RNA samples are free from contamination. A low A_{260}/A_{230} value ratio may be due to the present of phenolic compound in the sample. Concentration of the RNA can be calculated by using optical density at 260 nm, average extinction coefficient of RNA which is 40 µg/mL and also dilution factor (Farrell, 1993). Based on Table 4.1 and 4.2, all RNA samples have A_{260}/A_{230} ratios which fell between the ranges of 1.8 to 2.0 and this indicated the high purity RNA was isolated.

5.3 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

5.3.1 Principle of qRT-PCR

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) was used to quantify the LXRA expression level after 24 hours stimulation with varies concentration of ethanol or lauric acid in HepG2 cells. Instead of

two step qRT-PCR, one step qRT-PCR was used during the study to prevent the contamination of RNA and to ensure the accuracy of the results obtained (Thermo Fisher Scientific, 2018). RNA is the starting materials of qRT-PCR which will be converted into complementary DNA (cDNA) with the aid of reverse transcriptase before the amplification process. SYBR Green fluorescent dye was used for detection of amplification in this study. The SYBR Green dye will fluoresces once it bind to the minor groove of dsDNA and the emitted fluorescent light will be detected for quantitative purpose (Bustin and Mueller, 2005). Emission of fluorescent will slowly increase during the extension phase. Even though SYBR Green I-based detection of amplicons is cheap and reliable, it might also give non-sequence-specific PCR products such as primer dimer (Van der Velden et al., 2003). This is due to SYBR Green intercalating dye will bind to any dsDNA products present in the reaction tubes. Thus, melting curve analysis should be performed after amplification process to ensure the specifity of the PCR products obtained (Van der Velden et al., 2003).

5.3.2 Melting curve analysis

Melting curve analysis is a common tool that used for determining the specificity of the PCR products that obtained from the qRT-PCR. This is an extra step for qRT-PCR that is using non-probe assay or intercalating dye assay for amplicons detections. The melting curve analysis was performed at the end of the qRT-PCR within a range of temperature, 65 °C to90 °C. When

the temperature increased gradually, the double stranded DNA will slowly denature into single stranded DNA and loses intercalating dye which is bound on it. The release of SYBR Green dye will lead to loss of fluorescent light intensity (Van der Velden et al., 2003). As shown in Figure 4.5, a single sharp peak was observed which indicated that the amplicons have lost their double stranded structure at the same melting temperature, T_m. This proves that the primer used in qRT-PCR is specific enough to produce only one target amplicon (Downey, 2014).

5.3.3 Relative quantification

In order to estimate the gene expression level of LXRA in HepG2 cells under different treatment, relative quantification of target gene was used in this study. Relative quantification is also known as comparative threshold method or $2^{-\Delta\Delta Cq}$ method. Compared to absolute quantification, relative quantification is simple and easier to perform because it does not require a calibration curve. It is based on normalised target gene expression level against the housekeeping gene to estimate the physiological changes in gene expression levels (Pfaffl, 2004). The gene expression level in target gene is first normalised with housekeeping gene, then relative to the levels of treatment control RNA (Arya et al., 2005). The housekeeping gene which was used in this study is ACTB gene which is expressed constantly under different experimental conditions and tissues (Arya et al., 2005).

5.4 The dose response impact on LXRA mRNA expression

5.4.1 Effect of ethanol on LXRA expression

LXRA gene expression in ethanol dose response matter was shown in Figure 4.5. According to Figure 4.5, increasing ethanol concentration increased the gene expression of LXRA. However, there is a drop of LXRA expression level in 5% (v/v) ethanol treated HepG2 cells. This might probably due to the ethanol concentration added into HepG2 cells was too high that the cells underwent cell death. This can be indirectly supported by the low concentration of RNA obtained from 5% (v/v) ethanol treated HepG2 cells that shown in Table 4.1. Thus, 2% (v/v) ethanol was chosen for the second phase of stimulation in HepG2 cells because it provided a significant induction of LXRA expression.

This result suggests that LXRA does play a role in metabolising ethanol pathway. Watanabe et al. (2013) stated that LXRA was a positive regulator of cytochrome P450, CYP3A4 which is an important enzyme that help in metabolising ethanol. By increasing of LXRA expression level, this in turn would increase the expression level of CYP3A4 in the cells. Prior study by senior showed that CYP3A4 expression level raised in 2% (v/v) ethanol treated HepG2 cells (Lim, 2017). The hypothesis in this study is that the rise in CYP3A4 was moderated by LXRA, either in a positive or negative regulator of CYP3A4. From the results obtained in this study, LXRA is most likely the positive regulator of CYP3A4.

Besides that, chronic alcohol consumption will lead to the fat accumulation in liver (Sozio and Crabb, 2008; Seth et al., 2011). It will indirectly increase the LXRA gene expression too since LXRA is also a nuclear receptor which plays major function in cholesterol and lipid metabolism (Yoshikawa et al, 2003). Studies had proven that mechanism of alcohol-induced fat accumulation in liver includes the stimulation of fatty acids synthesis by sterol regulatory element-binding protein-1c (SREBP-1c) through LXRA activation (Repa et al., 2000; Steffensen and Gutafsson, 2004). LXRA activates the expression of SREBP-1c through LXR response element present on its promoter (Repa et al., 2000). Induction of SREBP-1c will cause lipid biosynthesis which leads to the formation of fatty liver. LXRA also regulates the lipogenic gene, fatty acid synthase (FAS) and stearoyl-CoA desaturase-1 (SCD-1), which eventually lead to increase lipogenesis in liver (Hegarty et al., 2005; Hebbachi et al., 2007). Thus, the data supports that increased of LXRA expression level in ethanol-treated HepG2 cells will not only increase CYP3A4 enzyme but will also induced the chronic alcohol-induced lipogenesis in the cells.

5.4.2 Effect of ethanol and lauric acid on LXRA mRNA expression and the correlation of CYP3A4

As shown in Figure 4.6, lauric acid treatment suppressed the gene expression level of LXRA in alcohol-induced HepG2 cells. Significant suppression of lauric acid on LXRA gene expression in alcohol-induced HepG2 cells was observed after 10 µM lauric acid treatment. Five µM of lauric acid did not

give any effect in reducing the LXRA gene expression level. Besides that, treatment with 20 µM lauric acid and resveratrol alone, did not give significant changes to LXRA gene expression as compared to untreated samples. This indicates that the lauric acid and resveratrol alone would not activate LXRA gene expression in normal physiology condition.

As mentioned by Watanabe et al. (2013), LXRA is said to regulate the CYP3A4 by either inhibiting function of Pregnane X Receptor (PXR) or by directly increasing the CYP3A4 gene expression during cholesterol homeostasis. PXR is the main transcription factor in regulating the CYP3A4 gene expression. Tompkins and Wallace (2007) showed that PXR activates CYP3A4 gene expression by binding on PXR response element present in CYP3A4 promoter region. In Figure 4.7, LXRA was increased 1.59-fold in gene expression when the cells were treated with 2% (v/v) ethanol. It showed that LXRA is not a negative regulator of CYP3A4 because if LXRA is a repressor of PXR, it is a repressor of CYP3A4. However, prior senior's study showed increasing of CYP3A4 gene expression level in 2% (v/v) ethanol treated HepG2 cells.

On the other hand, if LXRA is acting as a negative regulator, the gene expression level of LXRA should be increased while the lauric acid concentration increased. This is due to LXRA has a similar binding motif of PXR in the responsive element of CYP3A4 gene and also same binding partner (RXR) with PXR (Watanabe et al., 2013). This leads to the

competition between LXRA and PXR for their binding promoter region (Miao et al, 2006; Watanabe et al., 2013). In short, the increase in LXRA should reversibly inhibit PXR function in activating CYP3A4 gene expression and thus decrease the CYP3A4 formation.

In contrast, the LXRA gene expression level decreased when the concentration of the lauric acid was increased. This showed similar trend to that of CYP3A4 gene expression (Lim, 2017). Thus, the similarity in patterns could confirm that LXRA acts as a positive regulator to induce the activation of CYP3A4. In detail, the lauric acid will decrease the gene expression of LXRA through suppress the CYP3A4 in 4β-hydroxylation of cholesterol thus decrease the formation of 4β-hydroxycholesterol in the liver (Bodin et al, 2001; Honda et al, 2011). The metabolite, 4β-hydroxycholesterol, is the ligand of LXRA and it will help in regulating the cholesterol homeostasis. Cholesterol formation in the alcohol-induced HepG2 is due to the high formation of acetyl-CoA during oxidation of ethanol which is initiated by alcohol dehydrogenase (ADH). Acetyl-CoA is the basic component that used in synthesis the cholesterol compound (King, 2017).

Furthermore, since lauric acid is an antioxidant or a dietary saturated fat, it may play an important role in alteration of fatty acid synthesis which results from chronic alcoholic consumption (Ronis et al., 2004). As suggested by previous study, alcohol steatosis might be induced by the activation of SREBP-1c that lead to synthesis of lipogenic enzyme such as fatty acid

synthases (You et al., 2012). According to Ronis et al. (2004), the increase in lauric acid intake will not only improve the liver resistance towards oxidative stress but it should also decrease the fatty acid synthesis in the liver by increasing the fatty acid oxidation and lipid export.

Compared to 20 µM lauric acid treatment in alcohol-induced HepG2 cells, 20 µM resveratrol had no effect on inducing significant changes of LXRA gene expression level in alcohol-induced HepG2 cells. Resveratrol, a well-known antioxidant in treating the alcoholic fatty liver disease, could protect the hepatocytes from oxidative stress due to chronic alcohol consumption (Ajmo et al., 2008). This can be supported by previous senior study that showed that down regulation of CYP3A4 gene expression in HepG2 cells treated with 20 µM resveratrol and 2% (v/v) ethanol (Lim, 2017). However, in my study showed no significance different in LXRA gene expression level in 2% (v/v) ethanol induced HepG2 cells co-treated with 20 µM resveratrol. Therefore, it is assumed that resveratrol do not act on LXRA in regulating the CYP3A4 gene expression as lauric acid did. This can be supported by Deng et al. (2014), showed that resveratrol down regulate CYP3A4 through inhibiting the function of PXR, the main inducer that activate CYP3A4 gene expression.

5.5 Future studies

In this study, the exact mechanism of changes of LXRA gene expression was not studied clearly in every treatment. The LXRA function is still not identified in regulating the CYP3A4 gene expression especially with the treatment of 20 μM resveratrol and 2% (v/v) ethanol in HepG2 cells. Thus, the mechanism of LXRA in alcohol-induced cells can be further studied in details. Besides that, the relationship of CYP3A4, cholesterol metabolism, alcohol metabolism and LXRA function can be studied to identify the exact pathway of alcohol-induced cholesterol biosynthesis that involved CYP3A4 and LXRA. These studies can be done by knockdown LXRA gene in alcohol-induced HepG2 cells and identify the gene expression of CYP3A4 and also SREBP-1c. By observing physiological and molecular changes in both CYP3A4 and SREBP-1c, the mechanism of changes of LXRA gene expression in alcohol induced HepG2 cells can be identified.

In addition, a time dependent experiment can be done to identify the LXRA gene expression level in alcohol-induced HepG2 cells with the presence or absence of lauric acid. The main purpose of this experiment is to determine the gene expression level of LXRA before the ethanol was fully metabolised in HepG2 cells. When the ethanol was fully eliminated in HepG2 cells, there may have some changes in the gene expression of LXRA. Thus, a time dependent experiment on alcohol-induced HepG2 cells can be done to investigate the changes of LXRA gene expression level.

CHAPTER 6

CONCLUSION

A study regarding the effect of lauric acid on Liver X Receptor α (LXRA) mRNA expression in alcohol-induced HepG2 cells was done. This study showed that alcohol induced LXRA mRNA expression in HepG2 cells at the concentration of 1%, 2% and 5% (v/v). The highest induction of LXRA mRNA expression was shown in 2% (v/v) ethanol-induced HepG2. This result tallied with previous senior's study which showed CYP3A4 mRNA expression was increased in alcohol-induced HepG2 cells. In the presence of lauric acid, LXRA mRNA expression in alcohol-induced HepG2 cells was reduced. The most significant reduction was shown at 20 μM lauric acidco-treatment. The reduction in LXRA correlated with reduction in CYP3A4 in previous study. This hypothesises that LXRA mRNA expression level might affect the CYP3A4 mRNA expression in alcohol-induced HepG2 cells and LXRA is the direct regulator of CYP3A4 in alcohol-induced HepG2 cells. In a nut shell, lauric acid can act as potential antioxidant in reducing liver injury that is caused by ethanol consumption.

REFERENCES

Ajmo, J.M., Liang, X., Rogers, C.Q., Pennock, B., and You, M., 2008. Resveratrol alleviates alcoholic fatty liver in mice. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 295(4), G833–G842.

American Type Culture Collection, 2018.*Hep G2 [HEPG2] (ATCC® HB-8065™).*[online] Available at: <https://www.atcc.org/products/all/HB-8065.aspx#generalinformation> [Accessed 23 June 2018].

Aranda, P.S., LaJoie, D.M. and Jorcyk, C.L., 2012. Bleach Gel: A Simple Agarose Gel for Analyzing RNA Quality. *Electrophoresis.* 33(2), pp. 366–369.

Arya, M. et al., 2005.Basic principles of real-time quantitative PCR.*Expert Review of Molecular Diagnostics*, 5(2), pp. 209-212.

Baraona, E. and Lieber, C.S., 1979. Effects of ethanol on lipid metabolism. *The Journal of Lipid Research*, 20(3), pp.289–315.

Bodin, K. et al., 2001. Antiepileptic drugs increase plasma levels of 4beta-hydroxycholesterol in humans: evidence for involvement of cytochrome p450 3A4. *Journal of Biological Chemistry*, 276 (42), pp. 38685–89.

Bustin, S.A. and Mueller, R.,2005.Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis.*Clinical Science*, 109, pp. 365–379.

Ceni, E, Mello, T. and Galli, A., 2014. Pathogenesis of alcoholic liver disease: role of oxidative metabolism.*World Journal of Gastroenterology*, 20(47), pp.17756-17772.

Cha, J.Y. and Repa, J.J., 2007. The liver X receptor (LXR) and hepatic lipogenesis The carbohydrate-response element-binding protein is a target gene of LXR. *Journal of Biological Chemistry*, 282(1), pp.743-751.

Cheah, H.Y., Wong, Y.Y., Wong, H.K., Lim, W.S. and Chew, C.H., 2014. Nicotinic acid, lauric acid and kaempferol abolish ATP-binding cassette transporter subfamily A member 1 (ABCA1) down-regulation by TNF-α in hepatocarcinoma HepG2 cell line. *Biomedical Research*, 25(3),pp 419-425.

Chen, M., Bradley, M. N., Beavena, S. W. and Tontonoza, P., 2006.Phosphorylation of the liver X receptors.*Federation of European Biochemical Societies Letters*, 580(20), pp. 4835-4834.

Chomczynski, P. and Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.*Analytical Biochemistry*, 162(1), pp. 156-159.

Chomczynski, P. and Sacchi, N., 2006. The single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction: twenty-something years on. *Nature Protocols*, 1(2), pp. 581–585.

Dayrit, F.M., 2014. Lauric Acid is a Medium-Chain Fatty Acid, Coconut Oil is a Medium-Chain Triglyceride. *Philippine Journal of Science*, 143(2), pp. 157-166.

Dayrit, F.M., 2015. The Properties of Lauric Acid and Their Significance in Coconut Oil.*Journal of the American Oil Chemists' Society*, 92(1), pp. 1-15.

De, R.N., Schouten, E. and Katan, M., 2001. Consumption of a solid fat rich in lauric acid results in a more favorable serum lipid profile in healthy men and women than consumption of a solid fat rich in trans-fatty acids. *Journal of Nutrition*, 131(2), pp. 242-245.

Deng, R. et al., 2014. Resveratrol suppresses the inducible expression of CYP3A4 through the pregnane X receptor. *Journal of pharmacological sciences*, 126(2), pp.146-154.

Donohue, T.M. et al., 2012. Early growth response-1 contributes to steatosis development after acute ethanol administration. *Alcoholism: Clinical and Experimental Research*, 36(5), pp. 759-767.

Downey, N., 2014. *Interpreting melt curves: An indicator, not a diagnosis*. [online] Available at: < http://www.science.smith.edu/cmbs/wp-content/uploads/sites/36/2015/09/Interpreting-melt-curves.pdf> [Accessed 23 June 2018].

Duniec-Dmuchowski, Z., Ellis, E., Strom, S.C. and Kocarek, T.A., 2007.Regulation of CYP3A4 and CYP2B6 expression by liver X receptor agonists. *Biochemical pharmacology*, 74(10), pp.1535-1540.

Fan, X., Kim, H.J., Bouton, D., Warner, M. and Gustafsson, J.A., 2008 Expression of liver X receptor beta is essential for formation of superficial cortical layers and migration of later-born neurons. *Proceedings of the National Academy of Sciences of the United States of America*, 105 (36), pp. 13445–13450.

Farrell, R.E., 1993. *RNA Methodologies: A Laboratory Guide for Isolation and Characterization*. [e-book] California: Acadamic Press. Available at: Google Books <books.google.com> [Accessed 26 June 2018].

Faucette, S.R. et al., 2007. Relative activation of human pregnane X receptor versus constitutive androstane receptor defines distinct classes of CYP2B6 and CYP3A4 inducers. *Journal of Pharmacology and Experimental Therapeutics*, 320(1), pp.72-80.

Fife, B., 2013. Health properties of coconut oil.*Agro Food Industry Hi Tech*, 24 (3), pp. 5-7.

Fleige, S. and Pfaffl, M.W., 2006.RNA integrity and the effect on the real-time qRT-PCR performance.*Molecular Aspects of Medicine*, 27(2-3), pp. 126-139.

Friedman, S.L., 2008. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver.*Physiological Reviews*, 88(1), pp.125-72.

Germain, P., Altucci, L., Bourguet, W., Rochette-Egly, C. and Gronemeyer, H., 2003. Nuclear receptor superfamily: principles of signaling. *Pure and applied chemistry*, 75(11-12), pp.1619-1664.

Ghisletti, S. et al., 2007. Parallel SUMOylation-dependent pathways mediate gene-and signal-specific transrepression by LXRs and PPARγ. *Molecular cell*, 25(1), pp.57-70.

Grant, B.F., Dufour, M.C. and Harford, T.C., 1988. Epidemiology of alcoholic liver disease.*Seminars in Liver Disease*, 8(1), pp.12–25.

Guillouzo, A. et al., 2007. The human hepatomaHepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. *Chemico-biological Interactions*, 168(1), pp. 66-73.

Hall, J.M., Couse, J.F. andKorach, K.S., 2001 The multifaceted mechanisms of estradiol and estrogen receptor signaling. *Journal of Biological Chemistry*, 276(40), pp. 36869-36872.

Hebbachi, A.M., Knight, B.L., Wiggins, D., Patel, D.D. and Gibbons, G.F., 2007. Peroxisome Proliferator-activated Receptor α Deficiency Abolishes the Response of Lipogenic Gene Expression to Re-feeding Restoration of The Normal Response by Activation of Liver X Receptor α. *The Journal of Biological Chemistry*, 283(8), pp. 4833-4876.

Hegarty, B.D. et al., 2005. Distinct roles of insulin and liver X receptor in the induction and cleavage of sterol regulatory elementbinding protein-1c.*Proceedings of the National Academy of Sciences*, 102 (3), pp. 791-796.

Henry, G.E., Momin, R.A., Nair, M.G. and Dewitt, D.L., 2002. Antioxidant and Cyclooxygenase Activities of Fatty Acids Found in Food.*Journal of Agricultural and Food Chemistry*, 50(8), pp. 2231-2234.

Honda, A. et al., 2011. Cholesterol 25-hydroxylation activity of CYP3A.*Journal of lipid research*, 52(8), pp.1509–1516.

Janowski, B.A., Willy, P.J., Devi, T.R., Falck, J.R. and Mangelsdorf, D.J., 1996. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature*, 383 (6602), pp. 728–731.

Joseph, S.B., Castrillo, A., Laffitte, B.A., Mangelsdorf, D.J. and Tontonoz, P., 2003. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nature Medicine*,9 (2), pp. 213–219.

Jump, D.B., Tripathy, S. and Depner, C.M., 2013. Fatty acid–regulated transcription factors in the liver. *Annual review of nutrition*, 33, pp.249-269.

Kalaany, N.Y. and Mangelsdorf, D.J., 2006. LXRS and FXR: the yin and yang of cholesterol and fat metabolism. *Annual Review of Physiology*, 68, pp.159-191.

Katzung, B.G., Masters, S.B., and Trevor, A.J., 2012. *Basic and Clinical Pharmacology*. 12th ed. New York: McGraw-Hill.

King, M.K., 2017. *Introduction to Cholesterol Metabolism*. . [online] Available at: <https://themedicalbiochemistrypage.org/cholesterol.php#top/> [Accessed 30 June 2018].

Knowles, B.B., Howe, C.C. and Aden, D.P., 1980. Human Hepatocellular Carcinoma Cell Lines Secrete the Major Plasma Proteins and Hepatitis B Surface Antigen. *Science*, 209(4455), pp. 497-499.

Laudet, V. and Gronemeyer, H., 2002.*TheNuclear receptors facts book.*London: Academic Press.

Lefkowitch, J.H., 2005. Morphology of alcoholic liver disease.*Clinical Liver Disease*, 9(1), pp.37-53.

Lieberman, S., Enig, M.G. and Preuss, H.G., 2006.A Review of Monolaurin and Lauric Acid.*Alternative and Complementary Therapies*, 12(6), pp. 310-314.

Lim, H.L., 2017. *A study on the effect of lauric acid on CYP3A4 mRNA expression and cell viability in alcohol-induced HepG2 cells.*Bachelor.UniversitiTunku Abdul Rahman.

Lim, W.S., Gan, M.S.Y., Ong, M.H.L and Chew, C.H., 2015. Lauric acid abolishes interferon-gamma (IFN-γ)-induction of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression in human macrophages. *Asian Pacific Journal of Reproduction*, 4(3), pp. 217-221.

Lu, T.T., Repa, J.J. and Mangelsdorf, D.J., 2001. Orphan Nuclear Receptors as eLiXiRs and FiXeRs of Sterol Metabolism.*The Journal of Biological Chemistry*, 276(41), pp. 37735–37738.

Makishima, S., 2006. Nuclear receptors sense bile acid metabolism: a hormonal action of bile acids. *In Functional and Structural Biology on the Lipo-Network*, pp. 17–35.

Mangelsdorf, D.J. et al., 1995. The nuclear receptor superfamily: the second decade. *Cell*, 83(6), pp.835-839.

Mauch, T.J., Donohue, T.M. J.r, Zetterman, R.K., Sorrell, M.F. and Tuma, D.J., 1986. Covalent binding of acetaldehyde selectively inhibits the catalytic activity of lysine-dependent enzymes. *Hepatology*, 6(2), pp.263-269.

McKillop, I.H. and Schrum, L.W., 2009. Role of alcohol in liver carcinogenesis.*Seminar Liver Disease*, 29(2), pp. 222-232.

Mensink, R.P., Zock, P.L., Kester, A.D.M. and Katan, M.B., 2003. Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. *The American Journal of Clinical Nutrition*, 77(5), pp. 1146-1155.

Miao, J., Fang, S., Bae, Y. and Kemper, J.K., 2006. Functional inhibitory cross-talk between constitutive androstane receptor and hepatic nuclear factor-4 in hepatic lipid/ glucose metabolism is mediated by competition for binding to the DR1 motif and to the common coactivators, GRIP-1 and PGC-1alpha. *Journal of Biological Chemistry*, 281(21), pp.14537–14546.

Michael, W.K., 2017. *Ethanol Metabolism*. [online] Available at :<https://themedicalbiochemistrypage.org/ethanol-metabolism.php> [Accessed 28 July 2018].

Molecular Research Centre, 2007.*Tri Reagent® LS - RNA / DNA / Protein Isolation Reagent for Liquid Samples*. [online] Available at: <https://search.cosmobio.co.jp/cosmo_search_p/search_gate2/docs/MOR_/TS120.20100414.pdf> [Accessed 26 June 2018].

Murray, M.T., Pizzorno, L. and Pizzorno, J., 2005.*Encyclopedia of Healing Foods*. New York: Atria Book.

Nakatsuji, T. et al., 2009. Antimicrobial Property of Lauric Acid against Propionibacterium acnes: Its Therapeutic Potential for Inflammatory Acne Vulgaris. *Journal of Investigative Dermatology*, 129(10), pp. 2480-2488.

Nevin, K.G. and Ramajohan, T., 2004.Beneficial effects of virgin coconut oil on lipid parameters and in vitro LDL oxidation.*Clinical Biochemistry*, 37(9), pp. 830-835.

Ng, D.L. et al., 2011.Rapamycin pre-treatment abrogates Tumour Necrosis Factor-α down-regulatory effects on LXR-α and PXR mRNA expression via inhibition of c-Jun N-terminal kinase 1 activation in HepG2 cells. *Electronic Journal of Biotechnology*. [online] Available at :<http://www.ejbiotechnology.info/index.php/ejbiotechnology/article/view/v14n3-9/1311> [Accessed 2 February 2018].

Olefsky, J.M., 2001. Nuclear Receptor Minireview Series.*The Journal of Biological Chemistry Papers.* [online] Available at:<http://www.jbc.org/content/early/2001/07/17/jbc.R100047200.full.pdf> [Accessed 16 July 2018].

Ong, W.W., 2017. *A study on the effects of lauric acid on ROS formation and CYP2E1 mRNA expression in alcohol-induced HepG2 cells.*Bachelor.UniversitiTunku Abdul Rahman.

Oosterveer, M.H., Grefhorst, A., Groen, A.K. and Kuipers, F., 2010. The liver X receptor: Control of cellular lipid homeostasis and beyond: Implications for drug design. *Progress in lipid research*, 49(4), pp.343-352.

Orman, E. S., Odena, G. and Bataller, R., 2013. Alcoholic liver disease: Pathogenesis, management, and novel targets for therapy. *Journal of Gastroenterology and Hepatology*, 28(1), pp. 77–84.

Osna, N. A., Donohue, T. M. and Kharbanda, K. K., 2017. Alcoholic Liver Disease: Pathogenesis and Current Management. *Alcohol Research : Current Reviews*, 38(2), pp. 147–161.

Palmer, M. and Prediger, E., 2018.*Assessing RNA Quality*. [online] Available at: <https://www.thermofisher.com/us/en/home/references/ambion-tech-support/rna-isolation/tech-notes/assessing-rna-quality.html > [Accessed 26 June 2018].

Pfaffl, M.W., 2004. Quantification strategies in real-time PCR.*A-Z of quantitative PCR,*3, pp. 87-112.

Ream, W. and Field, K., 1999. *Molecular biology techniques*. 2nd ed. San Diego: Academic Press.

Rehm, J., Samokhvalov, A.V. and Sheild, K.D., 2013. Global burden of alcoholic liver diseases.*Journal of Hepatology*, 59(1), pp. 160-168.

Repa, J.J. et al., 2000. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXR alpha and LXR beta.*Genes & development,* 14(22), pp.2819-2830.

Ronis, M.J.J., Korourian, S., Zipperman, M., Hakkak, R. and Badger, T.M., 2004. Dietary Saturated Fat Reduces Alcoholic Hepatotoxicity in Rats by Altering Fatty Acid Metabolism and Membrane Composition.*The Journal of Nutrition*, 134(4), pp. 904–912.

Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989.*Molecular Cloning: A Laboratory Manual*. 2nd edition. NY: Cold Spring Harbor Laboratory Press.

Sassa, S., Sugita, O., Galbraith, R.A. and Kappas, A., 1987.Drug metabolism by the human hepatoma cell, HepG2. *Biochemical and Biophysical Research Communiactions*, 143(1), pp. 52–57.

Seth, D., Haber, P.S., Syn, W., Diehl, A.M. and Day, C.P., 2011. Pathogenesis of alcohol-induced liver disease: Classical concepts and recent advances. *Journal of Gastroenterology and Hepatology*, 26(7), pp.1089-1105.

Shenoy, S.D. et al., 2004. CYP3A induction by liver x receptor ligands in primary cultured rat and mouse hepatocytes is mediated by the pregnane X receptor. *Drug metabolism and disposition*, 32(1), pp. 66-71.

Shrestha, S., 2011.*Morphology of Alcoholic Liver Disease*. [blog] 26 July 2011. Available at: <http://medchrome.com/basic-science/pathology/morphology-alcoholic-liver-disease/>[Accessed 27 January 2018].

Sozio, M. and Crabb, D.W., 2008.Alcohol and lipid metabolism.*American Journal of Physiology-Endocrinology and Metabolism*, 295 (1), pp. 10 –16.

Steffensen, K.R. and Gustafsson, J., 2004. Section II: Nuclear Receptors and Islet Function. Putative Metabolic Effects of the Liver X Receptor (LXR).*Diabetes*, 53(1), pp. 36-42.

Tall, A.R., Costet, P. and Luo, Y., 2000. Orphans' meet cholesterol. *Nature Medicine*, 6(10), pp. 1104–1105.

Teli, M.R., Day, C.P., Burt, A.D., Bennett, M.K. and James, O.F., 1995.Determinants of progression to cirrhosis or fibrosis in pure alcoholic fatty liver. *Lancet*, 346(8981), pp.987–990.

Thermo Fisher Scientific, 2018.*Basic Principles of RT-qPCR*. [online] Available at: <https://www.thermofisher.com/my/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/basic-principles-rt-qpcr.html/> [Accessed 5 July 2018].

Tirabassi, R., 2017. *How to quality control check your RNA samples*. [online] Available at: <https://bitesizebio.com/13527/how-to-quality-control-check-your-rna-samples/> [Accessed 26 June 2018].

Tompkins, L.M. and Wallace, A.D., 2007. Mechanisms of cytochrome P450 induction. *Journal of biochemical and molecular toxicology*, 21(4), pp.176-181.

Uday, K.D., Christopher, V., Sobarani, D. and Nagendra, S.Y., 2014. Lauric Acid as Potential Natural Product in the Treatment of Cardiovascular Disease: A Review. *Journal of Bioanalysis& Biomedicine*, 6(5), pp. 37-39.

Van der Velden, V.H.J., Hochhaus, A., Cazzaniga, G., Szczepanski1, T.,Gabert, J. and van Dongen, J.J.M., 2003. Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. *Leukemia*, 17(6), pp. 1013-1034.

Wada, T., Kang, H.S., Jetten, A.M. and Xie, W., 2008.The emerging role of nuclear receptor RORα and its crosstalk with LXR in xeno-and endobiotic gene regulation. *Experimental Biology and Medicine*, 233(10), pp.1191-1201.

Walsh, K. and Alexander, G., 2000. Alcoholic liver disease.*Postgraduate Medical Journal*,76(895), pp. 280-286.

Watanabe, K., Sakurai, K., Tsuchiya, Y., Yamazoe, Y. and Yoshinari, K.,2013. Dual roles of nuclear receptor liver X receptor a (LXRa) in the CYP3A4 expression in human hepatocytes as a positive and negative regulator. *Biochemical Pharmacology*, 86, pp. 428-436.

Wei, X. et al., 2013. Chronic alcohol exposure disturbs lipid homeostasis at the adipose tissue-liver axis in mice: analysis of triacylglycerols using high-resolution mass spectrometry in combination with in vivo metabolite deuterium labeling. *PLoS One*, 8(2), pp.e55382.

Wikening, S., Stahl, F. and Bader, A., 2003.Comparison of Primary Human Hepatocytes and Hepatoma Cell Line Hepg2 with Regard to Their Biotransformation Properties.*Drug Metabolism and Disposition*, 31(8), pp. 1035-1042.

Wójcicka, G., Jamroz-Wiśniewska, A., Horoszewicz, K. and Bełtowski, J., 2007.Liver X receptors (LXRs). Part I: Structure, function, regulation of activity, and role in lipid metabolism Receptorywątrobowe X (LXR). Część I: Budowa, funkcja, regulacjaaktywności i znaczenie w metabolizmielipidów. *PostępyHigienyMedycznyDoświadczalnej*, 61, pp.736-759.

World Health Organization, 2014.*Global status report on alcohol and health.*[online] Available at: < http://www.who.int/substance_abuse/publications/global_alcohol_report/msb_gsr_2014_1.pdf > [Accessed 27 January 2018].

Yoshikawa, T. et al., 2003.Cross-Talk between Peroxisome Proliferator-Activated Receptor (PPAR) α and Liver X Receptor (LXR) in Nutritional Regulation of Fatty Acid Metabolism. I. PPARs Suppress Sterol Regulatory Element Binding Protein-1c Promoter through Inhibition of LXR Signalling. *Molecular Endocrinology*, 17(7), pp. 1240–1254.

You, M., Fischer, M., Deeg, M. A. and Crabb, D. W., 2002. Ethanol induces fatty acid synthesis pathways by activation of sterol regulatory element binding protein (SREBP). *Journal of Biological Chem*istry, 277, pp. 29342–29347.

Zelcer, N. and Tontonoz, P., 2006. Liver X receptors as integrators of metabolic and inflammatory signalling.*Journal of Clinical Investigation*, 116(3), pp.607-614.

Zhao, C. and Dahlman-Wright, K., 2010. Liver X Receptor in cholesterol metabolism. *Journal of Endocrinology*, 204(3), pp. 233-240.