IN VITRO ANTI-OXIDATION, ANTI-HEPATIC STEATOTIC AND ANTI-INFLAMMATORY EFFECTS OF *Auricularia nigricans* (SYN A. polytricha)

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IN VITRO ANTI-OXIDATION, ANTI-HEPATIC STEATOTIC AND ANTI-INFLAMMATORY EFFECTS OF *Auricularia nigricans* (SYN *A. polytricha*)

ABSTRACT

At present, non-alcoholic fatty liver disease (NAFLD) is one of the most frequent liver diseases worldwide. In general, NAFLD is asymptomatic, and can slowly progress to serious complications such as cirrhosis, hepatocellular carcinoma, cardiovascular disease and death. Although there are no approved treatment regimens for NAFLD at this moment, lifestyle modifications through diet is the mainstay of treatment. *Auricularia nigricans* is a popular edible mushroom known to possess medicinal properties such as antioxidant, antitumor and anti-inflammatory. The present study was conducted to determine the beneficial effects of *A. nigricans* against NAFLD through investigation of the *in vitro* free radical scavenging activities, anti-inflammatory capacity, hypolipidemic effect and mechanism triggered by bioactive fractionated samples from *A. nigricans* towards palmitic acid-induced HepG2 cells. Evaluation of total phenolic content in ethanolic crude, hexane, ethyl acetate and water fractions from *A. nigricans* indicated that ethyl acetate fraction was found significantly higher than other fractions ($p<0.05$). Furthermore, ethyl acetate fraction showed the strongest scavenging peroxyl, hydroxyl, DPPH radicals effects. In addition, ethyl acetate fraction also demonstrated significant high capacity in ferric reducing antioxidant power (FRAP). Among all, water fraction was the only fraction capable of scavenging all radicals tested. Therefore, ethyl acetate and water fraction were subjected to gas chromatography mass spectrometry and liquid chromatography mass spectrometry analysis for assessing the bioactive compounds profile. Linoleic acid ethyl ester, Butyl 9,12-octadecadienoate, 9,12-Octadecadienoic acid, Ergosta-5,7,22-trien-3-ol, 2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzene propanoic acid and 3,30-di-O-methyl ellagic acid were identified in the ethyl acetate

fraction, while oxooctadecanoic acid and 2(3,4-dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid were identified in the water fraction. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay showed that water fraction was non-cytotoxic to HepG2 cells, whereas ethyl acetate fraction was cytotoxic to HepG2 cells at 100 µg/mL. Assay on anti-hepatic steatotic, ethyl acetate fraction showed significant inhibitory effects on lipid accumulation ($p<0.05$), but not water fraction. Furthermore, ethyl acetate fraction exhibited significant capacity ($p<0.05$) in attenuating intracellular reactive oxygen species (ROS) level, but not water fraction. Hence, the anti-inflammatory effect of ethyl acetate fraction was investigated. Co-incubation of 25 and 50 µg/mL ethyl acetate fraction with palmitic acid, markedly decreases tumour necrosis factor alpha (TNF-α), intereukin-6 (IL-6), interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) levels. Both concentrations also showed no significant different in the reduction of pro-inflammatory cytokine levels. Further investigation of antioxidant and anti-inflammatory effects at signalling pathway using palmitic acid-induced HepG2 cells co-incubated with 25 µg/mL ethyl acetate fraction showed suppression of stress-activated protein kinases/jun amino-terminal regulated kinases (SAPK/JNK), p-38 mitogen-activated protein kinases (p-38 MAPK), nuclear factor-kappa B (NF-κB) and signal transducer and activator of transcription *3 (*STAT-3) signalling pathways. In conclusion, the present findings suggest that *A. nigricans* demonstrated anti-steatotic effects that involved antioxidant capacity, hypolipidemic effect and anti-inflammatory capacity in the palmitic acid-induced NAFLD pathological cell model. Combined together, ethyl acetate fraction shows potential to be used in the development of anti-hepatic steatotic agent and *A. nigricans* may be considered to be promoted to be used in diet intervention for NAFLD and NASH patients.

Keywords:
Mushroom, non-alcoholic fatty liver disease (NAFLD), palmitic acid-induced HepG2 cells

KESAN ANTI-OKSIDAKSI, ANTI-HEPATIK STEATOTIK DAN ANTI-INFLAMASI *Auricularia nigricans* (SYN *A. polytricha*) *IN-VITRO*

ABSTRAK

Penyakit hati berlemak bukan berpunca dari alkohol (NAFLD) merupakan salah satu penyakit hati yang kini paling kerap dihidapi oleh manusia. Umumnya, NAFLD adalah asimptomatik, dan berkembang menjadi serius, contohnya sirosis, hepatosellular karsinoma, penyakit kardiovaskular dan kematian. Walaupun pada masa kini tiada rejimen rawatan untuk NAFLD yang diluluskan, modifikasi cara kehidupan melalui pemakanan pilihan merupakan bantuan rawatan utama. *Auricularia nigricans* adalah cendawan boleh dimakan dan diminati ramai. Ia terkenal dengan ciri-ciri perubatan seperti antioksida, antitumor dan anti-radang. Dalam kajian ini manfaat *A. nigricans* terhadap NAFLD dikaji dengan penyiasatan secara *in vitro* seperti penghapusan aktiviti radikal bebas, kapasiti anti-radang, kesan hipolipidemik dan mekanisme yang dicetuskan oleh fraksi pelarut bioaktif dari *A. nigricans* terhadap sel-sel HepG2 terinduksi-asid palmitik. Dalam penilaian jumlah kandungan fenolik dalam ekstrak mentah etanol, fraksi heksana, fraksi etil asetat dan fraksi air dari *A. nigricans*, fraksi etil asetat menunjukkan secara ketara lebih tinggi daripada sampel lain ($p<0.05$). Tambahan pula, fraksi etil asetat didapati menunjukkan kesan penghapusan radikal peroksil, hidroksil, DPPH paling tinggi. Selain itu, fraksi etil asetat juga menunjukkan kapasiti yang tinggi dalam kuasa antioksidan penurunan ferrik (FRAP). Antara semua sampel, fraksi air sahaja berupaya menghapuskan semua radikal yang diujikan. Oleh itu, fraksi etil asetat dan fraksi air dianalisis seterusnya dengan menggunakan kromatografi gas mass spektrometri and kromatografi cecair spektrometri jisim untuk penilaian profil kompaun bioaktif. Linoleik asid etil ester, Butil 9,12-oktadekadienoate, 9,12-Oktadekadienoik asid, Ergosta-5,7,22-trien-3-ol, 2(3,4-Dihidroksifenil)-7-hidroksi-5-benzen propanoik asid and 3,30-di-O-

metil ellagic asid dikenalpasti dalam fraksi etil asetat, manakala oxooktadecanoik asid and 2(3,4-dihidroksifenil)-7-hidroksi-5-benzenpropanoik asid dikenalpasti dalam fraksi air. Esei 3-(4,5-dimetillthiazol-2-yl)-2,5-difeniltetrazolium bromide (MTT) menunjukkan fraksi air adalah tidak sitotoksik terhadap sel-sel HepG2 manakala fraksi etil asetat adalah sitotoksik kepada sel-sel HepG2 pada kepekatan 100 µg/mL. Dalam esei anti-hepatik steatotik, fraksi etil asetat menunjukkan perencatan ketara pengumpulan lipid ($p<0.05$), tetapi, bukan fraksi air. Tambahan lagi, fraksi etil asetat mempamerkan kapasiti ketara ($p<0.05$) menurunkan paras spesies reaktif oksigen intrasel (ROS), tetapi, bukan fraksi air. Dengan itu, analisis kesan anti-inflamasi fraksi etil asetat dijalankan. Pengeraman bersama asid palmitik, kehadiran 25 dan 50 µg /mL fraksi etil asetat menurunkan paras faktor tumour nekrosis alfa (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8) and "monocyte chemoattractant protein-1" (MCP-1) dengan jelas. Kedua-dua kepekatan tidak menunjukkan perbezaan yang ketara dalam penurunan paras pro-inflamasi sitokina. Siasatan lanjutan pada peringkat isyarat-isyarat laluan dengan menggunakan sel-sel HepG2 terinduksi dieramkan bersama dengan 25 µg/mL fraksi etil asetat menunjukkan penindasan "stress-activated protein kinases/jun amino-terminal regulated kinases" (SAPK/JNK), "p-38 mitogen-activated protein kinases" (p-38 MAPK), "nuclear factor-kappa B" (NF-κB) and "signal transducer and activator of transcription 3" (STAT-3). Sebagai kesimpulan, hasil kajian ini mencadangkan bahawa *A. nigricans* menunjukkan kesan anti-steatosis yang melibatkan kapasiti antioksida, kesan hipolipidemik dan kapasiti anti-radang dalam model sel patologi NAFLD-terinduksi asid palmitik. Secara bersama, fraksi etil asetat bernilai dijadikan ejen anti-hepatik steatotik dan *A. nigricans* boleh disaran sebagai makanan tambahan dalam diet pencegahan bagi pesakit-pesakit NAFLD dan NASH.

Kata kunci:
Cendawan, penyakit hati berlemak bukan alkohol (NAFLD), sel-sel HepG2 terinduksi asid palmitik

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
^{o}C	Degree Celsius
≥	greater than or equal to
µg/mL	Microgram per milliliter
µmol TE/g	micromoles Trolox equivalents per gram sample
µL	Microliter
µM	Micromolar
AAPH	2,2′-azobis(2-amidino-propane) dihydrochloride
ABC	ATP-binding cassette
ACOX	Acyl-CoA oxidase
ADP	Adenine diphosphate
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AUC	Area under the fluorescence decay curve
ATP	Adenine triphosphate
BSA	Bovine serum albumin
CAT	Catalase
CT	Computed tomography
CPT	Carnitine palmitoyl transferase
CRP	C-reactive protein
CYP	Cytochrome
CXC	Cysteine X cysteine
CO_2	Carbon dioxide
DCFH-DA	2'-7' dichlorofluoresceindiacetate
DCF	2',7'-dichlorofluorescein
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DNL	*De novo* lipogenesis
DPPH	1,1-diphenyl-2-picrylhydrazyl
EDTA	Ethylenediaminetetra acetic acid
EGF	Epidermal growth factor
ERK	Extracellular-signal regulated kinases
ETC	Electron transport chain
FADH2	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FDA	Food and drug administration
FFA	Free fatty acid
FOA	Fatty acid oxidation
g	Gram
GAE	Gallic acid equivalent
GCMS	Gas chromatography mass spectrum
gp	Glycoprotein
GPx	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HDL	High density lipopratein
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HRP	Horseradish peroxidase
IC_{50}	50% inhibitory concentration
IκB	Inhibitor of nuclear factor kappa B

IKK	Inhibitor of nuclear factor kappa B kinase
IL	Interleukin
JNK	Jun N-terminal kinase
LCMS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LDL	Low density lipoprotein
MDA	Malondialdehyde
M	Molar
mg	Milligram
MAPK	Mitogen activated protein kinase
MAPKKs	MAP kinase kinases
MEK	MAP/ERK kinase
MAPKKs	MAP kinase kinases
JAK	Janus kinases
MAPKKKs	MAP kinase kinase kinases
MCP-1	Monocyte chemoattractant protein-1
MKK	MAP kinase kinase
MKPs	MAPK phosphates
mM	milimolar
MRI	Microscopy resonance imaging
MRS	Magnetic resonance spectroscopy
MTT	3-[4,5-Dimethylthiazol-20yl]-2,5-diphennyltetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamideadenine dinucleotide phosphate
NAFLD	Non- alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NF-κB	Nuclear factor kappa B
nm	Nanometer
nM	NanoMolar
NO·	Nitric oxide
NOX	NADPH oxidase
$O_2{\cdot}^-$	Superoxide anion radical
OD	Optical density
$OH{\cdot}^-$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
ORAC	Oxygen radical absorbance capacity
OXPHOS	Oxidative phosphorylation
PBS	Phosphate buffered saline
PKC	Protein kinase C
PDGF	Platelet-derived growth factor
PMS	Phenazine methosulfate
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
ROO·	Peroxyl radicals
ROS	Reactive oxygen species
SD	Standard deviation
SOD	Superoxide dismutase
STAT	Signal transducer and activator of transcription
TCA	Tricarboxylic acid
TE	Milligram trolox equivalents
TG	Triglyceride
TGF	Transforming growth factor
TLR	Toll-like receptor

TNF	Tumour necrosis factor
TZDs	Thiazolidinediones @ Glitazones
w/v	Weight per volume
VDAC	Voltage-dependent anion-selective channel
VLDL	Very low density lipoprotein

CHAPTER 1: INTRODUCTION

1.1 Introduction

Liver disease is any disease that lead to abnormal liver function. A liver can be affected by several diseases, such as viral hepatitis (hepatitis B and/or C), hepatic steatosis, non-alcoholic steatohepatitis, inherited diseases (Wilson's disease), autoimmune hepatitis (Limdi & Hyde, 2003), alcoholic liver disease (Bruha *et. al.,* 2012) and toxic hepatitis (caused by solvents) (Malaguarnera *et. al.,* 2012). Non-alcoholic fatty liver disease (NAFLD) was first described in 1980 by Ludwig when a poorly understood liver disease similar to alcohol hepatitis and with a potential of progression to cirrhosis was found in twenty patients with non-alcoholic steatohepatitis (NASH) (Erickson, 2009; Jennings *et al.,* 2018). NAFLD is known as a condition of fat deposition in the liver (hepatic steatosis) and can develop to non-alcoholic steatohepatitis (NASH), which is an aggressive form of steatosis with significant inflammation. Eventually, NASH can progress to cirrhosis, end-stage liver failure and hepatocellular carcinoma (Hui *et al.,* 2013; Antonucci *et al.,* 2017).

NAFLD is associated with obesity, insulin resistance, hypertension, and dyslipidemia and is considered the hepatic manifestation of metabolic syndrome (Banini & Sanyal, 2016). NAFLD involves a cluster of liver disease pathologies ranging from liver lipid accumulation (steatosis) through inflammation (nonalcoholic steatohepatitis) to fibrosis and finally, irreversible cirrhosis (Walenbergh *et al.,* 2013) that are linked to oxidative stress, increased release of inflammatory cytokine, necrosis and apoptosis (Tan *et al.,* 2015). However, the pathogenesis of NAFLD remains a poorly understood liver disease because it is complex and multifactorial (Wang *et al.,* 2014; Bashirades *et al.,* 2016; Buzzetti *et. al.,* 2016). During β-oxidation of fatty acid in the mitochondria, electrons removed from fatty acids are used to produce ATP, CO_2 and water through mitochondrial respiratory chain, simultaneously, ROS such as superoxide, hydrogen

peroxide and hydroxyl radical are generated as a result of some oxygen that are not fully reduced to water at physiological state (Basaranoglu *et. al.,* 2013). Reports indicated that over-eating causes excessive FA loaded in the liver, which induced high levels of β-oxidation (Pettinelli *et. al.,* 2011), subsequently increased flow of electron through the electron transfer chain resulted in an increased mitochondrial ROS (Gusdon *et. al.,* 2014), therefore leading to oxidative stress (Pettinelli *et. al.,* 2011). ROS production within the hepatocytes are further increased through peroxisomal β-oxidation and ω-oxidation of fatty acids (Basaranoglu *et. al.,* 2013) while metabolic adaptations occur to compensate for the increased liver fat load, mitochondrial dysfunction eventually occurs. The mechanisms of it remains poorly understood, however, ROS are likely responsible for the impairing of electron transfer chain activity (Gusdon *et. al.,* 2014). Mitochondrial dysfunction leads to further over-production of ROS that resulted in lipd peroxidation and protein oxidation, which has a detrimental effect on fat homeostasis in the liver (Ucar *et. al.,* 2013). The cellular oxidative stress from hepatocytes and mitochondrial dysfunction contribute to hepatic inflammation through induction of pro-inflammatory cytokines, such as TNF-α, IL-6, IL-8 (Takaki *et. al.,* 2014; Ucar *et. al.,* 2013).

Clinical burden of NAFLD not only restricted to liver-related morbidity and mortality but increasing evidence indicates that NAFLD is multisystem disease, the effects of NAFLD contributed to extra-hepatic chronic complications (Byrne & Targher, 2015; Mikolasevic *et al.,* 2016). NAFLD is associated with accelerated atherosclerosis (Magosso *et al.,* 2010), also increase prevalence of prediabetes and the risk of developing type 2 diabetes mellitus (Leita *et al.,* 2014; Byrne & Targher, 2016) as a result of increased FFA level leading to impairment of muscle and liver insulin sensivity (Ortiz-Lopez *et. al.,* 2012). Furthermore, evidence linking NAFLD and its severity to the development of chronic kidney disease (Jang et al., 2018) are the similarity in risk factors such as hypertension, obesity, dyslipidemia, and insulin resistance, as well as linked to

an increased risk of cardiovascular disease events (Targher *et al.,* 2011; Marcuccilli & Chonchol, 2016). In addition to hepatocellular carcinoma, NAFLD is also associated with colorectal cancer and breast cancer (Kim *et al.,* 2017). As the prevalence of NAFLD grow, it become a significant economic burden to medicare. The economic burden of NAFLD in the United States and Europe, including Germany, France, Italy and United Kingdom, was estimated to be approximately $ 103 billion and € 35 billion, respectively (Younossi, 2017) by charging of US$ 1,613 per patient in the United States, and from € 354 to 1,163 per patient in Europe (Younossi *et al.*, 2018). The projected cost of caring for patients in United States is expected to increase by 18 % from 2000 to 2035 (Perumpail *et al.*, 2017).

Nowadays NAFLD is known as one of the most important causes of liver disease throughout the world and will probably become the predominant cause of end-stage liver disease in the coming decades, affecting both adults and children (Younossi *et al.*, 2018). Global prevalence of NAFLD is 24 % highest prevalence in the Middle East and South America and lowest in Africa (Younossi, 2018). Likewise, Europe has a high prevalence of NAFLD, approximately of 20–30 % (Zhang & Lu, 2015). Estes *et al.* (2018) projected that the prevalent of NAFLD cases in USA will increase by 21 %, from 83.1 million (2015) to 100.9 million (2030), while prevalent NASH cases will increase by 63 % from 16.52 million to 27.00 million cases. A study recruited 1621 Malaysian consisted of Chinese, Malay and Indian indicated that males and age group of $\geqslant$ 45 years had high prevalence of NAFLD, with the highest in Indian (68.2 %) followed by Malay (64.7 %) (Goh *et al.*, 2013). A recent report indicated that the prevalence of NAFLD in Malaysia was 82.8 % with Indian ethnics had the highest risk of developing NAFLD (90.9 %) (Suppiah *et al.*, 2016).

The rising prevalence of NAFLD worldwide may be accounted for by changes in dietary habits and an increase in sedentary lifestyle (Satapathy & Sanyal, 2015). The global growth of western diet and lack of physical activity play a crucial role in the rise

in the prevalence of NAFLD (Perumpail *et al.*, 2017). The western diet contained high saturated fat and fructose is believed to be the culprit (Ferolla *et al.*, 2015; Manopriya *et al.*, 2016). Since dietary patterns and nutrients are the important contributors to the development, progression, and treatment of NAFLD and associated metabolic comorbidities (Fan & Cao, 2013), the correction of unhealthy lifestyle is the best mode of prevention and treatment of NAFLD. Only when such intervention fail, then drug therapy becomes the second strategy (Hernandez-Rodas *et al.*, 2015).

To date, modification through dietary change, physical activity, and exercise to facilitate weight loss and weight loss maintenance is the most effective prevention and treatment for NAFLD (Zelber-Sagi *et al.*, 2016; Avery *et al.*, 2017). Mediterranean diet is characterized by high intake of monounsaturated-fat, nuts, fruits, legumens, vegetables, and fish and a low intake of red meat, processed meats and sweet, is the dietary pattern recommended for NAFLD patients by the recent European Association for the Study of the Liver (EASL)-European Association for the Study of Diabetes (EASD)-European Association for the Study of Obesity (EASO) Clinical Practice Guidelines (Romero-Gómez *et. al.*, 2017). Several medications and supplements that are used in the treatment of NAFLD (Benedict & Zhang, 2017) are focus primarily on metabolic comorbidities such as type 2 diabetes mellitus and obesity (Lorbek *et al.*, 2016) because NAFLD patients are frequently obese and/or diabetic (Romero-Gómez *et. al.*, 2017). However, those medications and supplements are not effective in curing NAFLD (Piacentini *et al.*, 2018) and have adverse effects such as manifestations due to hepatoxicity, increased risk of death, heart failure or pro-arrhythmic potential (Liu *et al.*, 2013).

Due to limited current NAFLD therapies; thus, much attention has been focused on identification of potential dietary substances from fruits, vegetables, and edible plants to provide a new strategy for NAFLD treatment (Pan *et al.*, 2014). As food, mushrooms are known as a delicacy with high nutritional and functional value, they are also accepted

as nutraceutical foods because they produce a large array of nutrients and other natural phytochemicals with useful biological properties that have wide ranges of nutritional and health benefits (Zhang *et al.*, 2014; Valverde *et al.*, 2015). Many mushrooms have long been used throughout Asia for medicinal purpose (Jayachandran *et al.*, 2017), such as *Agaricus blazei* Murrill, *Coriolus versicolor* (L.) Quél., *Ganoderma lucidum* sensu auct. asiatic., *Lentinus edodes* (Berk.) Singer, *Taiwnofungus camphoratus*, and *Cordyceps sinensis* (Berk.) Sacc. (Lee *et al.*, 2012). Mushrooms have various pharmacological activities including anti-bacterial, anti-fungal, anti-viral, immunomodulating, anti-inflammatory, anti-oxidative, anti-allergic, anti-depresive, anti-hyperlipidemic, anti-diabetic, digestive, hepatoprotective, neuroprotective, nephroprotective, osteoprotective, and hypotensive activitites (Gargano *et al.*, 2017; Jayachandran *et al.*, 2017). Notably, edible mushrooms should not be considered merely as foods because there is no clear differentiation between edible and medicinal mushrooms (Soares *et al.*, 2013; Valverde *et al.*, 2015). Many of common edible species have been shown to be rich in bioactive compounds and have therapeutic properties, several mushrooms used for medical purposes are also edible (Soares *et al.*, 2013; Valverde *et al.*, 2015). For instance, *Agaricus bisporus* (J.E. Lange) Imbach, *Pleurotus ostreatus* (Jacq.) P. Kumm. and *Lentinus edodes* were reported to reduce the cholesterol level in serum and/or liver, whereas mukitake mushroom (*Panellus serotinus* (Pers.) Kühner) was recently reported to alleviate nonalcoholic fatty liver disease in db/db mice (Kanaya *et. al.*, 2011).

In the light of this, *A. nigricans* has been regarded with several known biological activities including anti-oxidant activity, immunomodulatory, anti-tumor activities, anti-dementia properties, attenuation of the inflammatory response, oxidative stress and lipid deposition and hypocholesterolemic effects (Liang *et. al.*, 2019). Hence, attenuation of lipid deposition and inflammatory response as well as anti-oxidation capacity of *A. nigricans* are likely to make it a good source of anti-hepatic steatosis agent. However,

there is limited scientific reports of *A. nigricans* on NAFLD with regards to the anti-hepatic steatosis effects both *in vitro* and *in vivo* including the involved underlying mechanisms. To validate these therapeutic benefits, *in vitro* scientific experiments need to be carried out to clarify the beneficial effects of *A. nigricans* on NAFLD.

1.2 Research objectives

This study aims to evaluate the beneficial values of solvent-partitioned extracts fractions of *A. nigricans* in the prevention and treatment of non-alcoholic fatty liver. Therefore, the objectives of the present study include,

- To evaluate the *in vitro* antioxidant capacity of the solvent extracts and fractions of *A. nigricans*
- To profile the phytoconstituents present in the bioactive extract and fractions of *A. nigricans*
- To evaluate the anti-hepatosteatosis capacity and to evaluate intracellular reactive oxygen species scavenging capacity of selected solvent partitioned extracts and fractions of *A. nigricans*
- To evaluate the anti-inflammatory capacity and to elucidate the anti-inflammatory signalling pathways of selected solvent partitioned extracts and fractions of *A. nigricans*

CHAPTER 2: LITERATURE REVIEW

2.1 Non-alcoholic fatty liver disease (NAFLD)

Liver is one of the most pivotal organs for sustaining energy supply via performing carbohydrate, protein and fat metabolisms in the body (Wei *et al.*, 2008; Gu *et al.*, 2016). Under physiological condition, liver is not a storage depot for fat, the presence of triglyceride in hepatocytes is low (Kawano & Cohen, 2013), less than 5 % by its weight (Kani *et al.*, 2014). However, it is a central organ responsible for trafficking in and out of both triglycerides and fatty acids during fasting and feeding states (Kawano & Cohen, 2013). It gains input from intestine through the portal vein, the general circulation via hepatic artery, and the lymphatic system (Bradbury, 2006). Furthermore, it acts as the metabolic gatekeeper between the intestines and blood circulation. Also, it ensures that toxins are broken down into harmless compounds to protect the body from harm (Auger *et al.*, 2015).

Non-alcoholic fatty liver disease (NAFLD) is a broadly defined term for fatty liver-related diseases (Park et al., 2015), characterized by an accumulation of liver fat content exceeding 5 % of liver weight (Nassir & Ibdah, 2014; Yki-Järvinen, 2015), in the absence of significant amount of alcohol consumption, less than 20 g of ethanol per day (< 20 g / day) (Zivkovic *et al.*, 2007; Freidoony & Kong, 2014). Triglycerides is the main lipid that accumulate in hepatocytes due to an imbalance between its synthesis and transformation (Cao *et al.*, 2016). Therefore, an accumulation of triglyceride in the cytoplasm of hepatocyte is the hallmark of NAFLD (Kawano & Cohen, 2013). It is a chronic inflammatory disease (Hijona et al., 2010) that include a wide spectrum ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) (Das & Balakrishnan, 2011; Fan & Cao, 2013), which is characterized by the presence of inflammation and tissue injury (Rodriguez-Ramiro *et al.*, 2016). Hepatic steatosis is a benign and non-

progressive condition of liver injury, conversely, NASH may progress to liver fibrosis, cirrhosis (Fargion *et al.*, 2014) and hepatocellular carcinoma (Takahashi *et al.*, 2015; Yilmaz *et al.*, 2015). Furthermore, NAFLD becomes an independent risk factor for the occurrence and progression of cardiovascular diseases, including coronary heart disease and stroke (Fargion *et al.*, 2014; Rodriguez-Ramiro *et al.*, 2016) because these diseases share several risk factors and surrogate markers (Kim *et al.*, 2014). In addition, CVD represents the leading cause of mortality among NAFLD patients (Fotbolcu & Zorlu, 2016; Lonardo *et al.*, 2016). To date, non-alcoholic liver disease (NAFLD) is recognized to be the most common liver disease world-wide (Puri & Sanyal, 2012; Kanuri & Bergheim, 2013; Freidoony & Kong, 2014). It is a major global public health concern because of its high prevalence worldwide and potentially critical sequelae (Kawano & Cohen, 2013).

Histologically, NAFLD is characterized by the presence of fat droplets in hepatocyes that are either quite large and dislocate the hepatocyte nuclei to the periphery of cells or are small and form a "foamy" appearance of hepatocytes (Neuschwander-Tetri, 2005; Wei *et al.*, 2008). Hepatocytes with large droplet of hepatocytes fat is known as macrovesicular, whereas the small droplet of hepatocytes fat is called microvesicular (Neuschwander-Tetri, 2005). Morphologically, NASH is distinguished from NAFLD by the presence of macrovesicular steatosis, lobular inflammation, hepatocytes ballooning, fibrosis, apoptotic cells and Mallory's hyaline (Wei *et al.*, 2008; Kneeman *et al.*, 2012).

The pathogenesis of NAFLD remains incompletely understood (Yang *et al.*, 2014) and still highly debated (Vacca *et al.*, 2015). NAFLD is a multifactorial disease with a complex pathophysiology, such as lipid metabolism alteration, mitochondrial dysfunction, inflammation and oxidative stress (Serviddio *et al.*, 2013; Ragab *et al.*, 2015). The "two-hit hypothesis" is broadly accepted model to elucidate the development of NAFLD and progression from simple steatosis to NASH (Zhu *et al.*, 2014; Ai *et al.*, 2015). The first

hit is development of hepatic steatosis through accumulation of triglyceride in hepatocytes due to excessive delivery of free fatty acids (FFAs) from the adipose tissue, and imbalance of lipid synthesis and export in hepatocyte, whereas the second hit incorporates hepatic injury, inflammation, and fibrosis, which are closely associated with oxidative stress in the liver (Luo *et al.*, 2012; Liang *et al.*, 2015; Park *et al.*, 2015). Fatty degeneration of hepatocytes is more inclined to second hit, such as oxidative stress, cytokines, than normal one, which further promote the generation of inflammation that is the development of NAFLD (Gaemers *et al.*, 2011; Cao *et al.*, 2016).

2.2 Incidence and prevalence of NAFLD

The development of NAFLD is in a strong association with obesity, type 2 diabetes, hypertension and dyslipidemia, collectively termed as "metabolic syndrome (Fan & Cao, 2013; Ferramosca & Zara, 2014). This disease is associated with the growing incidence of metabolic syndrome (Wit *et al.*, 2012). The prevalence of NAFLD increase to 50 % in subjects with diabetes, 76 % in those with obesity and 100 % in those morbidly obese with type 2 diabetes; besides, 50-100 % of NASH persons are overweight, 50-60 % have systemic hypertension, and 50-60 % have dyslipidemia (Vacca *et al.*, 2015). Moreover, 10-15 % of normal weight individuals are found to have fatty liver (Fotbolcu & Zorlu, 2016). Interestingly, children are also found to be at risk of developing NAFLD (Wu *et al.*, 2011; Fargion *et al.*, 2014): the prevalence was about 3-10 % in lean subjects and about 53 % in obese pediatric population (Fargion *et al.*, 2014). Increased caloric intake and a sedentary life style are associated with metabolic syndrome, insulin resistance, dyslipidemia, and NAFLD (Sá *et al.*, 2015). Diet is an important contributor to the pathogenesis of NAFLD (Wit *et al.*, 2012).

2.3 Diagnosis of NAFLD

NAFLD is "a silent disease" because it is present in the body without any symptom. Patients may have non-specific clinical features such as fatigue, exercise intolerance, or unspecific vague abdominal pain in the right upper quadrant (Wei *et al.*, 2008; Farrell *et al.*, 2012; Clemente *et al.*, 2016). Only those who developed NASH with more severe liver damage will have some symptoms of chronic liver disease (Clemente *et al.*, 2016).

Laboratory investigations were carried out for the diagnosis of NAFLD by ruling out the other causes of liver damage (Jamali, 2013) such as viral hepatitis (De Alwis *et al.*, 2016). In clinical practice, NAFLD is generally proposed when an elevation of hepatobiliary enzymes is detected (Vajro *et al.*, 2012). Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and gamma-glutamyl transpeptidase (GGT) are among the most common serum parameters used to evaluate liver function (Jamali, 2013; Dyson *et al.*, 2014). Usually, a mild increase in ALT, AST and/or GGT is observed if liver function test is abnormal (Dyson *et al.*, 2014). Serum ALT is a broadly used and it is an inexpensive test for the screening and initial evaluation of NAFLD, but, the sensitivity of this biomarker is low (Vajro *et al.*, 2012) because approximately 78-80 % patients with NAFLD have normal range of ALT level (Obika & Noguchi, 2012; Dyson *et al.*, 2014). Moreover, ALT values do not correlate with the presence or severity of histological findings of NAFLD (Vajro *et al.*, 2012; Clement *et al.*, 2016). Hence, over reliance on abnormal liver enzymes to identify patients with NAFLD result in potentially missing opportunities for intervention, overlooking of patients with significant liver disease (Dyson *et al.*, 2014).

Abdominal ultrasound is the most common method to be used for screening asymptomatic patients with an incidental elevation of liver enzymes and suspected NAFLD (Mishra & Younossi, 2007; Obika & Noguchi, 2012). Typically, ultrasound

evaluation of hepatic steatosis is a qualitative visual assessment of hepatic echogenicity (Lee & Park, 2014). Based on echogenicity, a structure can be characterized as hyperechoic (white on the screen), hypoechoic (gray on the screen) and anechoic (black on the screen) (Ihnatsenka & Boezaart, 2010). Fatty liver exhibits hyperechogenicity because it scatters the beam of ultrasound more than a normal liver (Jamali, 2013). The echogenicity is needed to be compared with internal organs recognized to be clear of fat, for instance the kidneys or spleen because there is no absolute echogenicity that designated liver fat (Jamali, 2013; Lee & Park, 2014). Nonsteatotic hepatic parenchyma exhibits an echotexture similar to that of renal parenchyma, but becomes "brighter" when infiltrated with fat (Obika & Noguchi, 2012). Ultrasound has some limitations even though it is greater ease of use and low cost (Obika & Noguchi, 2012; Khov *et al.*, 2014). It is operator dependent and subject to significant intra- and interobserver variability (Obika & Noguchi, 2012; Lee & Park, 2014). Furthermore, it does not provide quantitative information about the degree of fat deposition in liver and varying stages of NASH (Bhatia *et al.*, 2012; Obika & Noguchi, 2012), thus, this method cannot establish the diagnosis of NASH or stage of hepatic fibrosis (Mishra & Younossi, 2007). The sensitivity for detecting mild degrees of steatosis is low, ranging from 55-90 % (Khov *et al.*, 2014) because ultrasound can only detect steatosis when 30 % of the liver is affected (Obika & Noguchi, 2012; Dyson *et al.*, 2014). Therefore, the finding of a normal liver on ultrasound does not rule out mild fatty infiltration of the liver (Dyson *et al.*, 2014). Additionally, sensitivity of ultrasound is low in obese patients (Obika & Noguchi, 2012; Jamali, 2013). Other non-invasive imaging modalities such as magnetic reasonance spectroscopy (MRS), microscopy resonance imaging (MRI) and computed tomograpy (CT) are marvellous at detecting steatosis, but similar to ultrasound, are not reliably distinguished between NASH and other degree of NAFLD (Khov *et al.*, 2014; Thrasher and Abdemalek, 2016). Furthermore, neither CT nor MRI are routinely used in the

assessment of steatosis (Dyson *et al.*, 2014). Among the non-invasive imaging modalities, abdominal ultrasound is the most commonly used imaging method as a result of it is being easy to perform and less costly than other imaging methods (Lee & Park, 2014).

At present, liver biopsy is the gold standard for diagnosing NAFLD and staging of NASH and fibrosis, accompanied by histological assessment (Bhatia *et al.*, 2012; Machado & Cortez-Pinto, 2013). Histology is important for distinguishing between simple seatosis, which is generally non-progressive and readily reversible, versus steatohepatitis, which is potentially to progress to severe fibrosis or cirrhosis (Thrasher & Abdelmalek, 2016). The key histological features to distinct NASH from simple steatosis consist of the presence of macrovesicular fatty changes of hepatocytes with displacement of the nucleus to the edge of the cell, ballooning degeneration of hepatocytes, and a mixed lobular inflammation (Vajro *et al.*, 2012; Dyson *et al.*, 2014). Performing liver biopsy, in general, is to confirm or exclude the diagnosis, diagnose other liver disease and to determine degree of damage to the liver for treatment and prognosis (Nalbantoglu & Brunt, 2014). Liver biopsy approach is an invasive with small risk but expensive (Spengler & Loomba, 2015), thus, it is uncommonly practised in hospitals (Jamali, 2013; Rodriguez-Ramiro *et al.*, 2016). As an invasive procedure, liver biopsy has a minor risk of complications including pain, bleeding (0.3 %), and rarely, death (0.01 %) (Obika & Noguchi, 2012; Nalbantoglu & Brunt, 2014). Other limitations of liver biopsy are due to sampling variability and intra- and interobserver variabiity on histological analysis (Obika & Noguchi, 2012). Despite these limitations, liver biopsy remains the gold standard to confirm NASH and assess fibrosis due to the lack of reliable noninvasive methods (Rinella *et al.*, 2014). It is also important to determine the presence of NASH and liver fibrosis for close monitoring and follow-up are necessary for these patients (Obika & Noguchi, 2012).

2.4 Mechanisms contributing to NAFLD

Mechanisms that contribute to the pathogenesis of NAFLD is associated to dietary high fat intake, uptake from circulating free fatty acids and *de novo* lipogenesis (Gusdon *et al.*, 2014; Machado & Cortez-Pinto, 2014), as shown in Figure 2.1. The hallmark of NAFLD is ascribed as accumulation of triglyceride in cytoplasm of hepatocyte as a result of imbalance between pathways of fatty acid input and removal (Kanuri & Bergheim, 2013; Kawano & Cohen, 2013). Free fatty acid influx from adipose tissue contributed to approximately 60 % of triglyceride in liver of NAFLD patients, whereas 26 % of triglyceride derived from De novo lipogenesis and 15 % from diet (Dowman *et al.*, 2010; Hazlehurst & Tomlinson, 2013).

In general, nutrition has been linked to human health and disease (Sá *et al.*, 2015). Diet is an important determinant of lifestyle and is a major contributor to the pathogenesis and high prevalence of NAFLD (Wit *et al.*, 2012; Freidoony & Kong, 2014). Dietary effects on whole-body metabolism and its regulation through effects on hormones, transcription factors, and lipid metabolic pathways are considered to play a central role in NAFLD (Zivkovic *et al.*, 2007; Piano *et al.*, 2014). Overnutrition or inappropriate diet are thought to lead to chronically elevated glucose, insulin, and free fatty acid concentrations in the blood in most patients (Zivkovic *et al.*, 2007). Commonly, hypercaloric diets, especially rich in trans/saturated fat and cholesterol, and fructose-sweetened beverages, seem to exacerbate NAFLD toward nonalcoholic steatohepatitis (NASH) (Freidoony & Kong, 2014).

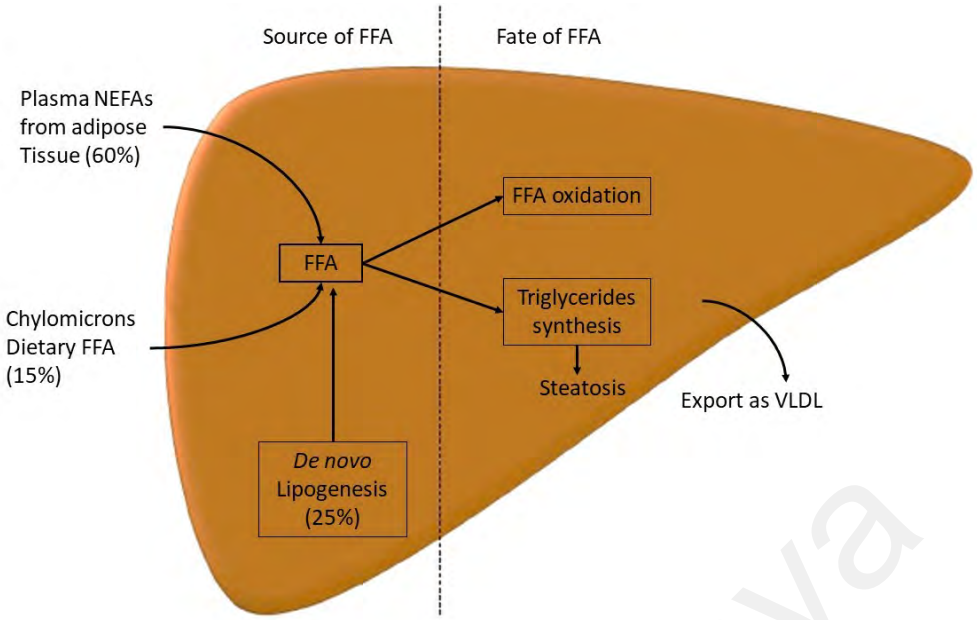

Figure 2.1: Pathogenesis of liver steatosis

Hepatic steatosis can result from an increased influx of lipids, free fatty acids (FFA), to the liver or a decreased lipid disposal. Three main sources of FFA in the liver are the plasmatic nonesterified fatty acids (NEFAs), which originate predominantly from lipolysis in the adipose tissue, from *de novo* lipogenesis, mainly from glucose or other carbohydrates, and from FFA that come in chylomicrons from the gut (dietary FFA). In the liver, FFA can either be oxidized, mainly in the mitochondria, beta-oxidation, or can be used to produce triglycerides. The latter can be exported as very low-density lipoproteins (VLDL) to the circulation or can accumulate in lipid droplets in the hepatocyte leading to steatosis (Source: Machado & Cortez-Pinto, 2014).

2.4.1 Dietary fat

Fats is one of the major components in the normal human diet. It has been regarded to be sources of energy and components of biological membranes (Juárez-Hernández *et al.*, 2016). Furthermore, it plays many vital functions in the body. For instance, it is crucial for facilitating absorption of fat-soluble vitamins, such as Vitamin A, D, E and K, and carotenoids; and shelter major organs from shock during normal activities and offer insulation that prevent heat loss (Welch-White *et al.*, 2013). Fatty acids are an important constituent of the cell membrane because they involve in modulation of the activity of membrane-bound transporters and enzymes, modification of the membrane fluidity, serve as intracellular messenger and alter intracellular functions (Piano *et al.*, 2014). Saturated free fatty acid derived from fats, such as the 18-carbon stearic acid, the 16-carbon palmitic acid, and the 14-carbon myristic acid are needed for

various biological functions such as production of hormone and cellular membrane signalling (Binienda *et al.*, 2013). Essential fatty acids (EFAs), also known as polyunsaturated fatty acids (PUFAs), which cannot be synthesized by the body but only obtain it in diet source, such as omega-6 fatty acid and omega-3 fatty acid, desaturated in the body to form precursors of respective prostaglandins, thromboxanes, leukotrienes, and prostacyclin, which have tremendous effect on the brain's blood flow, immune system and the neurotransmitter system (Chang *et al.*, 2009).

Diets with adequate energy (30 % originating from fat) are sufficient to promote normal growth and normal sexual maturation; however, diets that exceed this amount may lead to excessive weight gain. Dietary fat intake and excessive caloric intake has been proposed as a causative factor in the development of metabolic syndrome (Welch-White *et al.*, 2013).

In the fed state, dietary lipids are efficiently absorbed by the small intestine, incorporated into triglyceride-rich lipoproteins, named chylomicrons, and secreted into lymphatics, entered the plasma and transported in the circulation to various tissues and liver (Kawano & Cohen, 2013; Dash *et al.*, 2015). Mainly, 70 % of the fatty acid in chylomicrons are delivered to adipose tissue whereas the remaining being taken up by the liver (Kawano & Cohn, 2013). Additionally, free fatty acid derived from the spillover mechanism due to the action of lipoprotein lipase in peripheral tissues, especially from adipose tissue on chylomicrons release fatty acids that mixed with circulating free fatty acids (Almandoz *et al.*, 2013; Gambino *et al.*, 2016), which contribute 59 % of hepatic triglyceride (Nobili *et al.*, 2011). In fact, spillover from chylomicrons is the key source of free fatty acid under postprandial condition (Gaggini *et al.*, 2013). High fat diet increases the total amount of chylomicrons in the intestine (Kesh *et al.*, 2016), which contribute to dyslipidemia seen in common metabolic disorders (Dash *et al.*, 2015).

2.4.2 Dietary carbohydrate (*de novo* lipogenesis)

Carbohydrates are a major source of body energy for both children and adults. It is linked to NAFLD (Freidoony & Kong, 2014) because fatty acids are synthesized *de novo* within liver (Kawano & Cohen, 213). Hepatic *de novo* lipogenesis (DNL) is a fundamental biosynthetic pathway within the liver, which is activated by insulin after a high carbohydrate meal (Nassir & Ibdah, 2014). DNL leads cells to convert excess carbohydrate into fatty acids through acetyl-CoA subunits produced during glycolysis (Nassir & Ibdah, 2014; Sanders & Griffin, 2016). DNL has been suggested to contribute to the pathogenesis of NAFLD (Sanders & Griffin, 2016).

Volk *et al.* (2014) reported that changes of plasma saturated fat (proportion of palmitoleic acid in plasma triglyceride and cholesteryl ester) is associated to the intake of carbohydrate, not dietary saturated fat. This is because carbohydrate-induced insulin secretion stimulates DNL and suppresses lipolysis and fat-oxidation (Volk *et al.,* 2014). Also, a low carbohydrate diet led to a reduction in serum triglycerides, insulin, and glucose but increase in high density lipoprotein (Santos *et al.*, 2012). A post hoc analysis of alanine aminotransferase (ALT) level was carried out on 52 obese subjects showed that diet moderately lower in carbohydrate decreased serum alanine aminotransferase level than a higher carbohydrate/low fat diet (Ryan *et al.,* 2007).

High consumption of fructose and glucose as simple sugars induce the *de novo* synthesis of fatty acids, mainly in individuals with insulin resistance and in those who are overweight (Zivkovic *et al.*, 2007). Sugar sweetened beverages may increase type 2 diabetes mellitus, cardiovascular disease, and increase dietary glycemic load leading to inflammation, insulin resistance, impaired β-cell function (Malik *et al.*, 2010). Furthermore, high-fructose corn syrup enhanced DNL that result in increased oxidative stress, insulin resistance, increase blood pressure, promote accumulation of visceral adiposity, dyslipidemia and etopic fat deposition (Malik *et al.*, 2010; Asrih & Jornayvaz,

2014). Fructose is a monosaccharide, a sweet tasting sugar, sweeter than either glucose or sucrose, and found naturally in fruits and some vegetables; it was limited in human diet before the development of the worldwide sugar industry (Basaranoglu *et al.*, 2015). Compared to glucose and sucrose, fructose is more lipogenic and may induce less insulin secretion (Sullivan, 2010). It is extensively metabolized by liver via bypassing the rate-limiting step of glycolysis, thus it provides more substrate for *de novo* lipogenesis, subsequently increase intrahepatic triglycerides and very low density lipoprorotein production more than glucose (Sullivan, 2010; Yki-Järvinen, 2015).

2.4.3 Lipolysis

Adipose tissue is not only the main site of storage of excess energy derived from food intake but also known as an active endocrine organ (Jung & Choi, 2014). It is the only organ with unlimited growth potential at any stage of life; 60 % to 85 % of adipose tissue account for lipid, and with 90 % to 99 % are triglyceride (Qureshi & Abrams, 2007). NAFLD is related with increased adipose tissue lipolysis (Cohen *et al.*, 2011; Jha *et al.*, 2014). Adipose tissue lipolysis is a catabolic process that results in the breakdown of triglycerides stored in fat cells and release of fatty acids and glycerol for utilization by other organs as energy substrate during times of energy deprivation (Duncan *et al.*, 2007; Lafontan & Langin, 2009).

During severe metabolic demand, such as fasting, starvation, exercise, triglyceride stored in lipid droplet is hydrolyzed and released as non-esterified fatty acid back to the circulation and transported to other tissues to be used as an energy source (Qureshi & Abrams, 2007; Jung & Choi, 2014). High levels of circulating free fatty acids can also cause peripheral insulin resistance in both animals and humans (Jung & Choi, 2014). Free fatty acids in circulation mainly derived from adipose tissue (Jha *et al.*, 2014). In liver, FFAs have three major fates: They can be oxidized in mitochondria to produce energy

and ketone bodies, reesterified to TG and stored in lipid droplets, or coupled to apolipoproteins and secreted as a constituent of very-low-density lipoproteins (VLDL) (Cohen *et al.*, 2011). Excessive accumulation of lipid droplet in hepatocytes lead to increased in very low-density lipoprotein (VLDL) secretion and some of the serum lipid abnormalities noted in metabolic syndrome and NAFLD, including hypertriglyceridemia, decreased high-density lipoprotein (HDL), and higher low-density lipoprotein (LDL) (Fotbolcu & Zorlu, 2016).

The eating habit of human is more inclined towards the consumption of dietary saturated fat due to the impresssion of western food, which give rise to the amount of saturated fat in diet leading to an elevation in the amount of chylomicron in the intestine. Subsequently, increased concentration of chylomicron promotes transportation of dietary lipid to the liver. Furthermore, increased concentration of chylomicron also contributes to the increase in the fatty acid derived from spillover meachanism. Therefore, dietary fat, gradually, becomes important mechanism that contributes to NAFLD.

2.5 Reactive oxygen species (ROS)

ROS are found in all biological system (Xu & Touyz, 2006). They are characterized by high chemical reactivity (reactive chemical entity) that derived from oxygen metabolism (Paravicini & Tuozy, 2008) through the reduction of molecular oxygen (Paravicini & Touzy, 2006). ROS consist of two major groups: free radicals, such as superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), nitric oxide ($NO^{\cdot}$) and non-radical derivatives of oxygen, which includes hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) (Paravicini & Tuozy, 2008).

Every ROS possess their own unique chemical character even though they are derived from molecular oxygen (Paravicini & Touyz, 2006). $OH^{\cdot}$ has relative short half-life, thus $OH^{\cdot -}$ is highly reactive and most unstable ROS (Paravicini & Touyz, 2006;

Cosentino-Gomes *et al.*, 2012), mediating it quickly with adjunct cells, therefore, $OH^{\cdot}$ has limited ability to transmit signals across any significant distance (Cosentino-Gomes *et al.*, 2012). Similar to $OH^{\cdot}$, $O_2^{\cdot -}$ has relatively short half-life, and its electrophilic property causes it to be hardly permeable through the cellular membrane, as an alternative, it goes through ion channels (Paravicini & Touyz, 2006). Compared to $O_2^{\cdot -}$, H_2O_2 is more stable as a result of its longer half-life. Furthermore, it is electrophobic, therefore, it's able to diffuse freely within and between cells across the lipid bilayer (Paravicini & Touyz, 2006; Cat *et al.*, 2013).

Generally, cells have evolved an antioxidant defense system viz. antioxidant enzymes, such as as catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutases (SOD) that tightly regulate the intracellular ROS level to maintain physiological level (Li & Shah, 2003; Frey *et al.*, 2009). For instance, SOD catalyzes the dismutation of $O_2^{\cdot -}$ to form H_2O_2. The H_2O_2 is further decomposed to water by CAT or GPx (Li *et al.*, 2006). However, enhanced activity of oxidant enzymes and /or decreased activity of antioxidant enzymes lead to oxidative stress (Wassmann *et al.*, 2004). Altered ROS level may activate some signaling pathways and inhibit others resulting in alteration of gene expression, which contribute to various pathophysiological responses (Mohora *et al.*, 2009). Therefore, exogenous antioxidants such as vitamins A, E and C, flavonoids, glutathione (GSH) are essential to support cells in curbing this oxidative stress (Lobo *et al.*, 2010).

ROS are produced by distinct sources in various cells of the body. Mainly, ROS are produced as products by mitochondrial respiration and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase of the NOX family (Yan *et al.*, 2015). ROS that are generated within a cell, may also be released extracellularly (Yang *et al.*, 2007). Extracellularly, ROS may damage surrounding tissues that possibly result in stimulating inflammatory process; whereas, intracellular ROS participate in host defense against

infectious agents and be a messenger for various redox-signaling transductions (Yang *et al.*, 2007; Zhang *et al.*, 2016). In fact, ROS have direct impact in many intracellular pathways, including changes through redox-sensitive protein kinase, such as mitogen activated protein kinase (MAPK), protein kinase C (PKC) (Dworakowski *et al.*, 2008; Yang *et al.*, 2007), alteration in the activity of redox-sensitive transcription factors such as activate protein-1, nuclear factor kappa B (NFκB) (Dworakowski *et al.*, 2008; Yan *et al.*, 2015), via changes in activity of redox-sensitive molecule such as thioredoxin and through direct effect on enzymes, receptors or ion channels (Dworakowski *et al.*, 2008).

In general, ROS were recognized as a hazard product of cellular metabolism because they deleteriously affect DNA, lipids and proteins, in high concentrations. Conversely, these reactant molecules, in lower doses, are now thought to act as essential mediators of cell growth, adhesion, differentiation, senescence, and apoptosis by modifying key elements in protein regulatory sites (Cosentino-Gomes *et al.*, 2012). Among numerous ROS; $O_2{\cdot}-$ and H_2O_2 are important because they are suggested to be involved in the cellular signal transduction as a signaling molecule, at physiological concentration (Matsushita *et al.*, 2005; Frey *et al.*, 2009). Differences in chemical properties between $O_2{\cdot}-$ and H_2O_2 lead to activativation of diverse signaling pathways which contribute to distinct biological responses. Nevertheless, H_2O_2 is known to be the key signaling molecule due to it is relatively stable compared to $O_2{\cdot}-$, thereby multiply its range of action (Lassègue & Griendling, 2010; Cat *et al.*, 2013). H_2O_2 induce signal transduction by activating MAPK family member: ERK1/2, p38, c-Jun NH2-terminal kinase (JNK), activate tyrosine kinases and inhibit tyrosine phosphatases and also mediates epidermal growth factor (EGF)-induced phosphorylation of its receptor and phospholipase C, platelet-derived growth factor (PDGF) stimulation of signal transducers and activators of transcription (STATs) (Cai *et al.*, 2002). Furthermore, some experiments suggested that ROS production due to induction of TNF-α also activates NFκB and

activator protein-1 (AP-1) pathways in various cells (Aharoni-Simon *et al.*, 2006; Kim *et al.*, 2012). ROS alter the structure and function of many redox-sensitive target proteins through modification of the thiols group of cysteine residues (Cat *et al.*, 2013; Chouchani *et al.*, 2011). Redox-sensitive cysteine undergoes reversible thiol modification in response to ROS lead to activation or inactivation of signaling protein in a range of physiological processes, such as the redox regulation of transcription factors, the regulation of tyrosine phosphatase activity (Brandes *et al*., 2009; Cat *et al.*, 2013).

Accumulation of fat in the cell might contribute to cytotoxicity neither directly or through sensitization to other agents. Metabolic dysregulation, mitochondrial impairment and oxidative stress cause hepatocyte damage and result in intensive changes in gene expression which ultimately promote to apoptosis and contributing to the inflammatory process (Marra *et al.*, 2008). In hepatocytes, cytochrome P450 system and mitochondria are the main source of ROS (Serviddio *et al.*, 2013).

2.6 Mitochondrial and peroxisomal beta (β)-oxidation

In human, ATP is produced in cytoplasm via glycolysis in the absence of oxygen as well as in the mitochondria through oxidative metabolism of fats, sugars and protein. In comparison, 95 % of ATP is generated through oxidative metabolism and yields 20 times the amount of ATP as its anaerobic counterpart (Nsiah-Sefaa & Mckenzie, 2016).

The main source of energy in human during fasting and well-fed condition is supplied by fatty acids (FAs) constitute because some organs, including heart have preference for FAs at all times (Wanders *et al.*, 2010). In liver, the uptake and *de novo* synthesis of free fatty acids (FFAs) are esterified into triglycerides, which are either liberated into circulation in the form of very low density lipoprotein (VLDL) or stored in hepatocytes as triglyceride vacuoles. On the other hand, FFAs that are not transformed into trigycerides via esterification will be metabolized in the liver by beta- oxidation (β-

oxidation) (Guturu & Duchini, 2012). In mammals, β-oxidation takes place in mitochondria as well as in peroxisomes (Poirier *et al.*, 2006). The two metabolic pathways that are central in the process of producing energy are mitochondrial fatty acid β-oxidation (FAO) and oxidative phosphorylation (OXPHOS) (Nsiah-Sefaa & McKenzie, 2016).

Mitochondrial fatty acid oxidation (FAO) exhibits a key role in maintaining body energy homoeostasis (Wajner & Amaral, 2016). FFAs for mitochondrial β-oxidation may derive from varied sources, including [1] the diet, [2] *de novo* synthesis, [3] release from adipose tissue, and [4] release from different intracellular sites, including the peroxisome and lysosome (Wanders *et al.*, 2010).

Relatively, mitochondria are large organelles (Rohlena *et al.*, 2012), it is the size of bacteria (2-4 μm) (Hüttemann *et al.*, 2007) and is sorrounded by double membrane (Cooper, 2000), consisting of a four-layer structure, including outer mitochondrial membrane, intermembrane space, inner mitochondrial membrane and matrix (Li *et al*., 2013). On the other hand, mammalian peroxisomes are 0.1-0.5 μm-sized single-membrane organelles (Vasko, 2016). The number of mitochondria is significantly distinct among cell types (Hüttemann *et al.*, 2007). Typically, hepatocytes are rich in mitochondria and each hepatocyte has approximately 800 mitochondria occupying about 18% of the entire liver cell volume (Vacca *et al.*, 2015), whereas peroxisomes are present in the majority of eukaryotic cells with the highest abundance in the liver and the kidney (Vasko, 2016).

Mitochondria plays a vital role in hepatocyte metabolism, as the primary site for the oxidation of fatty acids and oxidative phosphorylation (OXPHOS) (Vacca *et al.*, 2015). However, peroxisomal respiratory pathway does not coupled to oxidative phosphorylation (Schrader & Fahimi, 2006). Mitochondria catalyze β-oxidization of dietary FAs such as palmitic acid, oleic acid, and linoleic acid whereas peroxisomes catalyze the β-oxidation of a range of FAs and fatty acid derivatives that are not handled

by mitochondria, including very-long-chain FAs, pristanic acid, and the bile acid intermediates di- and trihydroxycholestanoic acid (Wanders & Waterham,2006; Wanders *et al.*, 2010).

Prior to β-oxidation in mitochondria, FAs are esterified into acyl-CoA in cytosol since it cannot directly cross the mitochondrial inner membrane. The activated acyl-CoA is transported to the mitochondrial matrix mediated by carnitine system, which composed of two acyl-transferases: the carnitine palmitoyltransferase (CPT) 1 and 2 and the carnitine acylcarnitine translocase (Bartlett & Eaton, 2004; Borgne & Demarquoy, 2012), as shown in Figure 2.2.

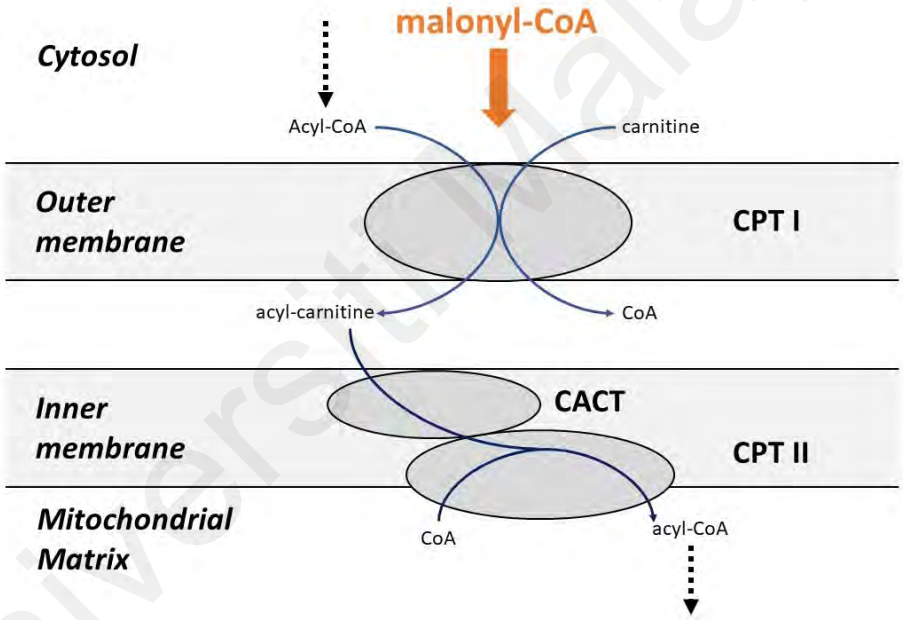

Figure 2.2: Transfer of acyl-CoAester into the mitochondrion by the combined activities of: carnitine palmitoyl transferase I (CPTI), carnitine acylcarnitine translocase (CACT) and carnitine palmitoyl transferase II (CPTII) (Source: Bartlett & Eaton, 2004)**.**

Upon entry to the inner mitochondria, acyl-CoA undergoes subsequent steps of dehydrogenation, hydration, another dehydrogenation, and finally thiolytic cleavage, with the four main enzymes involved the oxidation process in order, acyl-CoA dehydrogenase, enoyl-CoA hydratase, hydroxyacyl-CoA dehydrogenase, and ketoacyl-

CoA thiolase (Wanders *et al.*, 2010; Natarajan & Ibdah, 2018), as shown in Figure 2.3. The 4 steps order are described below:

(1) Acyl-CoA dehydrogenase (mitochondria have three such enzymes, specific for short, long, and medium acyl groups) removes two hydrogens between carbons 2 and 3, forming a trans enoyl-CoA and $FADH_2$.

(2) Water is added across the double bond by enoyl-CoA hydratase, forming 3-L-hydroxyacyl-CoA.

(3) 3-L-hydroxyacyl-CoA dehydrogenase removes hydrogens, forming 3-ketoacyl CoA, and generating NADH.

(4) The terminal acetyl-CoA group is cleaved in a thiolysis reaction with CoA catalyzed by Beta-ketothiolase (thiolase), forming a new acyl-CoA two carbons shorter than the previous one.

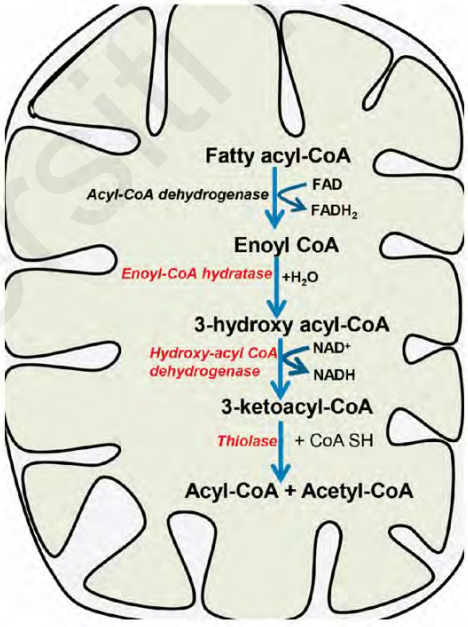

Figure 2.3: Mitochondrial fatty acid β-oxidation pathway.
Classical β-oxidtaion pathway involves dehydrogenase by acyl-CoA dehydrogenase and hydration, dehydrogenation and thiolytic cleavage is catalysed by enoyl-CoA hydratase, hydroxyl acyl-CoA dehydrogenase and thiolase activity. The straight arrows represent products and bent arrows represent the involvement of co-factor in this enzyme catalysed reaction (Source: Natarajan & Ibdah, 2018).

The resulting shortened fatty acyl-CoA ester back to the onset of the pathway and undergo the oxidation again until only two acetyl-CoA molecules remain (Nsiah-Sefaa & McKenzie, 2016). At the end of each β-oxidation process, the acyl-CoA esters can be β-oxidized to completion, and the end product acetyl-CoA then enters the mitochondrial tricarboxylic acid (TCA) cycle. NADH and $FADH_2$ are produced from TCA cycle. Meanwhile, NADH and $FADH_2$ produced by both fatty acid β-oxidation and the TCA cycle reduction of oxidized cofactors (NAD^+ and FAD) are used by the electron transport chain to generate ATP via oxidative phosphorylation (OXPHOS) system (Rolo *et al.*, 2012; Fillmore *et al.*, 2018; Nsiah-Sefaa & McKenzie, 2016), as shown in Figure 2.4.

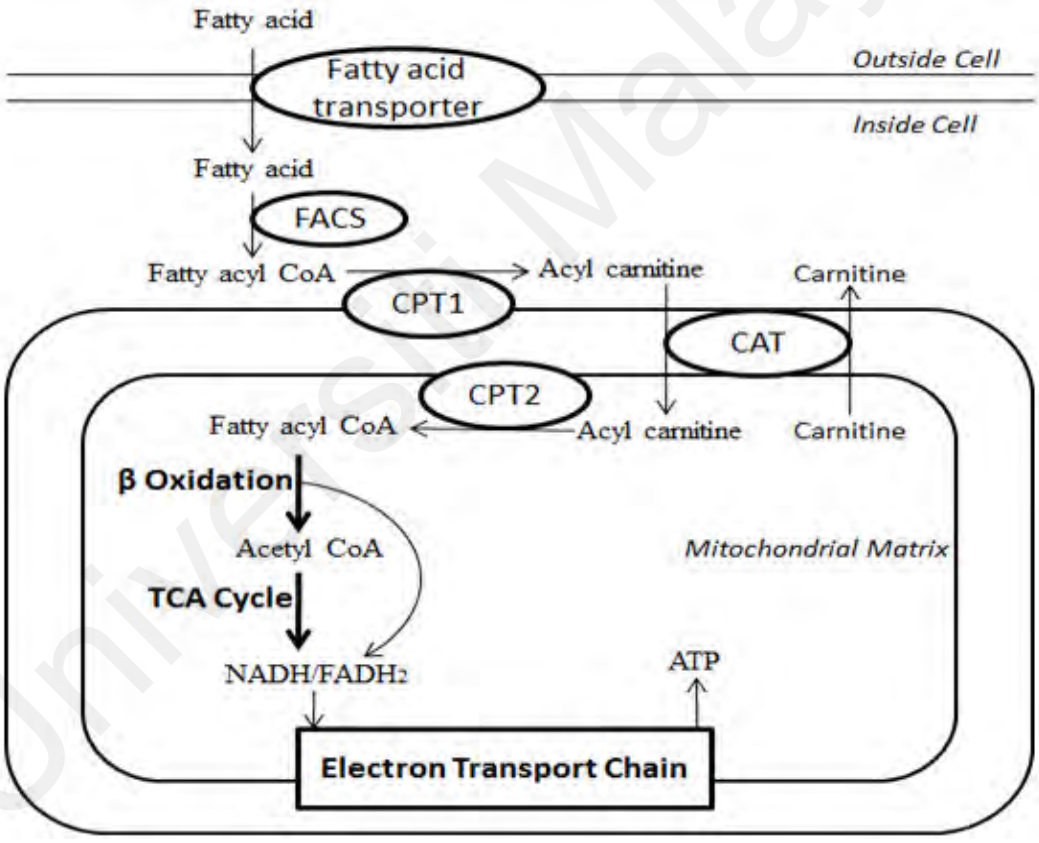

Figure 2.4: Fatty acid β-oxidation is the process by which fatty acids are broken down to produce energy.

Fatty acids primarily enter a cell via fatty acid protein transporters on the cell surface. Once inside, FACS adds a CoA group to the fatty acid. CPT1 then converts the long-chain acyl-CoA to long-chain acylcarnitine. The fatty acid moiety is transported by CAT across the inner mitochondrial membrane. CPT2 then converts the long-chain acylcarnitine back to long-chain acyl-CoA. The long-chain acyl-CoA can then enter the fatty acid β-oxidation pathway, resulting in the production of one acetyl-CoA from each cycle of β-oxidation. This acetyl-CoA then enters the TCA cycle. The NADH and $FADH_2$ produced by both β-oxidation and the TCA cycle are used by the electron transport chain to produce ATP (Source: Fillmore *et al.*, 2018).

Likewise, β-oxidation in peroxisomes begins with dehydrogenated of activated fatty acid (acylCoA), followed by hydration of double bond, and then dehydrogenated and finally cleaved (Borgne & Demarquoy, 2012). But, the enzymes involved in the peroxisomal beta-oxidation are different: The first step in peroxisomal β-oxidation is catalyzed by acyl-CoA oxidase (ACOX), followed by reactions catalyzed by bifunctional enzyme and 3-ketoacyl-CoA thiolase (Vasko, 2016). Also, ABC transporter (ABCD1) is responsible for the entry of acyl CoA in peroxisomes, instead carnitine system (Borgne & Demarquoy, 2012). In comparison to mitochondria, peroxisomes are more promptly in oxidizing long-chain FAs (Koek *et al.*, 2011), > 22 carbon atoms (C22), the branched fatty acids, some prostaglandins and leukotrienes (Borgne & Demarquoy, 2012). Peroxisomes are unable to fully degrade FAs, thus, the end product of peroxisomes, chain-shortened FAs either as free FAs or as carnitine ester, are then taken up by mitochondria for full oxidation to CO_2 and H_2O (Wanders & Waterham,2006; Wanders *et al.*, 2010; Borgne & Demarquoy, 2012). Furthermore, peroxisomal fatty acid oxidation does not involve in generating ATP because it does not integrate with oxidative phosphorylation, resulting in most of the energy is released as heat (Borgne & Demarquoy, 2012; Vasko, 2016). Another difference between mitochondria and peroxisome is the production of ROS. Superoxide ions are generated at the complex 1 in electron transport chain of mitochondria via oxidative phosphorylation, whereas H_2O_2 is released in peroxisomal metabolism (Borgne & Demarquoy, 2012). H_2O_2 is promptly converted to the highly hydroxyl radical (Koek *et al.*, 2011). Superoxide generated by mitochondria is converted to H_2O_2 mediated by SOD, successively, H_2O_2 is either converted to water by GPx or CAT, or undergo Fenton reaction to become OH^- in the presence of divalent cations such as iron (Paradies et al., 2014).

In liver, fatty acid oxidation (FAO) also occurs in endoplasmic reticulum via omega-oxidation (ω-oxidation) (Wei *et al.*, 2008) by cytochrome P450 system, which is

mainly for detoxification of endogenous and exogenous compounds, however, it become more predominant when defective β-oxidation and/or during periods of increased influx of fatty acids into the liver (Rolo *et al.*, 2012). Recent study performed on patient with simple steatosis suggested P450 system are involved in the development of NAFLD because a group of cytochrome P450 family proteins, including CYP2E1, CYP4A11, and CYP2C9, are linked with lipid droplets, and up-regulated in fatty liver (Su *et al.*, 2014). Microsomal CYP4A involved in metabolism of long chain and very long chain fatty acids and produced dicarboxylic acids, which is substrate for peroxisomal β-oxidation (Pacana & Sanyal, 2012). FFA induces the activation of CYP2E1 and CYP4A isoforms, caused an increase in ROS generation and uncoupling mitochondrial respiration (Varela-Rey, 2009). In humans and animals with NAFLD, activity and expression of CYP2E1 is increased, and this elevation correspond to the severity of NAFLD (Gracía-Ruiz *et al.*, 2015).

To sum up, the increased load of fatty acids in the hepatocytes up-regulate activity of both mitochondrial β-oxidation and in cytochrome P4504A and cytochrome P4502E1 levels, thereafter increase ROS generation. The increased mitochondrial oxidative stress trigger lipid peroxidation, cytokine production, and Fas ligand induction that resulted in second hit: progression of steatosis to steatohepatitis and fibrosis (Kumar *et al.*, 2013).

2.6.1 Mitochondrial oxidative phosphorylation

Cell respiration occurs in the mitochondria. Generation of ROS mainly occurs at the electron transport chain (ETC), also known as respiratory chain (Kalogeris *et al.*, 2014), located on the inner mitochondrial membrane during the process of oxidative phosphorylation (OXPHOS) (Li *et al.*, 2013) (Figure 2.5). Mitochondria uses oxygen and glucose, fatty acids, amino acids to perform oxidative phosphorylation, which is a cellular process that to generate cell's main energy source - adenosine triphosphate (ATP) (Li *et*

al., 2013; Wen *et al.*, 2013). The mitochondrial ETC complexes comprises five macromolecular protein complexes, named as complex I (NADH dehydrogenase (ubiquinone)), complex II (succinate dehydrogenase), complex III (ubiquinol-cytochrome c reductase), complex IV (cytochrome c oxidase), and complex V (ATP synthase) (Rohlena *et al.*, 2012; Li *et al.*, 2013). Electrons derived from nicotine adenine dinucleotide (NADH) at complex I and flavin adenine dinucleotide (FADH2) at complex II pass through ETC and ultimately reduce O_2 to water at complex IV. Meanwhile, mitochondrial membrane potential is generated by pumping positively charged protons (H+) from mitochondrial matrix into the intermembrane, caused an increased negative charge in the mitochondrial matrix and upregulated positive charges in the intermembrane space, thus generating a mitochondrial potential across the inner mitochondrial membrane (Li *et al.*, 2013). This proton-motive force allows complex V (ATP synthase) to generate ATP from adenosine diphosphate (ADP) and inorganic phosphate when protons re-enter the mitochondrial matrix through the complex V enzyme (Hüttemenn *et al.*, 2007; Li *et al.*, 2013).

During oxidative phosphorylation, electrons leak from complex I and complex III when electron transport in mitochondria, and react to oxygen that leads to partial reduction of oxygen to form superoxide ($O_2^{\cdot -}$) (Keane *et al.*, 2011; Li *et al.*, 2013). It has been estimated that 1 % to 2 % of O_2 consumed by mitochondria is converted to $O_2^{\cdot -}$ under normal physiological condition (West *et al.*, 2011; Nassir & Ibdah, 2014; Chen *et al.*, 2015). $O_2^{\cdot -}$ from complex 1 released into the mitochondrial matrix, whereas complex III leaks $O_2^{\cdot -}$ into both the intermembrane space and mitochondrial matrix (Li *et al.*, 2013; West *et al.*, 2011). Superoxide can then cross the outer mitochondria membrane via a voltage-dependent anion-selective channel (VDAC) or can be converted into hydrogen peroxide (H_2O_2) in the matrix by superoxide dismutase 2 (SOD2) or in the intermembrane space by superoxide dismutase 1 (SOD1) (West *et al.*, 2011).

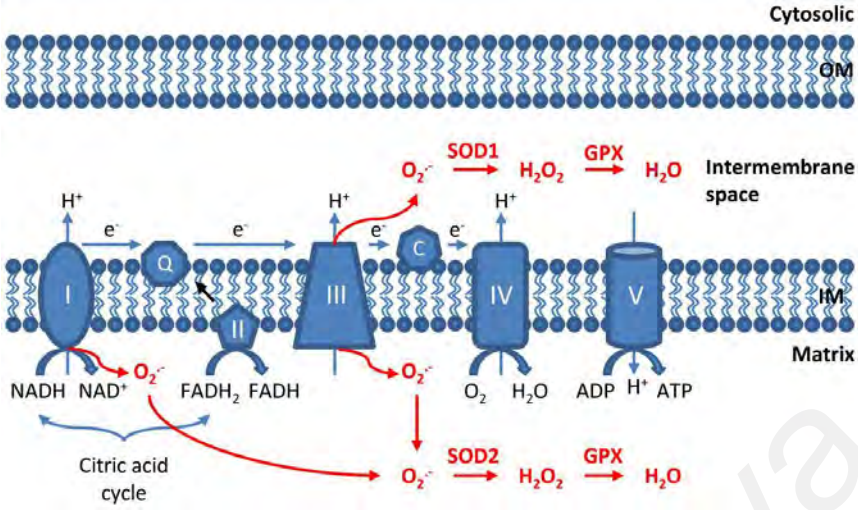

Figure 2.5: Production and disposal of mitochondrial ROS.

Electrons (e^-) donated from NADH and $FADH_2$ pass through the electron transport chain and ultimately reduce O_2 to form H_2O at complex IV. MtROS are produced from the leakage of e^- to form superoxide ($O_2{\cdot}-$) at complex I and complex III. $O_2{\cdot}-$ is produced within matrix at complex I, whereas at complex III $O_2{\cdot}-$ is released towards both the matrix and the intermembrane space. Once generated, $O_2{\cdot}-$ is dismutated to H_2O_2 by superoxide dismutase 1 (SOD1) in the intermembrane space and by SOD2 in the matrix. Afterwards, H_2O_2 is fully reduced to water by glutathione peroxidase (GPX). Both $O_2{\cdot}-$ and H_2O_2 produced in this process are considered as mtROS. OM: outer membrane; IM: inner membrane (Source from Li *et al.*, 2013).

2.6.2 Mitochondrial dysfunction

Under normal physiologic condition, liver disposes of FFAs through either oxidation or secretion as VLDL (Mashek, 2013). Despite that, mitochondrial β-oxidation is the principal oxidative pathway for the disposal of fatty acids (Rolo *et al.*, 2012; Mashek, 2013). However, this cascade of event is disrupted in NAFLD. Furthermore, studies reported that ATP level decreased in liver of NAFLD (Serviddo *et al.*, 2008; Jiang *et al.*, 2011). Loss of efficiency in the electron transport chain and reductions in the synthesis of ATP, is known to be the feature of mitochondrial dysfunction (Nicolson, 2014). Emerging evidence indicated that that mitochondrial dysfunction plays a key role in the physiopathology of NAFLD, although the mechanism(s) underlying this dysfunction are still unclear (Petrosilo *et al.*, 2007; Paradies *et al.*, 2014; Yu *et al.*, 2016). Mitochondrial dysfunction leads to impairment of fat homeostasis in liver (Fromenty *et*

al., 2004; Nassir & Ibdah, 2014; Paradies *et al.*, 2014), also over-production of ROS (Petrosillo *et al.*, 2007)

Excessive of FFAs in liver contribute to increased mitochondrial β-oxidation, which results in excess electrons are provided to the mitochondrial respiratory chain, subsequently leading to excess electrons are conveyed to oxygen. Hence, oxygen molecules are reduced to superoxide, in turn converted to hydrogen peroxide either by spontaneously or through superoxide dismutase (Kim *et al.*, 2008; Guturu & Duchini, 2012). The highest rate of ROS generated in high-fat or high-glucose states when the proton gradient in ETC is high and ATP demand is low (Kim *et al.*, 2008; Rolo *et al.*, 2012).

Mitochondria could also be the major target of ROS attack even though it is a key source of ROS (Petrosillo *et al.*, 2007). Over-production of ROS induce lipid peroxidation of mitochondrial membranes, which result in damaged mitochondrial function and continuous ROS generation, also trigger inflammatory cytokine overproduction and cell death (Fromentry *et al.*, 2004; Nassir & Ibdah, 2014). The loss of mitochondrial function inhibits β-oxidation of lipids, further increasing the steatosis and begin a brutal cycle (Ucar *et al.*, 2013). The presence of obesity and insulin resistance further complicate the disorder because both are the factors involved in uptake/synthesis and oxidation/export of fatty acids and accumulation of triglycerides in hepatocytes (Ucar *et al.*, 2013). Hence, mitochondria dysfunction might have important role in the induction of both "hits"(Gracía-Ruiz *et al.*, 2015). Therefore, mitochondrial dysfunction impairs fat homeostasis in liver, and leads to oxidative stress, the over-production ROS further induce lipid peroxidation, cytokine production and cell death (Takaki *et al.*, 2014).

2.7 Cellular signaling pathways

Cells react to changes in their extracellular environment either physical such as light, temperature and pressure, or chemicals that includes food, hormones and neurotransmitter (Schenk & Snaar-Jagalska, 1999) and transform (transduction) the extracellular stimuli to intracellular responses such as migration, proliferation and differentiation (Shankaran *et al.*, 2007). Cells sense the changes in their extracellular milieu via signal transduction (cell signalling) system and initiate responses (Wheeler-Jones, 2005). The cellular signalling pathways involved in NAFLD and NASH included Nuclear factor kappa B (NF-κB), JNK, p38 MAPK and STAT3 as shown in Figure 2.6.

2.7.1 Nuclear factor kappa B (NF-κB)

Nuclear factor kappa B (NF-κB) is an ubiquitous transcription factor family that is crucial central mediator in immunity and inflammatory responses by regulating gene encoding cytokines, chemokines, growth factors, cell adhesion molecules and acute phase proteins (Oeckinghaus & Ghosh, 2009; Zhang & Sun, 2015). It is also involved in regulation of expression of various genes that control cell growth, differentiation, development, apoptosis, autophagy, senescence (Morgan & Liu, 2011), redox status and tissue specific enzymes (Donato *et al.*, 2009). NF-κB pathways has at least two different ways for activation, termed canonical (classical) and non - canonical (non-classical) pathways (Rahman & McFadden, 2011), as shown in Figure 2.7.

The canonical NF-κB-activating pathway is induced by microbial products, stress, and proinflammatory cytokines, whereas, the noncanonical NF-κB-activating pathway is activated in response to B-cell activating factor, lymphotoxin β, CD40 ligand, CD27 ligand, human T-cell leukemia virus, and Epstein-Barr virus (Zhang *et al.*, 2016). NF-κB associated with a number of diseases related to acute and chronic inflammation and proliferation (Rodriguez-Porcel *et al.*, 2002).

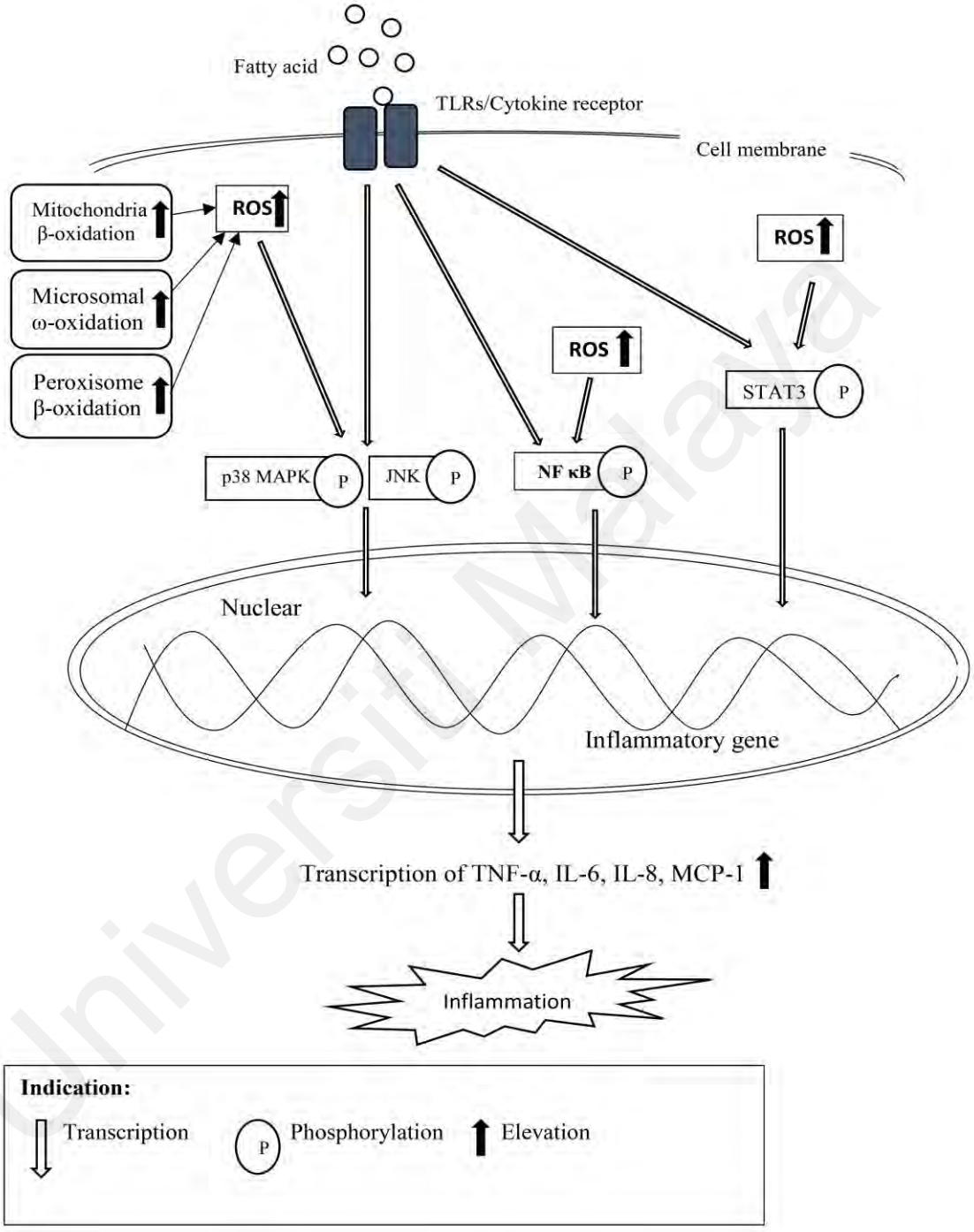

Figure 2.6: Schematic representation of the signalling pathways involved in NAFLD and NASH.

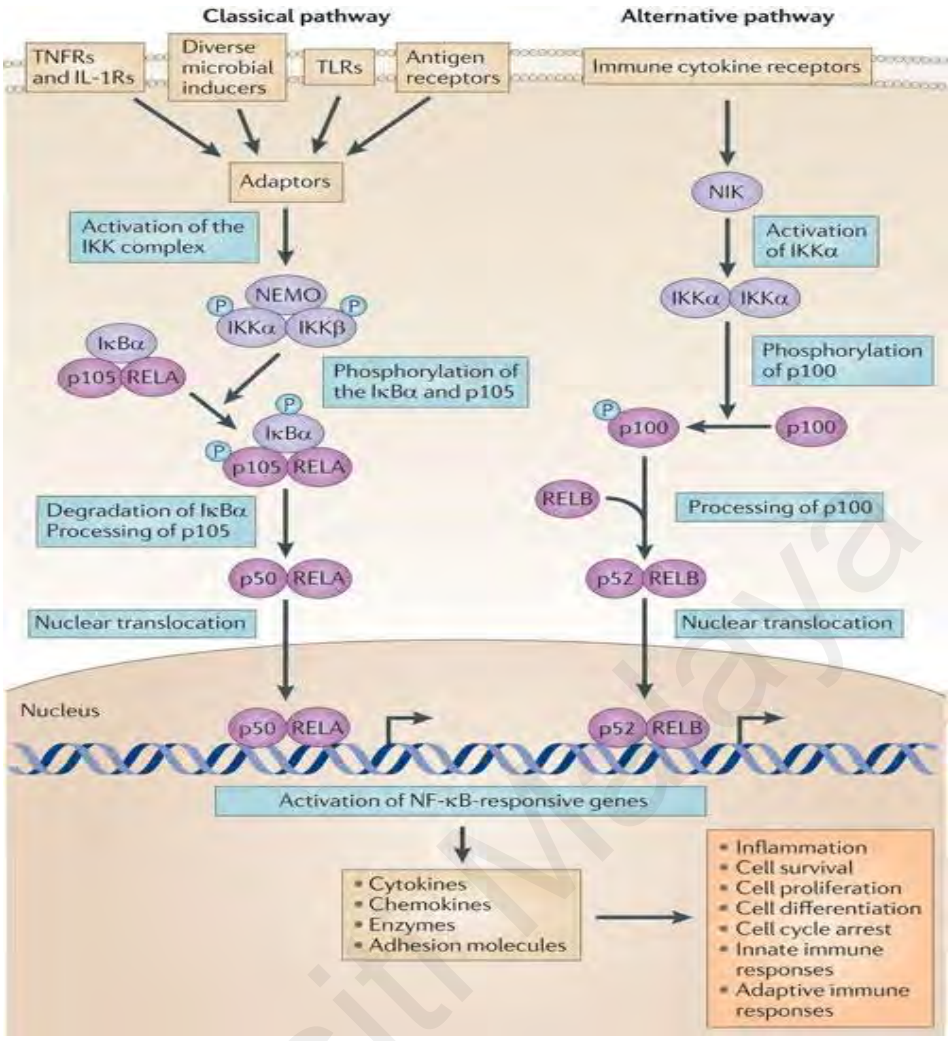

Figure 2.7: The classical and alternative NF-κB signalling pathways (Source from Rahman & McFadden, 2011).

In mammals, the NF-κB family of transcription factors contains five members: NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB, and c-Rel (Caamano & Hunter, 2002) that bind as a homodimer or heterodimer to 10 base pairs κB site (Morgan & Liu, 2011). NF-κB1 and NF-κB2 are synthesized as large polypeptides that are post-translationally cleaved to generate the DNA binding subunits p50 and p52, respectively (Caamano & Hunter, 2002). All family members share a highly conserved Rel homology domain (RHD; ~300 amino acids) responsible for DNA binding, a dimerization domain (Ahn & Aggarwal, 2005). RelA, RelB and c-Rel carry transcription activation domain (TAD) at the C-terminus that serves to positively regulate gene expression (Nishikori,

2005; Morgan & Liu, 2011). In contrast, both NF-κB1 (p105/p50) and NF-κB2 (p100/p52) have C-terminal ankyrin repeat that restrain DNA binding. Thus, they only transcribe when are paired with one of the Rel proteins (Morgan & Liu, 2011). All members are expressed ubiquitously with exception of RelB and c-Rel, which only found in lymphoid and hematopoietic cells, respectively (Hajra *et al.,* 2000). Typically, NF-κB is presented as a heterodimer of the p65/RelA and p50 or p52 subunits in the cytoplasm of cells. The p65/p50 heterodimer is considered to be the classic form of NF-κB (Sonis, 2002), it is ubiquitously expressed and comprises the most common inducible NF-κB binding activity (Caamano & Hunter, 2002).

NF-κB remain inactive in cytoplasm under physiological state (Rahman & McFadden, 2011). In unstimulating state, p65/p50 heterodimer exists as an inactive cytoplasmic complex and bound to ankyrin-riched region of inhibitory proteins, collectively termed as IκB (Caamano & Hunter, 2002). NF-κB signaling pathway can be activated by several of mechanisms due to the various ways of combination of members to form heterodimer and homodimer resulted in varies affinity to κB site in distinct DNA sequences (Morgan & Liu, 2011).

Activation of NFκB promotes IκB kinase (IKK) to phosphorylate IκB at position Serine-32 and Serine-36 (Caamano & Hunter, 2002; Gareus *et al.,* 2008), leading to their ubiquitination and consecutive proteasomal degradation (Berg *et al.,* 2001; Gareus *et al.*, 2008). The degradation of IκB disclose the nuclear localization sequence and liberates the NF-κB from complex, results in immediate translocation of the NF-κB heterodimer to the nucleus where it binds κB motifs present in the promoters of gene targets, and regulate gene transcription such as expression of cytokines, chemokines, adhesion molecule, and antiapoptotic and antioxidative protein (Caamano & Hunter, 2002, Gareus *et al.,* 2008). IκB kinase (IKK complex) constitutes two catalytic kinases named IKKα and IKKβ, and a regulatory subunit, IKKγ (also known as NEMO)

(Morgan & Liu, 2011). NEMO plays crucial role for IKK-mediated IκB phosphorylation and activation of NF-κB pathway (Gareus *et al.*, 2008).

Fundamentally, NF-κB is stimulated in every chronic liver disease, such as alcoholic liver disease, NAFLD, viral hepatitis and biliary liver disease. It is known to play a key role in transformation from simple steatosis to steatohepatitis (Chen *et al.*, 2016). NF-κB modulates numerous main functions in hepatocytes, Kuffer cells and hepatic stellate cells (Luedde & Schwabe, 2011). It exhibits crucial function in liver inflammation and oxidative stress (Bhatia *et al.*, 2012; Lin *et al.*, 2014). *In vivo* study showed that in high fat diet induced rodents resulted in hepatic steatosis, which activated NF-κB pathways and up-regulated pro-inflammatory cytokines (Cai *et al.*, 2005). Fatty acid involved in the progression from NAFLD to NASH, and disease severity is positively correlated with elevation of free fatty acid (Nehra *et al.*, 2001; Diraison *et al.*, 2003; Gentile *et al.*, 2011). Saturated fatty acid is reported to be more detrimental than unsaturated fatty acid (Gentile *et al.*, 2011), which induced liver cell inflammation/toxicity may due to saturated fatty acids trigger NF-κB in liver cells (Gentile *et al.*, 2011).

2.7.2 Mitogen activated protein kinase (MAPK)

Mitogen-activated protein kinase (MAPK) signal transduction pathways is broadly existed in mammalian cells (Zeng *et al.*, 2014) and are among the most widespread mechanisms of eukaryotic cell regulation, which are activated by various stimuli comprise of hormones, growth factors, transforming growth factor (TGF)-β-related polypeptides, inflammatory cytokines (e.g tumour necrosis factor (TNF) family) and environmental stresses such as osmotic shock, ionizing radiation and ischemic injury) (Kyriakis & Avruch, 2001). Besides, MAPK pathway is also activated by oxidative stress caused by ROS (Son *et al.*, 2011).

MAPK consists of a superfamily of serine/threonine protein kinases that are critical for various cellular functions in different cell types (Kulisz *at el.*, 2002). The four major MAPK pathways are extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPK, c-Jun N-terminal kinase or stress-activated protein kinase, and ERK5 (Figure 2.8) (Griendling *et al.*, 2000; Manna & Stocco, 2011; Morrison, 2012).

Extracellular stimuli induce MAPK cascade that contained three clear core unit mediated by specific upstream MAP kinase kinase kinases (MAPKKKs) and MAP kinase kinases (MAPKKs) (Maroni *et al.*, 2004; Morrison, 2012) (Figure 2.9).

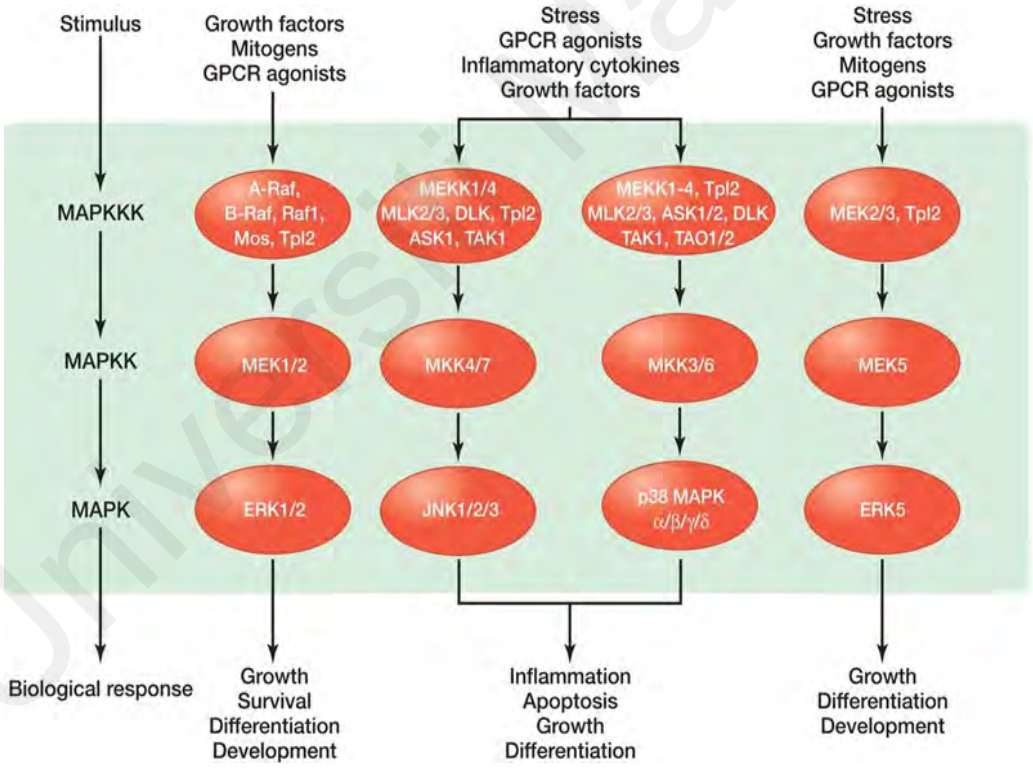

Figure 2.8: Mitogen-activated protein kinase (MAPK) pathway
(Source from Morrison, 2012).

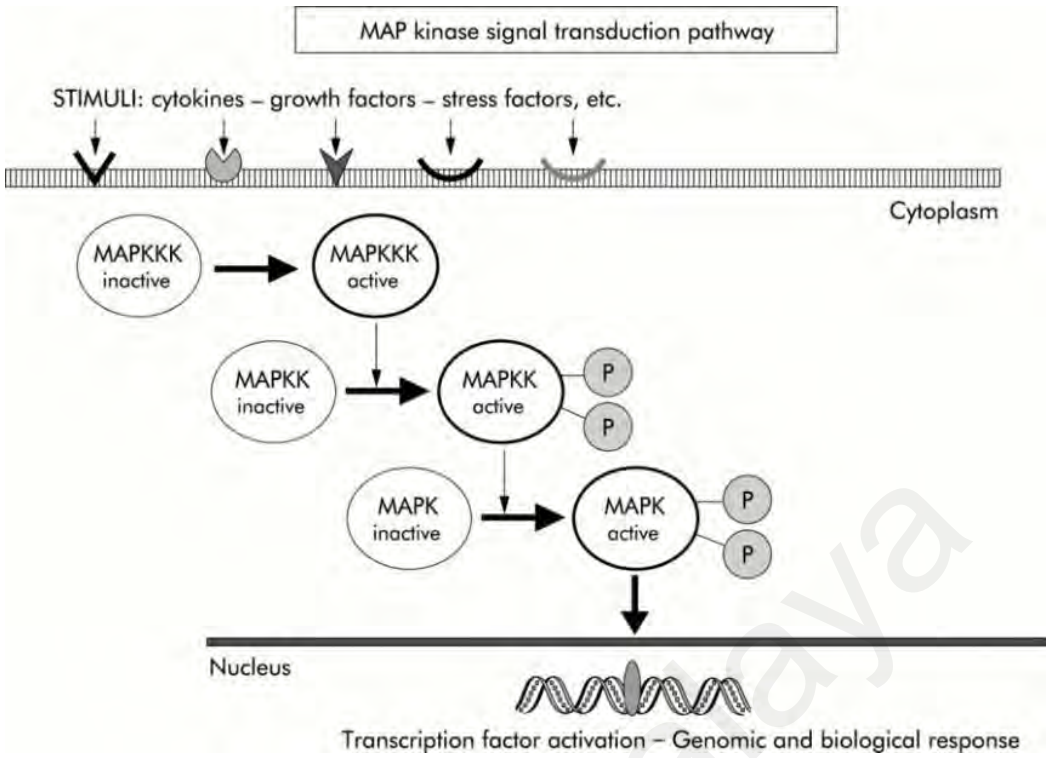

Figure 2.9: Different extracellular stimuli can activate the family of mitogen activated protein (MAP) kinases after receptor-ligand interactions.
Members of this family activate each other by adding phosphate groups to serine/threonine amino acids. MAPK, MAP kinase; MAPKK, MAP kinase kinase; MAPKKK, MAP kinase kinase kinase (Source from Hommes *et al.*, 2003).

Activation of MEKs is controlled by phosphorylation of MAPKKKs (Hommes *et al.*, 2003). MAPK is activated through a cascade of phosphorylation on threonine (T) and tyrosine (Y) residues within a T-X-Y phosphorylation motif and negatively regulated by MAPK phosphatases (MKPs) (Son *et al.*, 2011), where X is glutamate in ERK, proline or glycine in JNK or p38 MAPK (Moosavi *et al.*, 2017).

MAPK highly specifically interact to its immediate upstream kinase (MAPKK): for instance, MAP/ERK kinase (MEK) 1 and 2 only phosphorylates ERK (p42/p44) MAP kinases; p38 MAP kinase is selectively activated by MAP kinase kinases (MKK) 3 and 6, whereas JNK is activated by MKK7 and MKK4 in most conditions, however MKK4 can sometimes activate p38 MAP kinase when over expressed (Maroni *et al.*, 2004) and MEK5 phosphorylates and activates ERK5 (Nishimoto & Nishida, 2006).

Activation of MAPK through a sequential transduction of biological signal from cell membrane to the nucleus plays essential role in many transduction pathways, including cell growth, differentiation, proliferation, migration, apoptosis, and the regulation of various transcription factors and gene expression (Son *et al.*, 2011).

The ERKs play a crucial role in the cellular response induced by growth factor stimulation, whereas JNK and p38 are related with stress and inflammation (Zeng *et al.*, 2014). In the liver, over-activation of JNK signaling specifically has been recognized as a general mechanism underlying hepatocyte death including that from oxidant stress, tumor necrosis factor (TNF), ischemia reperfusion injury and fatty liver disease. JNK over-expression is also mechanistically associated to both hepatic steatosis and injury in nonalcoholic fatty liver injury (Schattenberg & Czaja, 2014). Sinha-Hikim *et al.* (2011) reported that hepatic steatosis in high-fat diet induced obese mice resulted in an increase in both phospho-JNK and phospho-p38MAPK levels than the normal control group and significantly reduced after antioxidants treatment. In addition, activation of JNK pathway in NAFLD rat model was found to be associated with inflammatory cytokine, free fatty acids, oxidative stress, which contribute to insulin resistance, hepatocyte fat accumulation and cell injury (Kodama & Brenner, 2009; Zeng *et al.*, 2014).

2.7.3 Signal transducers and activators of transcription (STAT) proteins

Signal transducers and activators of transcription (STAT) proteins consist of a big family of transcription with a dual role as signal transduction and transcription activators (Gkouveris *et al.*, 2015) that mediate intracellular signalling that is generally generated at cell surface receptors and thereby transmit to the nucleus (Siveen *et al.*, 2014). STAT family of transcription factors comprise seven proteins: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (Carpenter & Lo, 2014). Basically, STAT3 activation is detected in rodents' models of liver injury and human liver diseases (Wang *et al.*, 2011).

Furthermore, Min *et al.* (2015) reported that gp130-STAT3 pathway is activated and increased expression in either NAFLD or NASH, which is associated with the level of IL-6 in this population.

STAT3 is plentifully expressed in most tissues (Cong *et al.*, 2012) and is involved in variety of biological functions. It plays important role in normal development, acute response, chronic inflammation, autoimmunity, metabolism and cancer progression (Yang & Rincon, 2016) via regulating transcription genes, controlling cell survival and proliferation, regulating expression of anti - apoptotic, pro - proliferation and immune response genes (Gkouveris *et al.*, 2015). In normal physiology, STAT3 activation drives a well-organized gene regulation schedule (Gkouveris *et al.*, 2015). It is activated by various cytokines and growth factors (Herrmann *et al.*, 2003), such as IL-6 and epidermal growth factor family members, as well as hepatocyte growth factor (He & Karin, 2011). Furthermore, STAT3 in hepatocytes have been shown to be stimulated by various cytokines, including interleukin-6 (IL-6), the IL-6 family of cytokines (such as leukemia inhibitory factor, ciliary neurotrophic factor, oncostatin M, cardiotrophin-1, interleukin (IL)-11, and IL-22 (Wang *et al.*, 2011). Furthermore, STAT3 can also be activated in response to ROS accumulation, just as JNK (He & Karin, 2011).

In response to stimulation, STAT3 is phosphorylated at tyrosine 705 and serine 727 residues (as shown in Figure 2.10) (Gkouveris *et al.*, 2015). In general, phosphosylation of STAT3 at tyrosine 705 is mediated by Janus kinases (JAKs) (He & Karin, 2011); whereas serine 727 phosphorylation is mediated by ERK1, ERK2, p38, JNK and MAP kinases (Huang *et al.*, 2014; Yang & Rincon, 2016). Phosphorylation of STAT3 at tyrosine 705 is commonly believed to be essential for STAT3's transcriptional activity (Huang *et al.*, 2014). Upon activation, phosphorylated STAT3 molecules form dimers and translocate into the nucleus where it binds to enhancer sequences of target genes (Herrmann *et al.*, 2003; Gkouveris *et al.*, 2015). Min *et al.*, (2015) carried out a

study on activation of the GP 130-STAT3 axis and its potential implications in non-alcoholic fatty liver indicated that the STAT3 pathways is activated in NAFLD and can

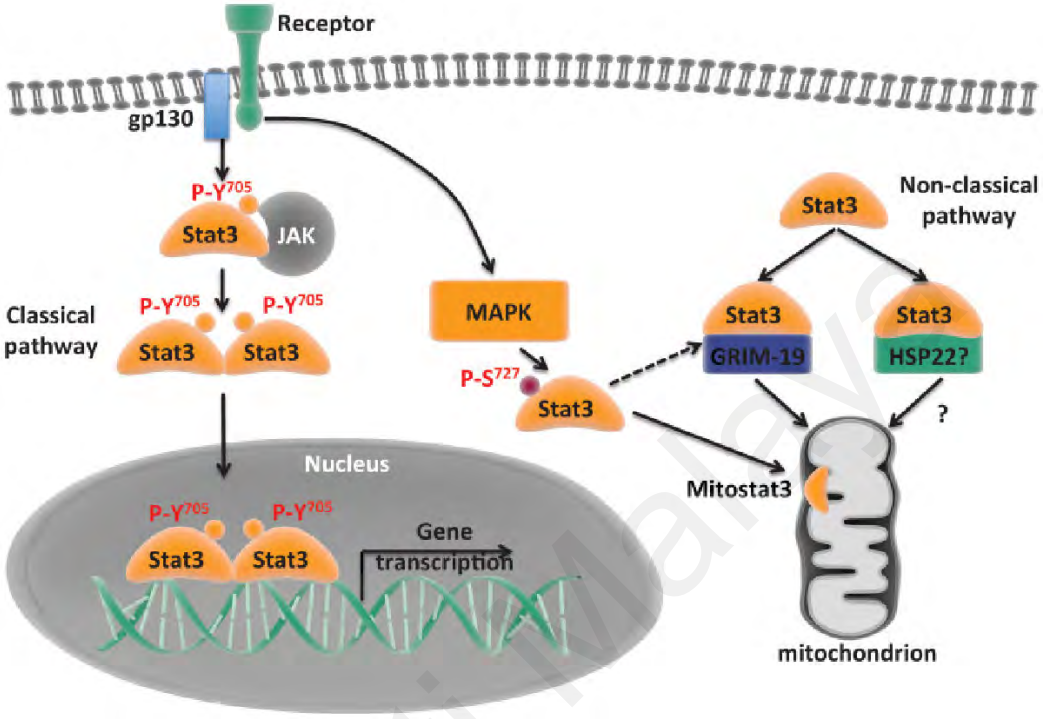

Figure 2.10: Classical and non-classical pathways of STAT3.
Classical pathway where STAT3 translocates to the nucleus and mediates gene transcription. Non-classical pathway where STAT3 is recruited to mitochondria and regulates functions alternative to transcription (Source from Yang & Rincon, 2016).

worsen insulin resistance. Furthermore, phosphorylated STAT3 is dramatically increased in patients with NASH, compared to NAFLD patients (Handa *et al.*, 2107).

2.8 Cytokines involved in NAFLD

Cytokines are small soluble proteins generated by a broad range of cells in the body, including majority types of liver cells, and it exert a specific intercellular communication between cells (Zhang & An, 2007; Braunersreuther *et al.*, 2012). They consist of several subfamilies, including interferons, interleukins, tumor necrosis factors (TNF), transforming growth factors (TGF), colony-stimulating factors, and chemokines (Braunersreuther *et al.*, 2012). Cytokines play physiological roles via mediating

processes such as growth, differentiation, hematopoiesis, as well as several inflammatory and immune responses (Suyavaran *et al.*, 2015). In general, there are pro- and anti-inflammatory cytokines (Cavaillon, 2001). The crucial pro - inflammatory cytokines are TNF-α, interleukin (IL)-1, IL-6 and interferon; whereas the main anti - inflammatory cytokines are IL-4 and IL-10 (Zahran *et al.*, 2013).

In addition, cytokines are known as autocrine if they exert influence on the cells that secrete them, if act on adjacent cells, it is called paracrine, and endocrine if exert influence on distant cells (Zhang & An, 2007; Duque & Descoteaux, 2014). Majority of cytokines are short-lived and show autocrine and paracrine actions (Duque & Descoteaux, 2014). Assorted cell types secrete the same cytokine or for a single cytokine to act on several different cell types, which is called as pleiotropy; cytokines action is often redundant, meaning different cytokines trigger the same function. They are usually produced in a cascade, as one cytokine stimulates its target cells to produce more cytokines (Zhang & An, 2007; Oliveira *et al.*, 2011). Cytokines can also act synergistically, meaning two or more cytokines acting together or antagonistically, indicating cytokines causing opposing activities (Zhang & An, 2007).

During normal physiological status, cytokines present in minimal level in hepatic circulation, and they are essential for hepatic homeostasis (Suyavaran *et al.*, 2015). In general, there is a balance between pro-inflammatory and anti-inflammatory cytokines (Das & Balakrishnan, 2011) because it is vital for maintenance of health (McBride & McBride, 1998). A balance between pro-inflammatory and anti-inflammatory cytokines seems to have a significant role in systemic, local metabolic and inflammatory processes involved in the development of NAFLD and insulin resistance (Stojsavljević *et al.*, 2014). Cytokines have been observed to responsible for mediating the inflammatory progression of NAFLD as characterized by apoptotic and necrotic lesion in liver leading to fibrosis (Suyavaran *et al.*, 2015). Circulating of pro-

inflammatory such as TNF-α and IL-6 are recognized to be the most significant factors in causing and maintaining insulin and closely associated to the development of NAFLD and other metabolic disorders (Chen *et al.*, 2014).

2.8.1 Tumour necrosis factor-alpha (TNF-α)

TNF-α is an important pro-inflammatory factor that is involved in systemic inflammation as well as stimulate acute inflammation (Liang *et al.*, 2015). TNF-α is one of the cytokines that have been studied in the pathogenesis of NAFLD (Stojsavljević *et al.*, 2014). It is a pro-inflammatory cytokine produced by a number of cell types such as neutrophils, macrophages, T and B lymphocytes, endothelial cells, mast cells, fibroblasts, hepatocytes and Kupffer cells in liver are also the principal contributors of TNF-α (Stojsavljević *et al.*, 2014; Suyavaran *et al.*, 2015). TNF-α is responsible for the development and progression of NAFLD (Gentile *et al.*, 2011) by playing a central role in the initiation of inflammatory cascade and its progression from steatosis to steatohepatitis (Suyavaran *et al.*, 2015). Human study of TNF-α and NAFLD has shown that severity of NAFLD is associated with high systemic level of TNF-α in obese patients (Paredes-Turrubiarte *et al.*, 2016).

Expression of TNF-α is strongly triggered in response to high fat diet (Chen *et al.*, 2014). Furthermore, increased levels of FFA can also induce TNF-α expression within the hepatocytes (Xirouchakis *et al.*, 2009). The likely sources of hepatic TNF-α are hepatocytes and Kupffer cells (Paradies *et al.*, 2014). Zahran *et al.* (2013) performed a clinical study on NAFLD subjects reported that TNF-α is significantly higher in NAFLD patients compared to control subjects. Suppression of TNF α signalling protect animals from dietary- and genetically-induced NASH via restraining hepatic steatosis without a considerable effect on weight gain (Gentile *et al.*, 2011; Chen *et al.*, 2014). Obesity-induced mice by high-fat diet revealed that the expression of TNF-α was inhibited and

the levels correlated with a significant reduction in the steatosis after treatment of thalidomide, an anti-TNF-α drug (Pinto *et al.*, 2010). In the liver, TNF-α activates stress-activated protein kinase (JNK), and inhibitory kappa B kinase beta (IKKβ). JNK activation further induces TNF-α, forming an autocrine/paracrine loop which potentiates insulin resistance (Xirouchakis *et al.*, 2009). Elevation of TNF-α may induce insulin resistance (IR), which then may further promote inflammation by impairing the anti-inflammatory effect of insulin (Plomgaard *et al.*, 2005; Seo *et al.*, 2013). TNF-α not only promotes insulin resistance, but also mediates cholesterol and TG metabolism (Wang *et al.*, 2014). TNF-α is known to attract inflammatory leukocytes to the liver and to promote the expression of sterol regulatory element binding protein-1c, which regulates *de novo* lipogenesis and is more highly expressed in NAFLD (Endo *et al.*, 2007; Seo *et al.*, 2013). Furthermore, TNF-α expression lead to harmful pro-atherogenic pathways partially through the reduction of high density lipoprotein (HDL) cholesterol, elevated expression of cholesterogenic genes, accompanied by an increase in potentially harmful precholesterol metabolites, and suppression of cholesterol elimination, also stimulates hepatic fatty acid synthesis, increases serum triglyceride levels and stimulates VLDL production from liver, as well as induce both hepatocyte cell death and hepatocyte proliferation (Das & Balakrishnan, 2011).

2.8.2 Interleukin-6 (IL-6)

IL-6 is a pro-inflammatory pleiotropic cytokine (Hijona *et al.*, 2010) produced by adipocytes, hepatocytes, immune and endothelial cells (Stojsavljević *et al.*, 2014). IL-6 involves in regulation of immune responses, acute phase reactions and hematopoiesis, and may play a central role ranging from inflammation to host defense to tissue injury (Das & Balakrishnan., 2011). In fact, IL-6 is key element in the acute phase response, mediating the synthesis of several acute phase proteins, for instance C-reactive protein

(CRP) and serum amyloid A (SAA) (Chen *et al.*, 2014). It is the second important pro-inflammatory cytokine that plays a role in the metabolic syndrome (Xirouchakis *et al.*, 2009).

Similar to TNF-α, high fat diet strongly induced expression of IL-6 in liver (Chen *et al.*, 2014). Also, accumulated FFAs in hepatocytes activate IKK-β and NF-κB, which lead to expression of various proinflammatory cytokines, including IL-6 (Stojsavljević *et al.*, 2014). Animal model and patients with NAFLD were found to have higher serum IL-6 level, which is positively correlated with IL-6 expression in hepatocytes and the severity of NAFLD (Wieckowska *et al*., 2008; Chen *et al.*, 2014, Wang *et al*., 2014). Besides playing a part in NAFLD, IL-6 also plays a role in stimulation of hepatic lipogenesis, associated with obesity and insulin resistance (Hijona *et al.*, 2010), impairs insulin signalling, and can alter insulin sensitivity by triggering different key steps in the insulin signalling pathway (Das & Balakrishnan, 2011). It is a prototypic glycoprotein 130 (gp130) cytokine and stimulates Janus-activated kinase (JAK)-signal transduction and activator of transcription 3 (JAK-STAT3), Src-Ras, and the phosphatidylinositol 3-kinase-Akt pathways via gp130 (Min *et al.*, 2015).

Contribution of IL-6 in the liver pathology is indeed complex, and its role in the development of NAFLD remains unclear and controversial (Braunersreuther *et al.*, 2012). Experiment performed by Sánchez-Garrido *et al.* (2009) showed that IL-6 ameliorated the mitochondria lipid disturbance in hepatocytes isolated from steatosis animals, which had increased liver fat content due to choline deficient diets. Conversely, Mas *et al.* (2009) reported, biochemical, histological and molecular analyses indicated that IL-6 contributes to the inflammation associated with the development of NASH in mice fed with methionine and choline-deficient diet-induced liver while IL-6 deficiency attenuates of NASH caused by methionine and choline-deficient diet. Mohamed *et al.* (2014) reported that IL-6 was found to be higher in NAFLD patients compared to healthy controls but the

difference was not significant. On the other hand, Wieckowska *et al.* (2008) showed a positive correlation between IL-6 hepatic expression and the severity of NAFLD.

2.8.3 Chemokines

A number of cell types, such as endothelial cells, smooth muscle cells, leukocytes, hepatocytes and stellate cells secrete chemokines, which are small heparin-binding proteins recognized to trigger mainly leukocyte trafficking, growth, and activation in inflammatory sites (Braunersreuther *et al.*, 2012). Majority of chemokines produced in the human body are inflammatory chemokines (Nedoszytko *et al.*, 2014). Chemokines are a family of cytokines that stimulate leukocyte chemotaxis and play crucial roles in the progression of systemic inflammation (Kitade *et al.*, 2017).

2.8.3.1 Monocyte chemoattractant protein-1 (MCP-1)

Monocyte chemoattractant protein-1 (MCP-1), also known as CCL2 (Xu *et al.*, 2015), is a small cytokine belonging to CC-chemokine family that is secreted by abundant of cell types but mainly by macrophages and endothelial cells and is a potent chemotactic factor for monocytes (Leach *et al.*, 2014). In an animal study carried out by Obstfeld *et al.* (2010), high-fat diet caused an increased in MCP-1 expression in hepatocytes that lead to recruitment of myeloid cells, such as monocyte, macrophage and resulted in hepatic steatosis. Furthermore, investigation performed by Kirovski *et al.* (2011) showed that increased MCP-1 serum levels, and elevated hepatic MCP-1 expression were detected in the state of absence of significant hepatic inflammation during early phase of hepatic steatosis, which leads to the progression to NASH from simple steatosis.

2.8.3.2 Interleukin-8 (IL-8)

IL-8, also recognized as CXCL8, is one of the most broadly studied chemokines and is a crucial inflammatory mediator (Turner *et al.*, 2014). It is a monomeric polypeptide and a member of cysteine X cysteine (CXC) chemokine family (Kim *et al.*, 2012). IL-8 is produced by various cell types such as adipocytes (Kim *et al.*, 2012), endothelial cells (Reinsberg *et al.*, 2000; Braunersreuther *et al.*, 2012), fibroblasts, and also monocytes/macrophages (Reinsberg *et al.*, 2000). In addition, Joshi-Batve *et al.* (2007) showed that exposing free fatty acid to hepatocytes cell lines, including HepG2 cells, rat primary hepatocytes, and human primary hepatocytes led to IL-8 secretion through activation of NF-κB and c-Jun N-terminal kinase/activator protein-1. Its main function is to recruit neutrophil to the inflamed tissues (Braunersreuther *et al.*, 2012) and has a role in induction and development of inflammatory processes (Cengiz *et al.*, 2014). Hepatocytes loaded with lipid may stimulate production of IL-8 (Joshi-Barve *et al.*, 2007; Perito *et al.*, 2017). Serum IL-8 was reported to be higher in NAFLD patients, as compared with both obese and non-obese controls (Jarrar *et al.*, 2008). Moreover, NASH patients were detected to have increased plasma IL-8 level (Cengiz *et al.*, 2014), which is significantly higher in comparison to hepatosteatosis or healthy control group (Braunersreuther *et al.*, 2012).

2.9 Treatment for NAFLD and NASH

To date, there are no effective drug therapies for NAFLD (Kawano & Cohen, 2013; Hernandez-Rodas *et al.*, 2015). Therefore, interventions in lifestyle, such as weight loss and increased physical activity remain to be primary treatment for NAFLD patients (Asrih & Jornayvaz, 2014; Hernandez-Rodas *et al.*, 2015). The optimum diet to treat NAFLD is not known. Diet rich in whole grains, such as Mediterranean diet is assumed prone to promote weight reduction, reduce liver fat, and protect against inflammation lead

to NASH (Dyson *et al.*, 2014; Milic *et al.*, 2015). Exercise is also a useful lifestyle-related intervention for NAFLD/NASH patients (Dyson *et al.*, 2014; Milic *et al.*, 2015). Patients with obesity-related NAFLD carried out an aerobic exercise session for 30-60 minutes, three to four times weekly for 4-12 weeks showed an improvement in fatty liver changes, even without accompanying body-weight reduction (Ahmed, 2015; Milic *et al.*, 2015). Furthermore, exercise ameliorated the metabolic syndrome, insulin resistance, and cardiovascular disease (Whitsett & Van Wagner, 2015) via enhancement of sensitivity of skeletal muscle insulin and improvement of liver enzymes (Dyson *et al.*, 2014). A weight loss of at least 3 % to 5 % of the total body weight has been found to improve steatosis, and a weight loss of more than 7 % of the total bodyweight is related with a decrease in necroinflammation (Spengler & Loomba, 2015). Patients, especially patients with biopsy-proven NASH are necessitated to consider to have liver-directed pharmacotherapy when sustaining compliance with such therapeutic measures as restrained food consumption is mentally, emotionally challenging, or lifestyle intervention failed to mitigate the condition of disease (Dyson *et al.*, 2014; Takaki *et al.*, 2014).

At present, there are no approved pharmaceutical treatment for NAFLD by The United State Food and Drug Administration (FDA) (Tolman & Dalpiaz, 2007; Thrasher & Abdemalek, 2016). Thus, a practice guideline developed by American Association for the study of liver diseases and approved by American College of Gastronentrology Association on the management of NAFLD (Ahmed, 2015). To date, there are few therapeutic options for this disease because the pathologies are incompletely understood (Park *et al.*, 2015). The principle for NAFLD therapies is based on the growing understanding of the disease pathogenesis and is directed towards treating metabolic risk factors associated, such as diabetes, dyslipidemia, hypertension, obesity (Fruci *et al.*, 2013; Ahmed, 2015; Thrasher & Abdemalek, 2016).

Metformin and Thiazolidinediones (TZDs) are the most popular drugs tested against NAFLD/NASH (Federico *et al.*, 2014). Metformin is an insulin sensitizing agent, which is commonly used as the first line therapy for diabetic patients (Fruci *et al.*, 2013; Ahmed, 2015). Metformin ameliorates insulin resistance through inhibiting the mitochondrial respiratory chain, inducing a transient reduction in cellular energy status that promotes the activation of adenosine monophosphate-activated protein kinase (AMPK), a key regulator of glucose and lipid metabolism, lead to reduction of hepatic gluconeogenesis, lipogenesis, and inhibition lipolysis and modulates adipokines synthesis and/or secretion, and increased fatty acid oxidation in liver and adipose tissue, promoting glucose uptake in the muscle (Fruci *et al.*, 2013; Milic *et al.*, 2015). Metformin also helps in increasing peripheral and hepatic insulin sensitivity, decreasing intestinal glucose absorption; and lowering serum lipid concentration (Milic *et al.*, 2015).

Metformin initially exhibited promising results in animal model of NASH (Tolman & Dalpiaz, 2007; Hardy *et al.*, 2015) and early pilot study of NAFLD/ASH patients by improving fatty liver disease and aminotransferase levels, as well as reversing hepatomegaly and steatosis (Tolman & Dalpiaz, 2007; Milic *et al.*, 2015). However it is not currently recommended as a targeted treatment for NAFLD because randomized control trial of metformin versus placebo failed to show major benefit for metformin on hepatic insulin sensitivity, aminotransferases, or liver histology (Filozof *et al.*, 2015; Hardy *et al.*, 2015).

Thiazolidinediones (TZDs) are a group of drugs that showed therapeutic effect of insulin sensitizer, consisting of troglitazone, rosiglitazone and pioglitazone (Fruci *et al.*, 2013; Takahashi *et al.*, 2015). They are peroxisome proliferator-activated receptor (PPAR)-γ agonists and increase insulin sensitivity (Takahashi *et al.*, 2015). (PPAR)-γ is largely expressed by adipose tissues (Federico *et al.*, 2014), also found in liver and muscle (Ahmed, 2015), it plays a central role in adipogenesis and glucose homeostasis (Federico

et al., 2014). TZDs treatment is associated with redistribution of lipid from liver and muscle cells to adipocytes that leads to enhance insulin sensitivity, also, increase adiponection expression, decrease pro-inflammatory cytokine expression such as TNF-α and increase beta-oxidation of fatty acids via stimulating adipocytes maturation (Tolman & Dalpiaz, 2007; Spengler & Loomba, 2015).

Troglitazone was approved to be used in the treatment of diabetes in 1997 due to its enhanced insulin sensitivity, however, it was withdrawn from the market in the year of 2000 because it caused hepatotoxicity resulted in liver injury and failure (Jaeschke, 2007). A randomized controlled trial reported that rosiglitazone was capable to alleviate steatosis and aminotransferase level in patients with NASH (Takakashi *et al.*, 2015). However, it was no longer in use for treatment because of an association with coronary events and decompensation of heart failure (Spengler & Loomba, 2015).

Randomized controlled trials of pioglitazones have been reported, all of which showed an improvement in serum aminotransferase level and ameliorate steatosis inflammation, ballooning, and Mallory-Denk bodies as well as insulin resistance compared to placebo in NASH patients (Tolman & Dalpiaz, 2007; Takahashi *et al.*, 2015), but, so far, no study has shown a convincing benefit to fibrosis (Tolman & Dalpiaz, 2007; Takahashi *et al.*, 2015). On the contrary, treatment with pioglitazone is also linked with some adverse effects, including weight gain, edema, heart failure, and bone density reduction (Fruci *et al.*, 2013; Takahashi *et al.*, 2015). Furthermore, pioglitazone use for more than 2 years may lead to an increase risk of bladder cancer (Federico *et al.*, 2014; Milic *et al.*, 2015). In the United States, the Food and Drug Administration recommends avoidance of pioglitazone in cases of active bladder cancer, and caution regarding its use in patients with history of bladder cancer (Milic *et al.*, 2015; Takahashi *et al.*, 2015).

As a common antioxidant, the therapeutic effect of vitamin E supplementation on NAFLD, is based on its free radical scavenging activity (Al-Busafi *et al.*, 2012; Chen *et*

al., 2016). *In vivo* animal studies revealed that vitamin E as a potential treatment for NAFLD, attenuating steatohepatitis by lowering serum aminotransferase levels, diminishing histological steatosis, necroinflammation through reduction in malondialdehyde (MDA) content, promoting superoxide dismutase activity, suppressing activity of NF-κB, down-regulation of gene associated with inflammation, apoptosis and fibrosis (Nan *et al.*, 2009, Pacana & Sanyal, 2012). A randomized controlled trial carried out by Sanyal *et al.* (2010) reported that vitamin E therapy was superior to placebo for the treatment in adults who had biopsy-confirmed nonalcoholic steatohepatits without diabetes, associated with reduction in aminotransferase level, hepatic steatosis and lobular inflammation, but no improvement in fibrosis. Two years or longer vitamin E treatment at dose of 300 mg/day for patients with biopsy-proven NASH proved a significantly improved transaminase activity, insulin resistance index, and even ameliorated NASH fibrosis (Sumida *et al.*, 2013). In addition, Fukui *et al.* (2015) reported that vitamin E treatment for 1 year improved not only laboratory values but also the non-invasive scores of hepatic fibrosis and liver stiffness in NAFLD patients. Nevertheless, some meta-analyses (Chalasani *et al*., 2012; Federico *et al.*, 2014) reported long term of vitamin E treatment continues the probability of an increase in all-cause mortality. Administration of vitamin E at dose of 400 IU/day might increase the risk of prostate cancer and haemorrhagic stroke (Chalasani *et al.*, 2012; Dyson *et al.*, 2014).

Long-term follow-up studies demonstrate cardiovascular mortality to be the most important cause of death in NAFLD patients (Francque *et al.*, 2016) because both NAFLD and cardiovascular disease share similar risk factors, including insulin resistance, hypertension, dyslipidemia, type 2 diabetes, and abdominal obesity (Misra *et al.*, 2009; Eklioğlu *et al.*, 2015). Dyslipidemia is very common in patients with NAFLD and effective treatment of this risk factor is important in the management of NAFLD to reduce patients cardiovascular risk profile (Dyson *et al.*, 2014). Statins are used to treat

dyslipidemia because it inhibits cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (Takahashi *et al.*, 2015). A randomized controlled trial consisted of 80 patients with NAFLD demonstrated that improving in hepatic steatosis after administration of atorvastatin (20 mg) combined with vitamin C and E (Foster *et al.*, 2011). A post-hoc analysis revealed, statin treatment is safe and can improve liver tests and reduce cardiovascular morbidity in patients with mild-to-moderately abnormal liver tests that are potentially attributable to non-alcoholic fatty liver disease (Athyros *et al.*, 2010). However, patients treated with simvastatin showed no significant improvement in serum aminotransferase levels, hepatic steatosis, necroinflammatory activity, or stage of fibrosis in a randomized placebo controlled trials carried out by Nelson *et al.* (2009). There are arguments about long term statin treatment could potentially exacerbate histology in NAFLD patients (Pastori *et al.*, 2015). Based on currently available data, Pastori *et al.* (2015) revealed that statins treatment should be carried out at low-moderate intensity to prevent from inducing liver toxicity. Nevertheless, physicians are still reluctant to prescribe statins for patients with NAFLD and NASH as a result of unexplained persistent elevation of liver enzyme (Chalasani *et al.*, 2012; Nseir *et al.*, 2012; Pastori *et al.*, 2015). Hence, more randomizied controlled trials with larger sample sizes and adequate duration are required to confirm their histological efficacy and the usefulness of statins for the treatment of NAFLD (Pastori *et al.*, 2015; Milic *et al.*, 2015; Takahashi *et al.*, 2015).

Polyunsaturated fatty acids (PUFA) are necessary in humans because the body cannot produce them (Patterson *et al.*, 2012; He *et al.*, 2016). Masterton *et al.* (2010) revealed that animal studies demonstrated omega-3 fatty acids reduce steatosis, improve insulin sensitivity and reduce markers of inflammation. Small size number of human trials (Tanaka *et al.*, 2008; Spadaro *et al.*, 2008) showed the advantage of omega-3 fatty acids in treating NAFLD, including lowering the level of alanine aminotransferase, triglyceride

and serum necrosis factor-alpha, serum ferritin aand thioredoxin. However, a randomized, placebo-controlled trial carried out by Sanyal *et al.* (2014) reported that a synthetic polyunsaturated fatty acid, ethyl-eicosapentanoic acid had no significant effect on the level of liver enzymes, insulin resistance, histological features of NASH. A recent meta analysis reported the benefits of omega-3 fatty acid supplement towards gamma-glutamytransferase level, but it is not significant in liver enzymes: alanine aminotransferase, aspartate aminotransferase, total cholesterol and low density lipoprotein in patint with NAFLD/NASH (Lu *et al.*, 2016). On the other hand, He *et al.* (2016) reported that omega-3 supplement gave beneficial changes in alanine aminotransferase, total cholesterol, high density lipoprotein, triglyceride and it is tended towards a beneficial effect on aspartate aminotransferase, gamma-glutamyl transferase and low density lipoprotein. Despite most studies suggested positive effect of omega-3 fatty acid in NAFLD/NASH treatment, it was also suggested that more high-quality, large randomized controlled trials to validate the current findings, quantify the magnitude of the effects of omega-3 fatty acid supplementation on the liver fat are needed (Hernandez-Rodas *et al.*, 2015; He *et al.*, 2016).

2.10 Mushrooms

The intake of complete foods rich in naturally-oocurring antioxidants, including nutrients (e.g vitamins) and phytochemicals (e.g polyphenols) has been widely recommended by many health organizations (Bouayed & Bohn 2010) because it is essential in mediating endogenous antioxidant for the neutralization of oxidative stress (Pham-Huy *et al.*, 2008). Eating habits can drastically reduce healthcare expenditures if individuals were to modify their diets based on an existing knowledge of nutrition (Saikia & Deka, 2011). In modern day, mushrooms are not only source of nutrients but also have been reported as therapeutic foods, useful in preventing diseases such as hypertension,

hypercholesterolemia and cancer (Usha & Suguna, 2014), antimicrobial, anti-inflammatory, immunomodulatory and antioxidant (Soares *et al.*, 2013). Furthermore, it has been reported that mushrooms provide beneficial effects such as strengthening vital energy, maintaining one's optimal weight, favouring longevity, and avoiding unnecessary aging (Wu *et al.*, 2016).

Mushrooms are named by referring to a fruiting body that developed by a few hyphae growing upwards and produce spores (Wu *et al.*, 2016). According to Chang & Miles (1992), the mushrooms are a macro fungus with distinctive fruiting bodies that could be hypogeous or epigeous and they are large enough to be seen by naked eyes and to be picked by hands. Mushrooms are a large and assorted group of macro-fungi classified to Basidiomycetes and Ascomycetes (Elsayed *et al.*, 2014), which have stem (stipe), a cap (pileus), and gills (lamellae, sing. lamella) on the underside of the cap (Wu *et al.*, 2016). Approximately 140,000 to 150,000 mushroom species exist on earth, but, only 10 % have been identified up to the present (Chatterjee *et al.*, 2011; Wu *et al.*, 2016). At present, of those identified species today, about 50 % are regarded to have varying degrees of edibility, more than 2000 are safe, and about 700 species are recognized to possess significant pharmacological properties (Lull *et al.*, 2005).

2.10.1 Medicinal mushrooms

Mushrooms have been known as gourmet cuisine (Patel & Goyal, 2012) and treated as special kind of food (Rahi & Malik, 2016) across the earth in olden days. Ancient Greek believed that mushrooms provide strength for warriors in battle, the Roman accredited them as the "Food of Gods", while the Pharaohs of Egypt and some European tribes considered mushrooms as a delicacy, and Mexican Indian eat mushroom during festive occasions as hallucinogens (Reddy, 2015; Rahi & Malik, 2016). In Chinese society, mushrooms were treated as the ―Elixir of life (Chatterjee *et al.*, 2011).

Apart from culinary ingredients, the fruiting body of mushrooms have a long history of use as a folk medicine throughout the world since ancient times, particularly China and Japan (Tako *et al.*, 2013). Traditionally, mushrooms have been used in many different cultures as folk medicines for the maintenance of health and also the prevention and treatment of diseases through boosting immune system of the human body (Thatoi & Singdevaschan, 2014; Valverde *et al.*, 2015). Frequently, Chinese include mushrooms in the main daily meal because they believe that mushrooms have a detoxification effect that are essential in cleansing liver and kidney, also mushrooms have healing wounds effect (Aung, 2005). Reports from China, since about 500 BC, the medicinal properties of *Ganoderma lucidum* sensu auct. asiatic. (known in China as reishi) extracts, especially its anti-cancer properties, have been passed on generation to generation (Dias *et al.*, 2004). After the development of the methods for cultivation of *Lentinula edodes* (Berk.) Singer (shiitake) mushrooms in the Ming dynasty (1620 AD), there have been reports on the medicinal properties of *L. edodes* (shiitake) mushrooms as a tonic that could counteract the quotidian aches, pains, and fatigue due to aging, considered an elixir of life and possessing the ability to enhance 'vital energy' and cure colds (Dias *et al.*, 2004; Money, 2016). *Cordyceps sinensis* (Berk.) Sacc., also known as Dong Chong Xia Cao which means "winter worm summer grass, is one of the valuable traditional medicinal fungi among Chinese medicines having long history. It has been treasured throughout Asia as one of the most effective natural tonics to strengthen vitality and promote longevity over the millennia (Shashidhar *et al.*, 2015). In Asia, *Auricularia auricula-judae* (Bull.) Quél is considered to be a source of antitumor compounds in the Chinese traditional medicine, being used for topical treatment of sore throat, ophthalmia, tonsillitis and laryngocele by applying fresh fungus to the sore body part (Kadnikova *et al.*, 2015).

Globally, researches on medicinal properties of mushrooms have been conducted intensively, particularly on medicinal mushrooms (Lindequist *et al.*, 2014; Glamočlija &

Soković, 2017). By using animal model in investigation the hypoglycemic properties of *Ganoderma lucidum* and *Agaricus brasiliensis* Wasser, M. Didukh, Amazonas & Stamets, Vitak *et al.* (2015) observed a decreased in blood glucose and glycosylated hemoglobin concentrations, an increase in the number of erythrocytes in bloodstream, erythrocyte resistance to acid hemolysis and the normalization of fetal hemoglobin concentration as well as an elevation in the red blood cells generation in streptozotocin-induced diabetic rats after orally treated with submerged culture mycelium powder of abovementioned mushrooms for two weeks, compared to placebo group. Vitak *et al.* (2016) conducted an animal model study to investigate the hypoglycemic effect of *G. lucidum* and *A. brasiliensis*. This group observed that administration of submerged culture mycelium powder of abovementioned mushrooms exhibited protective effect against oxidative-nitrosative stress in diabetic rats by recovery of total nitrite oxide synthase activity to control values (Vitak *et al.*, 2016). Griensven and Verhoeven (2013) suggested that polysaccharides extract from *Phellinus linteus* (Berk. & M.A. Curtis) Teng may be used for further study in age related neurodegenerative disease such as Alzheimer disease and Parkinson disease because hyperpolarizing compounds in *Phellinus linteus* might restore Ca^{2+} homeostasis and thereby prevent the loss of neuronal function. Besides that, *Hericium erinaceus* Pers. was considered as useful therapeutic agents in the treatment and/or management of neurodegenerative diseases, whereby polysaccharides from *H. erinaceus* was reported to stimulate the synthesis of nerve growth factor, promote the growth and differentiation of nerve cells and protecte the cells against oxidative stress (Gargano *et al.*, 2017). Schillaci *et al.* (2013) conducted antibacterial activity of medicinal mushroom by extracting cationic peptides from *Pleurotus nebrodensis* (Inzenga) Quél., *P. eryngii var. ferulae (Lanzi) Sacc., P. eryngii var. elaoselini* and *P. eryngii var eryngii*. All of *Pleurotus* species tested exerted antibacterial activity towards *Staphylococcus*

aureus ATCC 25923, *S. epidermidis* RP62A, *Pseudomonas aeruginosa* ATCC 15442, and *Escherichia coli* ATCC10536 at varying degrees (Schillaci *et al.*, 2013).

2.10.2 Nutritional value of mushrooms

To date, mushrooms continue to attract considerable interest in many areas of food and biopharmaceutical research (Ike *et al.*, 2012). It is widely known to be low in calories and rich in essential nutrients such as polysaccharides, protein, vitamins, minerals and a variety of secondary metabolite (Vieira *et al.*, 2012; Chiu *et al.*, 2014; Valverde *et al.*, 2015). In general, fruiting body of mushroom contains about 56.8 % carbohydrate, 25 % protein, and 5.7 % fat, and 12.5 % ash on dry weight basis (Hung & Nhi, 2012). The nine essential amino acids: lysine, methionine, tryptophan, threonine, valine, leucine, isoleucine, histidine, and phenylalanine, that are not endogenously synthesized can be obtained from mushroom; also 72 % of the total fatty acids were found to be unsaturated in mushroom, therefore making mushrooms a healthy food (Rahi & Malik, 2016). Moreover, edible mushrooms provide a nutritionally significant content of vitamins (B1, B2, B12, C, D, and E) (Valverde *et al.*, 2015). Secondary metabolites that are present in mushrooms, include phenolic and polyphenolics, polyketides, terpenoids, egosterol, and steroids, sesquiterpenes, glycoprotein, alkaloids, and volatile organic compounds (Oke & Aslim, 2011, Elsayed *et al.*, 2014; Valverde *et al.*, 2015). Over the past few decades, the consumption of mushrooms has even increased remarkably (Wang & Xu. 2014).

2.10.3 *Auricularia nigricans* (syn *Auricularia polytricha*)

Auricularia Bull., is a genus of jelly fungi in the family *Auriculariaceae* Fr. The genus *Auricularia* comprised of 10 to 15 species that are recognized globally (Sękara *et al.*, 2015) and are scattered in tropical, subtropical and temperate regions (Li *et al.*, 2014; Bandara *et al.*, 2015). All species within the Auriculariales are thought to be saprotrophs;

most of them are wood rotters. They are typically found on dead or fallen wood, though a few (*Guepinia* and *Tremellodendropsis* species) are normally found on the ground. *Auricularia* species is the fourth most crucial cultivated mushroom used by humans throughout the world after *Agaricus*, *Pleurotus*, and *Lentinula edodes* (Zhang *et al.*, 2011; Li *et al.*, 2014). In general, *Auricularia* species are edible; both *Auricularia auricula-judae* and *Auricularia polytricha* (Mont.) Sacc. are popular in the market (Bandara *et al.*, 2015) and are grown commercially in Asian countries (Sękara *et al.*, 2015)

Auricularia polytricha is classified within Basidiomycota, Agaricomucetes (Zhou *et al.*, 2014). *A. polytricha* is also recognized as wood ear, Jew's ear, or red ear (Yu *et al.*, 2014). However, it is known as Telinga Kera by local. In 2013, *A. polytricha* was proposed to be renamed to *Auricularia nigricans* (Sw.) Birebak, Looney & Sánchez-García after the discussion of taxanomy and nomenclature *Peziza nigricans* (Looney *et al.*, 2013). Figure 2.11 showed the physical appearance of cultivated *A. nigricans* and its scientific nomenclature. It is found growing on living and dead broad-leaved trees, decayed stumps or logs in temperate to the tropics regions (Du *et al.*, 2011). It is having abundant of assorted nutrients such as polysaccharides, edible fibers and proteins (Zhou *et al.*, 2014).

Kingdom: Fungi

Phylum: Basidiomycota

Class: Agaricomycetes

Order: Auriculariales

Family: Auriculariaceae

Genus: Auricularia Bull., 1780

Figure 2.11: The physical appearance of *A. nigricans* and its scientific nomenclature.

It is widely used as food or tonic agent in the Orient, particularly in China and Korea (Yu *et al.*, 2014). In traditional medicine, Chinese rely on wood ear mushrooms to stop pain, activate blood circulation, treat of hemorrhoids, boost energy, prevent from geriatric disorders, as anticoagulant, cholesterol and triglycerides lowering agents (Kushwaha *et al.*, 2006; Du *et al.*, 2011). In addition, *A. polytricha* is also known for its antioxidant capacity, antitumor capacity, anti-dementia properties, immunomodulatory and attenuation of inflammatory response (Liang *et al.*, 2019). Because of its delicacy and biological activities, consumer demand for *A. polytricha* has increased yearly in Taiwan (Liang *et al.*, 2019).

In general, studies on *Auricularia* species are mainly focus on polysaccharides. Studies were done to evaluate the efficiency of polysaccharides and ethanolic crude extract of *Auricularia auricula* on hypocholesterolaemic in animal model by observing the lipid profile, Hepatic 3-hydroxy-3-methylglutary CoA (HMG-CoA) reductase activity, hepatic antioxidant status determination and determination of fecal neutral cholesterol and bile acids (Chen *et al.*, 2011). Also, Zhang *et al.* (2011) and Zeng *et al.* (2012) studied the antioxidant capacity of the polysaccharide of *Auricularia auricular-judae*. Likewise,

polysaccharides of *A. polytricha* are well-studied on anti-cancer activity (Yu *et al.*, 2014). In addition, hypocholesterolaemic effect and anti-oxidative status of aqueous extract of *A. polytricha* was evaluated in animal model with non-alcoholic fatty liver (Chiu *et al.*, 2014). Also, a study carried out on the extraction of soluble polysaccharide from *A. polytricha* and evaluation of its anti-hypercholesterolemic effect in rats showed that soluble polysaccharide of *A. polytricha* decreased the serum concentrations of blood lipid, made them close to the normal level. (Zhao *et al.*, 2015). Furthermore, Chellappan *et al.* (2016) looked into the effect of aqueous extract of *A. polytricha* in paracetomal induced hepatotoxicity in rats. Chellappan *et al.* (2016) reported that the aqueous extract of *A. polytricha* has significant protective effect against paracetamol-induced liver toxicity in rats, due to its potent antioxidant activity. In addition to polysaccharides, mycelium of *A. polytricha* was being studied on its hypolipidemic and hypoglycemic effect in animal model, which showed exo-biopolymer produced from a submerged mycelial culture of *A. polytricha* significantly decreased the concentrations of the plasma triacylglycerols, total cholesterol, and low-density lipoprotein cholesterol (Yang *et al.*, 2002). Ton *et al.* (2017) reported that polyschharides derived from *A. nigricans* had the benefit effect on hyperlipidemia and may be an ideal medicinal material for the prevention of coronary heart diseases after observing a reduction in plasma triglycerides, total cholesterol, and low density lipoprotein-cholesterol and an elevation high density lipoprotein-cholesterol in triton WR-1339-induced hyperlipidemic mice. Most studies have been focused on polysaccharides of *Auricularia* species, however few studies were done on the solvent extracts.

CHAPTER 3: MATERIALS AND METHODS

3.1 Preparation of extract from *Auricularia nigricans*

3.1.1 Solvents for extraction

Organic solvents with analytical grade were used for extraction and fractionation process of mushroom. The organic solvents such as ethanol, hexane, ethyl acetate, dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich.

3.1.2 Source of *Auricularia nigricans*

Auricularia nigricans was supplied by Mycology laboratory, Institute of Biological Science, University of Malaya, Kuala Lumpur, Malaysia.

3.1.3 Extraction of mushroom

The fresh *A. nigricans* was dried under the sun. Then it was ground into powder when it was completely dried. Next, the powder of mushroom (100 g) was extracted by soaking with 95 % ethanol (ETOH) for two days in the dark at room temperature. Subsequently, the ethanolic extract was filtered through Whatman No. 1 filter paper (Whatman, England). The extraction was repeated twice. The EtOH filtrate was collected and subjected to evaporation with rotary evaporator (Buchi) under reduced pressure at 40 ^{o}C to dryness. A small portion of the dried ethanolic extract was kept for assays whereas the remaining portion was suspended in distilled water (100 mL) and partitioned successively with hexane (100 mL), and ethyl acetate (100 mL) in manner of mild shaking with separating funnel to fractionate the non-polar and polar compounds in the crude extract. This partitioning was conducted in thrice to give hexane, ethyl acetate and water fractions. Each hexane and ethyl acetate soluble fractions were filtered through Whatman No. 1 filter paper (Whatman, England). The resulting fractions were evaporated to

dryness under reduced pressure whereas aqueous fraction was freeze-dried. The dried samples were weighed and the yield of the extracts obtained were calculated. The ethanolic crude and hexane, ethyl acetate and water fractions were kept in dark at 4 ^{o}C prior to analysis. In all experiments, samples were dissolved in dimethylsulfoxide (DMSO), except for water fraction that was dissolved in water, as stock solution and stored at 4 ^{o}C.

3.2 Determination of antioxidant capacity

3.2.1 Chemicals and reagents

Folin-Ciocalteau reagent, sodium carbonate (Na_2CO_3), ferrous sulfate heptahydrate ($FeSO_4.7H_2O$), ferric chloride hexahydrate ($FeCl_3.6H_2O$), sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$) and 1,1-Diphenyl-2-picrylhydrazyl (DPPH), trolox, gallic acid, potassium ferricyanide, ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), phosphte buffered saline (PBS) were purchased from Sigm-Aldrich company. The other chemicals and solvents used were analytical of grade.

3.2.2 Determination of total phenolic content

The total phenolic content of all samples was determined using Folin-Ciocalteu reagent as described by Mayur *et al.* (2010) with modification. The extract and each fraction were oxidized with Folin–Ciocalteu's reagents, and then, the reaction was neutralized with 7.5 % (w/v) sodium carbonate. A standard calibration curve was prepared using different concentrations of gallic acid in methanol (0.2-1 mg/mL). Both mushroom extract and fractionated samples (30 µL) and gallic acid (30 µL) were mixed with 10 % of Folin-Ciocalteu reagent (100 µL) separately. After five minutes, 7.5 % (w/v) of sodium of carbonate (100 µL) was added. Blank was concomitantly prepared, containing methanol and 10 % Folin-Ciocalteu and 7.5 % of sodium carbonate. The

absorbance readings were taken at 750 nm after incubation in dark at room temperature for 30 minutes. The reduction of Folin Ciocalteu reagent by the samples were expressed in terms of milligrams gallic acid equivalent per gram of fractionated sample (mg GAE/g). All of the samples were analyzed in triplicate and the results expressed as mean values ± standard deviations.

3.2.3 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The scavenging activity of samples was evaluated according to the procedure described by Zhao *et al.* (2014) with some modifications. The DPPH radical scavenging activities of various fractions of *A. polytricha* were examined by comparison with that of known antioxidant, trolox. Trolox is water-soluble analog of vitamin E. Briefly, 50 µL of 0.2 mM DPPH methanol solution was added to 30 µL sample solutions at different concentrations, and the resulting mixtures were left at room temperature in the absence of light for 30 minutes. The absorbance values were then measured at 517 nm. The scavenging effect was calculated based on the following equation:

Scavenging effect (%) = 1- [(Absorbance $_{sample}$/ Absorbance $_{control}$) x 100] (**Formula 1**)

Where, absorbance $_{sample}$ is the absorbance of DPPH solution plus samples and absorbance $_{control}$ is the absorbance of DPPH solution without sample. The IC_{50} was determined by plotting scavenging effect (%) at y-axis against sample concentration (x-axis), and a regression line (y = ax + b) was drawn.

3.2.4 Ferric reducing antioxidant power (FRAP)

The reducing power of samples was determined by the method of Murugan & Iyer (2012) with some modifications. Aliquots of various concentrations of samples (100 µL) in corresponding solvents were mixed with 100 µL of 0.2 M phosphate buffer (pH 6.6) and 100 µL of 1 % (w/v) potassium ferricyanide. The mixture was incubated at 50

^{o}C for 30 minutes. 10 % (w/v) of trichloroacetic acid solution was added after the mixture had cooled down. Following this, 125 µL from the upper layer was transferred into a 96-well plate and 20 µL of 0.1 % (w/v) of freshly prepared ferric chloride was added. The absorbance at 620 nm was measured as the reducing power. The trolox standard curve was used to calculate the antioxidant activity of the samples in relation to trolox and was expressed as milligram trolox equivalents (TE) per gram of sample (mg TE/g).

3.2.5 Scavenging of superoxide anion radical ($O_2 \bullet^-$)

Scavenging of superoxide anion assay was carried out according to Chien *et al.* (2013) with a minor modification. In this assay, the reaction mixture contained 50 µL phosphate buffer (0.2 M, pH=7.4), 50 µL β-nicotinamide adenine dinucleotide, NADH (300 µM), 50 µL nitroblue tetrazolium, NBT (156 µM) and 50 µL of various concentrations of samples solutions, the reaction was initiated by adding 50 µL of 60 µM of phenazine methosulfate (PMS) and then incubated at room temperature for 5 minutes. Formation of blue formazan dye was measured at 560 nm. The results were expressed in percent of inhibition as compared to control, (formula 1).

3.2.6 Scavenging of hydroxyl radical ($OH^{\cdot}$)

The $OH^{\cdot}$ was generated by a Fenton system (ascorbic acid/ferric chloride anhydrous-ethylenediaminetetraacetic acid/H_2O_2) (Bajpai *et al.*, 2013). First, the hydroxyl radical was generated by incubating a reaction mixture containing 20 µL $FeCl_3$ (3.2 mM), 20 µL ascorbic acid (1.8 mM), 20 µL EDTA (1 mM), 20 µL H_2O_2 (1 mM), 20 µL of deoxyribose (5 mM), and 250 µL of samples of different concentrations in phosphate buffered saline (PBS, 0.2 M, pH 7.4) at 50 ^{o}C for 20 minutes. Then, trichloroacetic acid (10 %, w/v, 250 µL) and of thiobarbituric acid (1 %, w/v, 150 µL) were added, followed by heating in a waterbath at 95 ^{o}C for 15 minutes to allow for the

development of colour. Trolox was used as a positive control in this assay. The colour development was measured at 532nm. The results were expressed in percent of inhibition as compared to control (formula 1).

3.2.7 Oxygen radical absorbance capacity (ORAC)

The ORAC assay is based on the scavenging of peroxyl radicals (ROO·) generated by 2,2′ -azobis(2-amidino-propane)dihydrochloride (AAPH) (Dudonne *et al.*, 2009). In brief, 60 mM of AAPH and 70 nM of fluorescein were prepared in 75 mM phosphate buffer at pH 7.4. 60 µL of each mushroom sample and 60 µL fluorescein were pre-incubated at 37 ^{o}C for 15 minutes. Then, 60 µL AAPH was added to initiate the reaction. Immediately, the plate was placed in BioTek microplate reader and the fluorescence readings were taken at an interval of one minute for eighty minutes at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Trolox, a hydrophilic derivative of vitamin E, was used as a standard. The area under the fluorescence decay curve (AUC) was calculated as formula below by Microsoft Excel.

AUC= 1 + f1/f0 + f2/f0 + f3/f0 +…. f80/f0

Where f0 is the initial fluorescence reading at 0 minute and fi is the fluorescence reading at time i = 1, 2, 3, …, 80 minutes

Net AUC was calculated as follow:

Net AUC = AUC trolox or samples – AUC blank

Regression equation between net AUC and Trolox concentration was determined and ORAC values for each sample were expressed as micromoles Trolox equivalents per gram sample (µmol TE/g).

3.3 Profiling of bioactive constitutes

Selected bioactive extracts were further subjected for characterization. The potent fractions were subjected to gas chromatography mass spectrum (GCMS) and liquid chromatography mass spectrometry (LCMS) analysis for detection of bioactive compounds present in *A. nigricans*. GCMS analysis was performed by High Impact Research Center, whereas LCMS was carried out by Advanced Chemistry Solutions (The Scientific Consultation Company).

3.3.1 Gas chromatography mass spectrometry (GCMS) analysis

The ethyl acetate fraction was subjected to GCMS for assessing the bioactive compounds profile. The phytochemical analysis of ethyl acetate fraction was carried out using Network Gas Chromatography system (Agilent Technologies 6890N) equipped to an Inert Mass Selective Detector (Agilent Technologies 5975) (70eV direct inlet) with HP-5ms (5 % phenyl methyl siloxane), (30 m × 250 μm × 0.25 μm) capillary column. Experimental condition of GCMS system was as follows: In the mobile phase, helium was used as carrier gas at a flow rate of 1 mL per min. For ethyl acetate fraction, temperature was initially set at 60 ºC, then increased at 5 ºC per min to 300 ºC and held for 10 min. To identify the compounds, the total ion chromatogram obtained was auto-integrated by chemstation and the constituents were identified by comparison with the accompanying mass-spectra database (Wiley 9th with NIST 11 Mass Spectral Library, USA) wherever possible.

3.3.2 Liquid chromatography-tandem mass spectrometry (LCMS-MS) analysis

Both ethyl acetate and water fractions were subjected to LCMS analysis. It was conducted by AB Sciex 3200QTrap LCMS-MS with Perkin Elmer FX 15 uHPLC system. Full Scan LCMS-MS was performed in the negative mode at voltage IS of -4500 V. The

mobile phase was a mixture of water with 0.1 % formic acid, 5mM ammonium formate (solvent A) and acetonitrile with 0.1 % formic acid, 5mM ammonium formate (solvent B) with a rapid screening at 15 minutes run time, and using a Zorbax C18-150 mm x 4.6 mm x 5 µM column. The gradient elution program was: 10 % B to 90 % B from 0.01 minutes to 8 minutes, hold for 3 minutes and back to 10 % B in 0.1 minutes and re-equilibrated for 5 minutes.

3.4 Preparation for cell culture assays

The bioactive water and ethyl acetate fractions were further studied based on cell line work.

3.4.1 Chemicals for cell culture works

Dulbecco's Modified Eagle Medium (DMEM) and trypsin-EDTA (0.25 %) was purchased from Hyclone (GE Healthcare, UK). Foetal Bovine serum (FBS) was obtained from Gibco. penicillin-streptomycin (100X) (ATCC), palmitic acid was purchased from Acros organic), bovine serum albumin (fatty acid free) was obtained from Sigma - Aldrich, Pathscan Inflammation Multi-Target Sandwich ELISA kit, ELISA kit for IL-6, IL-8, MCP-1 from Cell Signalling, and ELISA kit for TNF-α from Qiagen.

3.4.2 Cell line

The human hepatoma cell line (HepG2) was purchased from American Tissue Culture Collection (ATCC, USA). HepG2 cell lines was grown in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L glucose. DMEM was supplemented with 10% (v/v) Foetal Bovine Serum (FBS) (Gibco, USA), 2 % penicillin-streptomycin (100X). The cells were cultured at 37 ^{o}C in a humidified atmosphere CO_2 incubator. HepG2 cells

were harvested in the exponential growth phase for experiments and subcultured every 3 days.

3.4.3 Preparation of stock solutions of samples

Ethyl acetate fraction was dissolved in DMSO whereas the water fraction was dissolved in distilled water to obtain a stock solution at concentration of 50 mg/mL prior to performing assays.

3.4.4 Preparation of palmitic acid

Stock of palmitic acid at concentration of 100 mM was prepared by dissolving 25.6 mg of palmitic acid in one millilitre of absolute ethanol. 10 % Free Fatty Acid free, low endotoxin Bovine Serum Albumin (FFA-free BSA) was prepared in deionized water and adjusted to pH 7.0 using sodium hydroxide. Then, a 6 mM palmitic acid stock solution was prepared by mixing palmitic acid to FFA-free BSA.

3.5 Cytoxicity assay - 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The cytotoxicity of samples and palmitic acid were determined by MTT in HepG2 cells. The MTT cytotoxicity assay was carried out as described by Hasiah *et al.* (2011) with modification.

3.5.1 Cytotoxicity of samples

Briefly, 100 uL of each HepG2 cells were seeded in a 96 well plate at density of 6000 cells/well. After 24 hours, cells were treated with increasing concentrations (6 - 100 µg/mL) of water and ethyl acetate fractions for 24 and 48 hours. Cells treated with 0.2 % DMSO was used as vehicle control (Bardi et al., 2014) for ethyl acetate fraction. After

treatment, 10 µL of 5 mg/mL of MTT (Sigma) was added, and the cells were incubated for another three hours at 37 ^{o}C. Following three hours' incubation, the medium was discarded, the intracellular formazan product was dissolved with 100 µL DMSO. The plate was gently agitated until the purple formazan crystals were fully dissolved. Absorbnces at 570 nm was read using Multiskan Go microplate spectrophotometer (Thermo Scientific, Tewksbury, MA, USA), and the reduction in optical density caused by samples, was used as a measurement of cell viability, normalized to cells incubated in control medium, which was considered 100 % viable. Cell viability was calculated using the following formula:

$$\text{Viability cell (\%)} = \left(\frac{\text{Absorbance 570 nm of treated sample}}{\text{Absorbance 570nm of control}}\right) \text{X } 100\ \%$$

3.5.2 Cytotoxicity of palmitic acid

Cytotoxicity of palmitic acid on HepG2 was tested with increasing concentration of 50, 100, 300, 500 µM for 24 hours. Then, cytotoxicity of various concentrations of palmitic acid were determined by MTT assay as the method described in Section 3.5.1.

3.6 Detection of lipid droplets in HepG2 cells

Oil Red O staining was carried out for imaging and quantification lipid droplet in HepG2 cell line. Oil Red O staining was conducted as Yao *et al.* (2011) with slight modification.

3.6.1 Palmitic acid induced HepG2 cells

In brief, HepG2 cells were seeded at 10,000 cells/well in a 96-well plate. After 24 hours, cells were treated with different concentrations of palmitic acid for 24 hours. The treatment media were then removed and the cells were rinsed with cold PBS followed by fixing with 50 µL of 10 % of formaldehyde for one hour. The fixed cells were then rinsed

and stained with 50 µL of Oil Red O solution (working solution, 0.5 g Oil Red O powder dissolved in 60 % ethanol) for 15 minutes at room temperature. After 15 minutes, the cells were rinsed with PBS to remove unbound staining. Cell morphology and the distribution of lipid droplet were then observed and image-recorded under inverted microscope. The quantification of Oil Red O content levels was carried out by adding 50 µL of DMSO to each well and after gentle shaking at room temperature for 5 minutes. The density of Oil Red O was read at 510 nm on a spectrophotometer.

3.7 Anti-hepatic steatotic and scavenging of intracellular ROS effects of water and ethyl acetate fractions

3.7.1 Anti-hepatic steatotic effect

Similarly, after 24 hours of cell seeding, cells were grouped into induced, non-induced and treatment. In the induced group, cells were induced by palmitic acid by adding 200 µL of medium containing only palmitic acid (positive control) to the cells, whereas in non - induced group, cells were incubated in 200 µL of medium only, which was known as negative control. In the treatment group, cells were exposed to 200 µL of mixture of sample in the presence palmitic acid. Each group cells were allowed to incubate for 24 hours at 37 ^{o}C, 5 % CO_2 incubator. Next, Oil Red O staining was performed and absorbance was read at wavelength of 510 nm as described in Section 3.6.1.

3.7.2 Scavenging of intracellular ROS effects

Detection of intracellular ROS was performed using the 2'-7' dichlorofluoresceindiacetate (DCFH-DA), as performed by Kim *et al.* (2013) with some modification. Briefly, cells were seeded in 96-well plate at a density of 100 µL of 10,000 cells per well. Cells were treated as described in Section 3.6.1. Then, cells were treated

with 300 µM palmitic acid in the presence or absence of sample for 24 hours. After treatment, sample was removed and 100 µL of 5 µM DCFH-DA was added to the well for 30 minutes, in dark at 37 ^{o}C. Then, cells were washed twice with PBS. Then, quantifying of fluorescent intensity was detected with an excitation of 485 nm and emission of 530 nm by multi-plate reader (BioTek) and fluorescent images were taken by fluorescence microscopy (Nikon TE 2000U, Tokyo, Japan) using green filter.

3.8 Anti-inflammatory effect of ethyl acetate fraction

3.8.1 *In vitro* assessment of cytokines secretion

HepG2 cells were seeded in T25 flask and allowed to grow to sub-confluent. Then, cells were grouped into positive control, negative control and treatment as described in section 3.6.2, and exposed to each particular condition for 24 hours. After 24 hours incubation, the cell culture supernatant of the positive control, negative control and treated HepG2 cells were collected, and centrifuged at 1000 x g for 10 minutes. The supernatants were either assayed immediately or aliquoted and stored at -20 ^{o}C prior to analysis.

3.8.1.1 Detection of TNF-α

The effect of sample on secretory level of TNF-α was measured using single-analyte ELISArray kit (Qiagen). All required reagents and solutions were provided in the ELISA kit. Determination of TNF-α level in the culture supernantant as conducted according to manufacturer's instruction.

After treatment, 50 µL of culture supernatant was added to the well pre-coated with the protein-specific capture antibody and incubated for two hours at room temperature. After that, culture supernatant was removed and washed three times with 350 µL of 1X wash buffer. This is followed by addition of 100 µL of antibody detection

solution to each well and incubated for one hour at room temperature. Then, the solution was removed and washed three times with 350 µL of 1x wash buffer, followed by addition of 100 µL of avidin-HRP to well and incubated it in dark for thirty minutes at room temperature. After thirty minutes of incubation, solution was discarded and washed four times with 350 µL of 1x wash buffer. After the final wash, 100 µL of development solution was pipetted into each well and further incubated for fifteen minutes in dark at room temperature. At last, 100 µL of stop solution was added to well to stop the colour development and the absorbance was immediately read at 450 nm using Multiskan Go microplate spectropotometer (Thermo Sientific). A standard curve, range from 0 to 2000 pg/ml, for determination of the concentration of TNF-α was constructed using the provided antigen standard in the ELISA kit

3.8.1.2 Detection of IL-6, IL-8 and MCP-1

Secretory levels of IL-6, IL-8 and MCP-1 were measured by enzyme linked immunosorbant assay (ELISA) kits (Quantikine ELISA, R&D Systems Inc, Mineapolis USA). Reagents and solutions needed for this assay were provided in the kits and assays were conducted according to instruction of manufacturer.

Briefly, after treatment, 100 µL of culture supernatant was added into 96-wells microplate coated with monoclonal antibody specific for human IL-6, whereas 200 µL of culture supernatant was added into 96-wells microplate coated with monoclonal antibody specific for human MCP-1. A slight variation in assay of IL-8, where the culture supernatant was required to be 100-fold diluted before transferring 50 µL of diluted culture supernatant into the well coated with monoclonal antibody specific for IL-8. The plates were then incubated for two hours at room temperature. Then, the culture supernatants were removed and washed with 400 µL of wash buffer for four times. After that, 200 µL of human IL-6 conjugate was added and allowed it to incubate for two hours

at room temperature. On the other hand, 200 µL of human MCP-1 conjugate and 100 µL human IL-8 conjugate were added to respective microplate strips, and the plates were incubated for one hour at room temperature. After the incubation period, the solution was discarded and washed four times with 400 µL of wash buffer before 200 µL of substrate solution was added into each well. The plates were incubated at room temperature, protected from light for 30 minutes for IL-8 and MCP-1 assays, whereas IL-6 assay only required 20 minutes' incubation in the dark. Lastly, 50 µL of stop solution was added to well to stop reaction and solution colour in wells changed from blue to yellow. Absorbances of each well was determined immediately at 450 nm by Multiskan Go microplate spectrophotometer (Thermo Scientific). The standard curves of IL-6, IL-8 and MCP-1 were constructed with the provided recombinant human proteins in the respective ELISA kits. Recombinant proteins in serial in dilutions from 0-2000 pg/ml for both IL-8 and MCP-1, 0-300 pg/ml for IL-6, were used to construct standard curve.

3.8.2 Anti-inflammatory transduction signaling pathways

To predict the pathways occurred in the induced HepG2 cells, PathScan Inflammation Multi-Target Sandwich ELISA kit (Cell Signaling Technology). All reagent and solution required for this assay were provided in the kit. Detection of phosphorylated signaling protein was carried out as described by the manufacturer's protocol.

3.8.2.1 Extraction of whole cell protein lysates

Similar to section 3.8, HepG2 cells were seeded in T25 flask and allowed to grow to sub-confluent. The cells were grouped into positive control, negative control and treatment as described in section 3.6.2, and exposed to each particular condition for 24 hours. The medium was then discarded, followed by rinsing the cells with ice-cold PBS.

The PBS was aspirated out and replaced by 0.5 mL of ice-cold 1X cell lysis buffer, which contained 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM disodium EDTA, 1 mM ethylene glycerol tetraacetic acid (EGTA), 1 % Triton, 20 mM sodium pyrophosphate, 25 mM sodium fluoride, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate (Na_3VO_4) and 1 µg/mL leupeptin (Cell Signaling Technology) plus 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, USA), and incubated on ice for five minutes. After five minutes, cells were scraped off, transferred to 2 mL microtube and sonicated on ice prior to microcentrifugation at 4 ^{o}C for 10 minutes at 10,000 X g. Then, the supernatant (cell lysate) was collected and stored at -80 ^{o}C.

3.8.2.2 Quantification of protein

The protein concentration of each cell lysate was determined by Coomassie Plus (Bradford) assay kit (Thermo Fisher Scientific), and compared to a protein standard. A Bovine Serum Albumin (BSA) standard curve, ranged from 0 to 2000 µg/mL was constructed. A total of 300 µL of Coomassie Plus Reagent was pipetted into 96-well plate. Then, 10 µL of BSA or cell lysate was added into each well and mixed on a benchtop for ten minutes at room temperature. The absorbance was taken at 595 nm by Multiskan Go microplate spectrophotometer (Thermo Scientific). The protein concentration of cell lysate was determined by comparing its absorbance value to the BSA standrad curve.

3.8.2.3 Detection of phosphorylated signalling protein

To predict the pathways involved in palmitic acid-induced HepG2 cells, the protein expressions of phosphorylated nuclearfactor kappa B p65 subunit (p-NF-κB p65), phosphorylated inhibitor kappa B-alpha (p-IκBα), phosphorylated stress-activated proteinkinase/Jun amino-terminal kinases (p-SAPK/JNK), phosphorylated p38 mitogen-activated protein kinases (p-p38 MAPK), phosphorylated signal transducer activator

target 3 (p-STAT3) were measured using PathScan Inflammation Multi-Target Sandwich Elisa kit (Cell Signalling Technology) according to the manufacturer's protocol. All reagents and solutions required for this assay were provided in the kit. Cell lysate from palmitic acid-induced HepG2 cells and treated HepG2 cells was prepared as described in Section 3.9.1. One-hundred microliter of cell lysates were incubated in microwells precoated individually with anti-NF-κB p65, anti-phosphorylated-NF-κB p65, anti-phosphorylated-SAPK/JNK, anti-phosphorylated-p38, anti-phosphorylated-IκBα and anti-phosphorylated-STAT3 antibodies, followed by incubation overnight at 4 ^{o}C. Subsequently, the medium was discarded, and the wells were washed four time with 200 µL of 1X washing buffer. Then, washing buffer was discarded and the plate was blotted onto fresh paper to get rid of excess washing buffer. After the washing step, 100 µL of reconstituted detection antibody was added to each well and incubated for one hour at 37 ^{o}C. Then, the solution was removed, and the wells were washed again prior to adding 100 µL of horseradish peroxidase-streptavidin-linked secondary antibody solution into each well. The plate was incubated for 30 minutes at 37 ^{o}C. After the final washing steps, 100 µL of TMB One-Step Substrate Reagent was added to each well and then incubated for 10 minutes at 37 ^{o}C in the dark. Subsequently, the reaction was stopped by adding 100 µL of stop solution to each well in order to stop the colour development. Then, the plate was read at absorbance 450 nm with Multiskan Go microplate spectropotometer (Thermo Sientific). The magnitude of absorbance for the developed colour is proportonal to the quantity of the p-NF-κB p65, p-IκBβ, p-SAPK/JNK, p-p38, p-STAT3, NF-κB.

3.9 Statistical Analysis

All bioassays were expressed as mean ± standard deviation (SD) of three independent experiment with three replicates in each experiment. The results were subjected to one-way analysis of variance (ANOVA) and the significance of the

difference was determined by the Duncan's multiple range tests at 95 % least significant difference ($p<0.05$).

CHAPTER 4: RESULTS

4.1 Yield of crude and fractions from *Auricularia nigricans*

The ethanol extraction of *A. nigricans* yielded 2 % of brown viscous substance from the dried mushroom (Table 4.1). Ethanolic crude was further partitioned to give water, ethyl acetate and hexane fractions. However, water, hexane and ethyl acetate fractions obtained from the dried mushroom were only 0.45 %, 0.44 % and 0.02 % respectively. The percentage yield of fractionated extracts was based on the weight of ethanolic crude extract as shown in Table 4.1. Among all extractions, the yield was most abundant in the water fraction (29.0 %) while the ethyl acetate fraction had the lowest yield, (1.0 %).

Table 4.1: Yield of ethanolic crude and fractions from *A. nigricans*

Samples	Yield (%), w/w of dried mushroom	Yield (%), in the percentage of total ethanol extract
Ethanolic crude	2.00	-
Water	0.45	29.0
Hexane	0.44	28.3
Ethyl acetate	0.02	1.0

4.2 Antioxidant capacity of *A. nigricans*

4.2.1 Total phenolic content of crude and fractions from *A. nigricans*

Table 4.2 presented the average concentration of total phenolic (mg GAE/g dry weight) for ethanolic crude and fractions from *A. nigricans* were analysed from Table 1.2 of Appendix. The highest amount of phenolic content was found in ethyl acetate fraction (105.50 ± 3.15 mg GAE/g) and decreased in the order of water > ethanolic crude >hexane.

Table 4.2: Total Phenolic Content of *A. nigricans*

Samples	Total phenolic content (mg GAE/g)
Ethanolic crude	11.38 ± 0.47^{c}
Hexane	10.62 ± 0.08^{c}
Ethyl acetate	105.50 ± 3.15^{a}
Water	15.10 ± 0.13^{b}

The capability of ethanolic crude and fractions from *A. nigricans* on DPPH scavencing activity, FRAP, $O_2 \cdot^-$ scavenging activity, $OH\cdot$ scavenging activity and ORAC were analysed and presented in Figures 1.2 to 1.13 and in Table 1.18 of Appendix and summarised in Table 4.3. Trolox, a water soluble vitamin E was used as control.

4.2.2 Scavenging of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical

The results of antioxidant activity (DPPH) of ethanolic crude and fractions from *A. nigricans* were presented as IC_{50} values in Table 4.3. In this study, the highest DPPH radical scavenging capacity was detected in ethyl acetate fraction (IC_{50} = 0.17 ± 0.01 mg/mL), followed by ethanolic crude (IC_{50} = 0.96 ± 0.02 mg/mL), water fraction (IC_{50} = 1.62 ± 0.03 mg/mL) and hexane fraction (IC_{50} = 1.82 ± 0.03 mg/mL). The Capacity of trolox (IC_{50} = 0.02 ± 0.00 mg/mL) in scavenging of DPPH radical was significantly higher than all the mushroom samples.

4.2.3 Ferric reducing antioxidant power (FRAP)

The reducing capacity of the samples were determined by the FRAP method. As illustrated in Table 4.3, the ethyl acetate fraction exerted highest antioxidant capacity at 616.76 ± 3.71 mg TE/mg extract. The ferric reducing antioxidant power of ethanolic crude and fractions from *A. nigricans* decreased in order of ethyl acetate > hexane > water > ethanolic crude. However, both ethanolic crude and water fraction were not significantly different.

4.2.4 Scavenging of superoxide anion radical ($O_2 \bullet^-$)

In this study, all samples of *A. nigricans* were not effective in scavenging $O_2 \bullet^-$, except for water fraction. Trolox, which was used as positive control was active in scavenging of the reactive radical, at concentration of 1.72 ± 0.06 mg/mL. Table 4.3

showed that the potential of water fraction in scavenging of superoxide anion, with IC_{50} of 4.25 ± 0.38 mg/mL.

4.2.5 Scavenging of hydroxyl radical (OH·)

The results showed that ethyl acetate fraction strongly scavenged OH·, exhibited the highest scavenging activity on hydroxyl with the IC_{50} of 0.17 ± 0.01 mg/mL which is not significantly ($P<0.05$) different from positive control, trolox (IC_{50} = 0.10 ± 0.00 mg/mL). Crude, water and hexane samples possessed significant difference in antioxidant capacity towards scavenging of OH·, with IC_{50} value of 0.54 ± 0.01, 0.44 ± 0.05 and 1.98 ± 0.07 mg/mL respectively (as shown in Table 4.3).

4.2.6 Oxygen radical absorbance capacity (ORAC)

It was found that all samples of *A. nigricans* and Trolox had the ability to scavenge peroxyl radical. The ORAC values of *A. nigricans* ranged from 188.57 to 2116.92 μmol TE/g extract as shown in Table 4.3. Ethyl acetate fraction (2116.92 ± 66.24 μmol TE/g extract) was significantly the most efficient in scavenging of ROO· compared to water fraction (188.57 ± 17.32 μmol TE/g extract), hexane fraction (372.40 ± 44.58 μmol TE/g extract) and ethanolic crude (752.51 ± 96.14 μmol TE/g extract).

4.3 Profiling of phytoconstituents in bioactive fraction of *A. nigricans*

Both ethyl acetate and water fractions from *A. nigricans* were potent in antioxidant effects towards various free radicals. Hence, they were selected in attempting to determine bioactive compounds availability via GCMS and LCMS.

Table 4.3: Antioxidant Capability of *A. nigricans*

Samples	DPPH IC_{50}, mg/ml	FRAP mg TE/g	$O_2^{\cdot -}$ IC_{50}, mg/ml	$OH^{\cdot}$ IC_{50}, mg/ml	ORAC µmol TE/g
Trolox	0.02 ± 0.00^{e}	-	1.72 ± 0.06^{b}	0.10 ± 0.00^{d}	-
Ethanolic crude	0.96 ± 0.02^{c}	60.02 ± 2.47^{c}	NA	0.54 ± 0.01^{b}	752.51 ± 96.14^{b}
Hexane	1.82 ± 0.03^{a}	105.66 ± 2.90^{b}	NA	1.98 ± 0.07^{a}	372.40 ± 44.58^{c}
Ethyl acetate	0.17 ± 0.01^{d}*	616.76 ± 3.71^{a}*	NA	0.17 ± 0.01^{d}*	2116.92 ± 66.24^{a}*
Water	1.62 ± 0.03^{b}	63.41 ± 2.41^{c}	4.25 ± 0.38^{a}	0.44 ± 0.05^{c}	188.57 ± 17.32^{d}

Results were expressed as mean ± standard deviation of triplicate measurements (n=3). The different superscript letter (a-d) within column represent means with significant difference ($p<0.05$). Trolox was used as a positive control in all antioxidant assays. GAE, Gallic acid equivalent. TE, Trolox equivalent. µmol TE/g, micromoles Trolox equivalents per gram sample. IC_{50} (mg/mL): inhibition concentration at which 50% of radicals are scavenged. NA: not active. * indicated fraction that exhibited the highest capacity in scavenging radical in respective assays.

4.3.1 GCMS profile of bioactive constitutes in *A. nigricans*

The major chemical components in bioactive ethyl acetate fraction were identified via GCMS. GCMS analysis of ethyl acetate fraction from *A. nigricans* led to the identification of linoleic acid ethyl ester, butyl-9,12-octadecadienoate, 9,12-Octadecadienoate and ergosta-5,7,22-trien-3-ol as shown in Table 4.4. The total amount of identified compound occupied 75.65 % of the total peak area.

Table 4.4: Phytoconstituents identified in ethyl acetate fraction from *A. nigricans* by GCMS analysis

No.	Probable compounds	Retention time (min)	Peak area (%)	Molecular formula	Compound nature
1.	Linoleic acid ethyl ester	32.572	2.12	$C_{20}H_{36}O_2$	Fatty acid
2.	Butyl 9,12-octadecadienoate	37.699	6.65	$C_{22}H_{40}O_2$	Fatty acid
3.	9, 12-Octadecadienoic acid	40.949	5.75	$C_{18}H_{32}O_2$	Fatty acid
4.	Ergosta-5,7,22-trien-3-ol	47.947	60.97	$C_{28}H_{44}O$	Steroid

4.3.2 LCMS-MS profile of bioactive constitutes from *Auricularia nigricans*

Further analytical LCMS-MS was undertaken to assess the various phytoconstituents present in the bioactive ethyl acetate fraction. Full LCMS-MS of ethyl acetate fraction of *A. nigricans* (Figure 4.1) showed the presence of fifteen peaks at retention times of 2.90', 3.52', 4.33', 4.72', 4.98', 5.39', 5.79', 6.30', 8.01', 8.41', 8.99', 9.36', 10.02', 10.66', 11.49'. Of the total, only two compounds were detected as 2(3.4-Dihydroxyphenyl)-7-hydroxy-5-benzene propanoic acid at 8.41', with average mass of 309.17 Da, and 3, 30-di-O-methyl ellagic acid at 6.30', average mass of 328.25 Da as in Table 4.5.

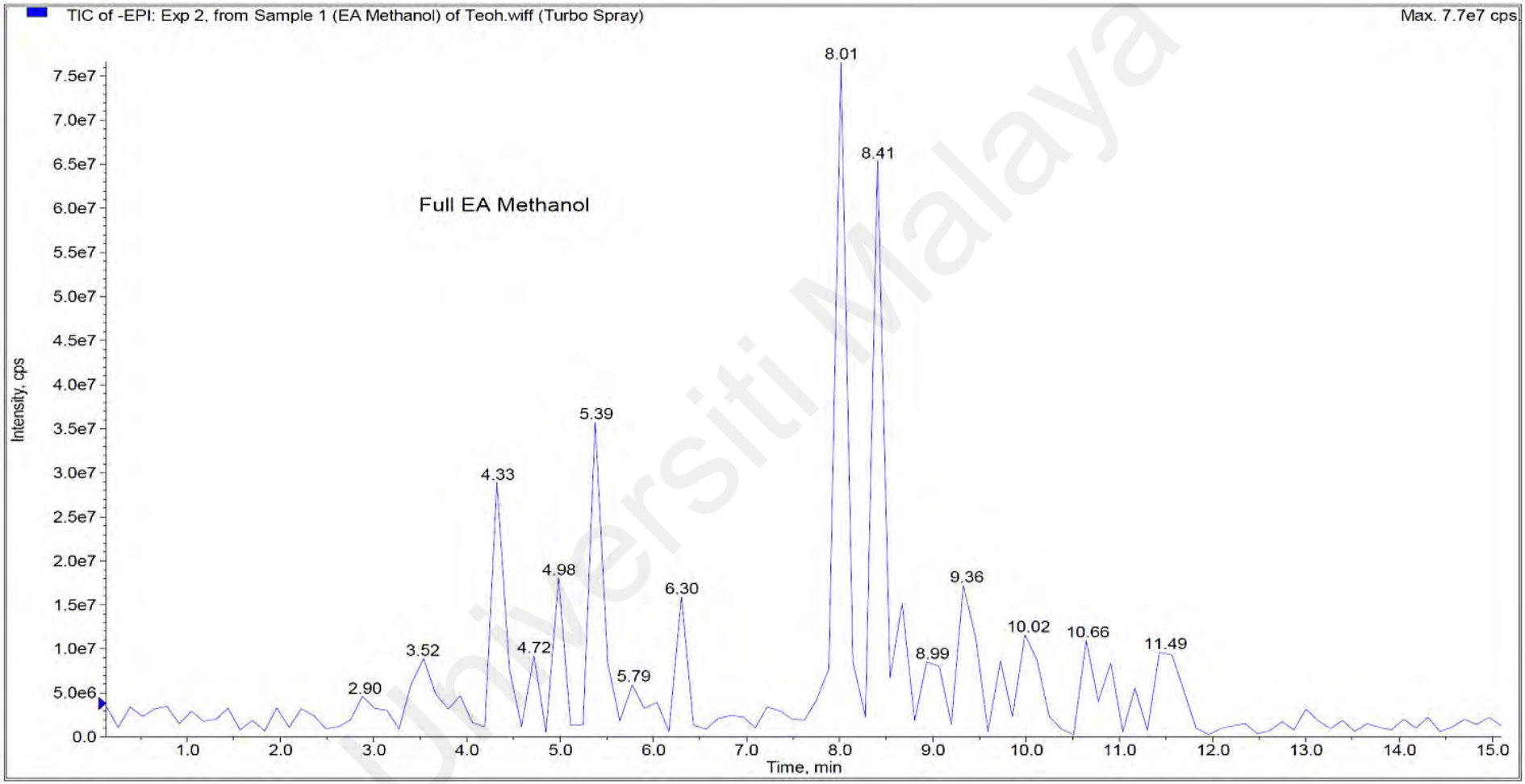

Figure 4.1: LCMS/MS profile of ethyl acetate fraction from *Auricularia nigricans*

Table 4.5: Phytoconstituents identified in ethyl acetate fraction from *Auricularia nigricans* by LCMS-MS

No.	Probable compounds from LCMS-MS analysis	Retention time (min)	Average mass (Da)
1	2(3.4-Dihydroxyphenyl)-7-hydroxy-5-benzene propanoic acid	8.41	309.17
2	3, 30-di-O-methyl ellagic acid	6.30	328.25

The identification of main bioactive phytohemicals presented in water fraction was carried out via LCMS/MS. Water fraction from *A. nigricans* also showed the presence of twenty peaks as illustrated in Figure 4.2. However, only four compounds were identified, including oxooctadecanoic acid, 2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid and two isomers of 2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid with retention time of 7.99', 8.25', 11.06' and 11.66' respectively. Average mass for oxooctadecanoic acid was 295.15Da, whereas 2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid and its isomers were 309.15 Da, 309.39 Da and 309.38 Da (Table 4.6) respectively.

Table 4.6: Phytoconstituents identified in water fraction from *Auricularia nigricans* by LCMS/MS

No.	Probable compounds	Retention time (min)	Average mass (Da)
1.	Oxooctadecanoic acid	7.99	295.15
2.	2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid	8.25	309.15
3.	2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid isomer	11.06	309.39
4.	2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid isomer	11.66	309.38

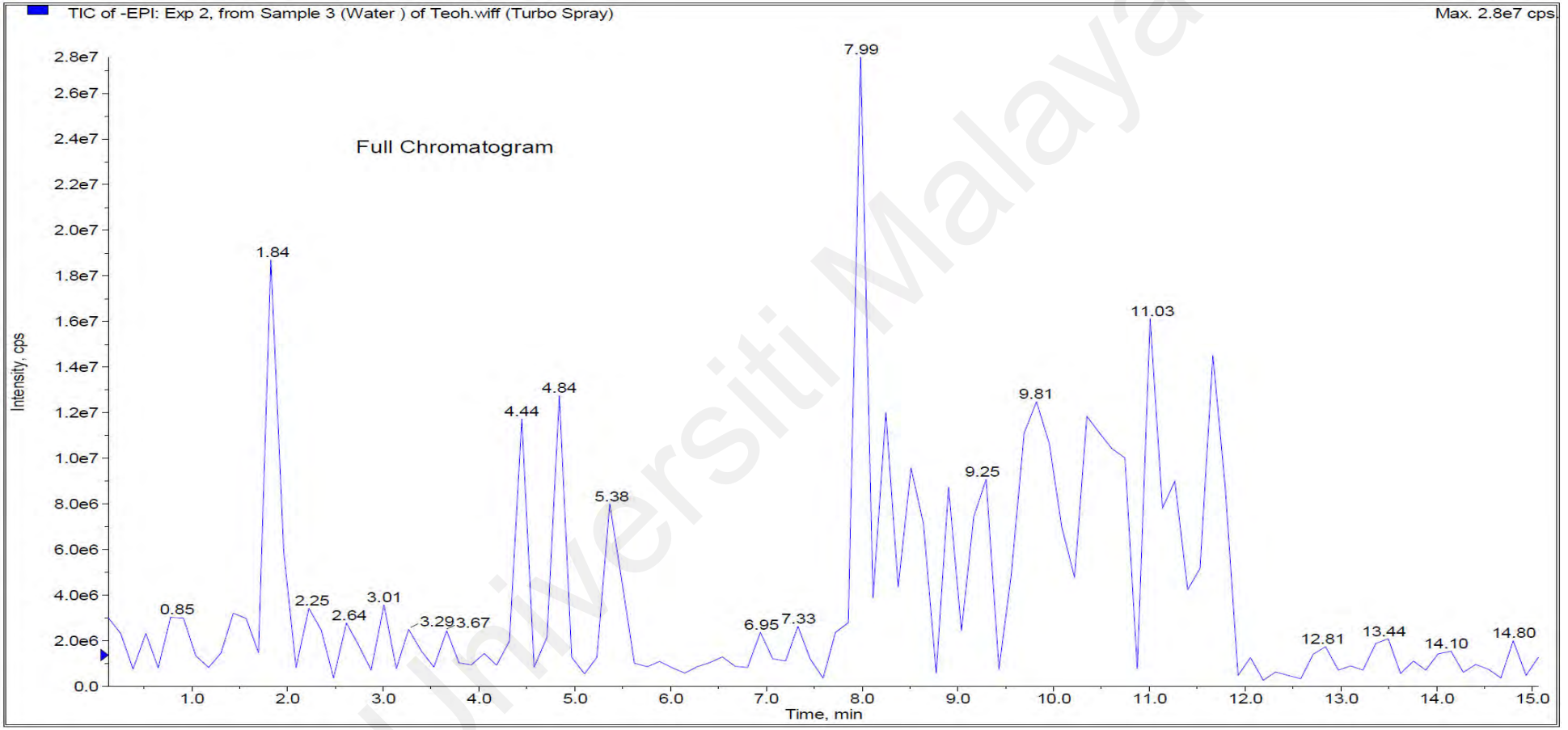

Figure 4.2: LCMS/MS profile of water fraction from *Auricularia nigricans*

4.4 Viability assay

4.4.1 Cytotoxicity effect of ethyl acetate and water fractions on HepG2 cells

To determine the effect of water and ethyl acetate fractions on HepG2 cells viability, MTT assay was performed to select a concentration that exhibited the least toxicity to the cells. HepG2 cells were exposed to various concentrations of water and ethyl acetate fractions (6 - 100 µg/ml), for 24 and 48 hours. The results are shown in Figures 4.3 and 4.4.

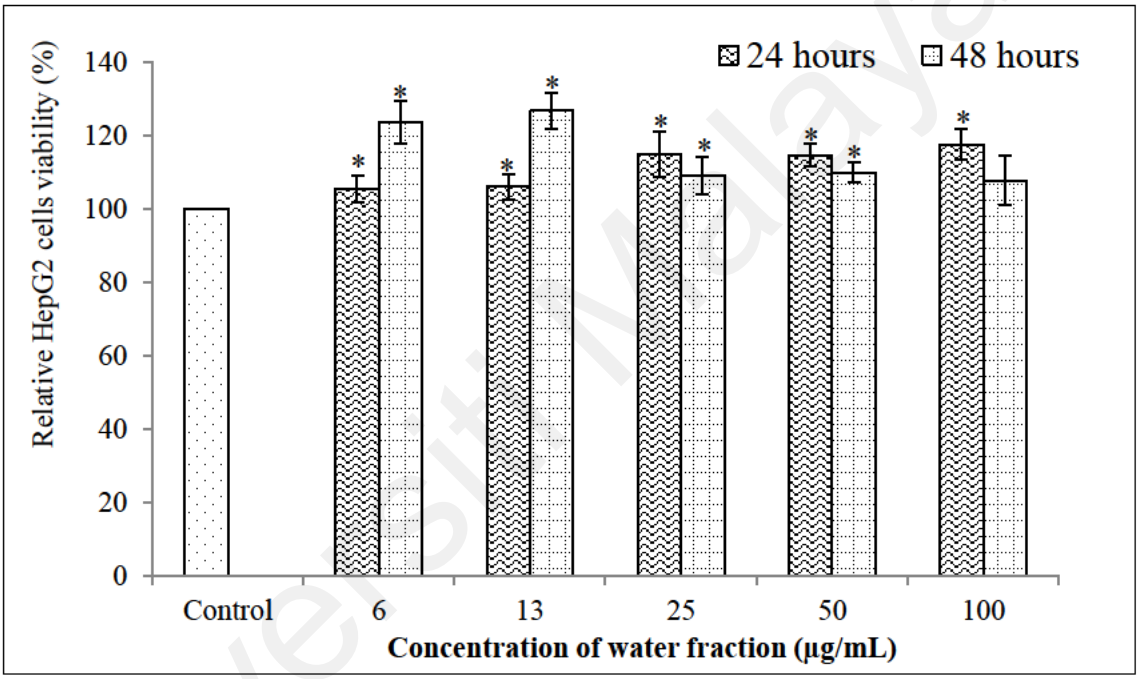

Figure 4.3: Water fraction from *A. nigricans* was tested for cytotoxicity on HepG2 cell line.

The treatment of water fraction from *A. nigricans* to HepG2 cells was carried out for 24 and 48 hours and cytotoxicity was performed using MTT assay. The water fraction concentrations were 100, 50, 25, 13 and 6 µg/mL. The mean absorbance obtained from wells contained only medium with HepG2 cells (untreated control) was designated as 100 % viable and readings of experimental well were expressed as percentage of untreated control. Data expressed were as mean ± standard deviation of three independent experiments (n=3) in triplicate. *$p<0.05$ significantly different from untreated control.

As shown in Figure 4.3, treatment of HepG2 cells at different concentrations (6–100 µg/mL) of water fraction did not decrease the cell viability, conversely, water fraction slightly induce proliferation of HepG2 cells. After 24 hours of incubation with 6, 12.5, 25, 50, 100 µg/mL of water fraction, cells viability was 105.52 ± 2.67, 106. 05 ± 3.45, 114.64 ± 4.39, 114.81 ± 2.13 and 117.70 ± 2.95 %, respectively. Viability of HepG2 cells

decreased slightly after 48 hours of exposure to water fraction at concentration of 25, 50 and 100 µg/mL, which gave, 109.19 ± 5.08, 109.87 ± 2.77, and 107.81 ± 6.77 % respectively. However, viability of HepG2 cell increased to123.64 ± 5.86, 126.72 ± 4.81 for 48 hours of incubation with 6 and 13 µg/mL of water fraction, respectively (Figure 4.3).

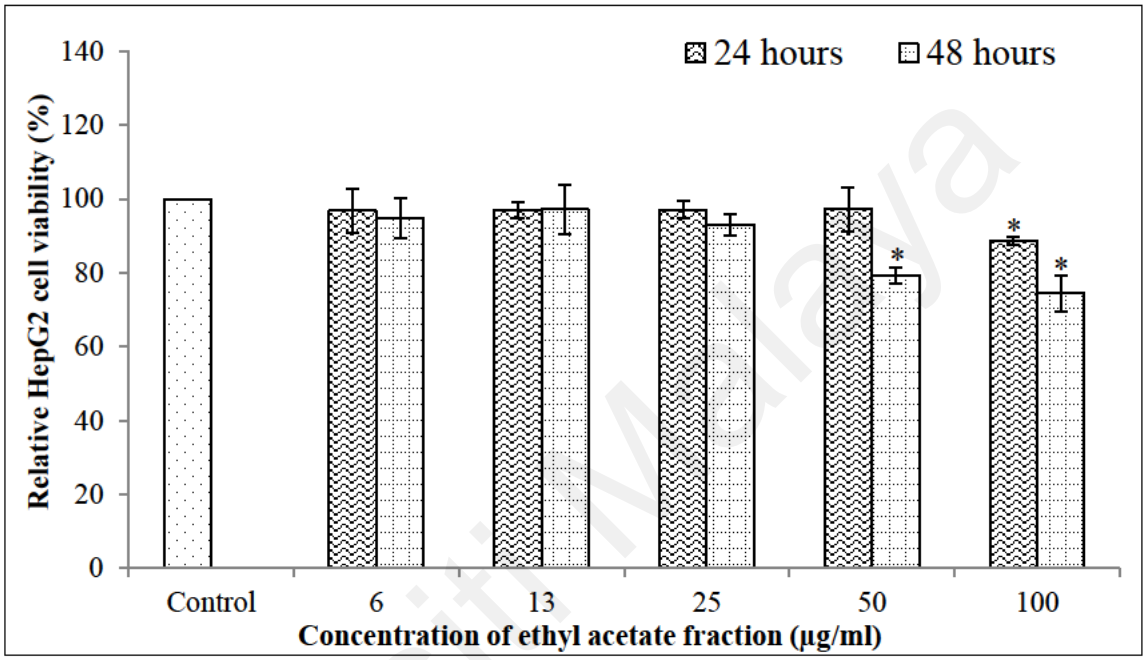

Figure 4.4: Ethyl acetate fraction from *A. nigricans* was tested for cytotoxicity on HepG2 cell line.

The treatment of ethyl acetate fraction from *A. nigricans* to HepG2 cells was carried for 24 and 48 hours and cytotoxicity was performed using MTT assay. The ethyl acetate fraction concentrations were 100, 50, 25, 13 and 6 µg/mL. The mean absorbance obtained from wells contained only medium with HepG2 cells (untreated control) was designated as 100 % viable and readings of experimental well were expressed as percentage of untreated control. Data expressed were as mean ± standard deviation of three independent experiments (n=3) in triplicate. *$p<0.05$ significantly different from untreated control.

Conversely, ethyl acetate fraction elicited some cytotoxicity at high concentration. At concentration of 100 µg/mL, ethyl acetate fraction significantly reduced HepG2 cells viability to 88.56 ± 1.03 % after 24 hours of exposure (Figure 4.4). Concentration of ethyl acetate fraction below 100 ug/mL showed no significant effect on the HepG2 cells viability. 50µg/mL of ethyl acetate fraction gave 97.23 ± 5.99 % cell viability. Incubation of HepG2 cells with 6, 13, 25 µg/mL of ethyl acetate fraction for 24 hours contributed to viability cell of 96.79 ± 5.85, 96.98 ± 2.15, and 97.00 ± 2.30 %. The effect of different concentrations of ethyl acetate fraction on HepG2 cells after 48 hours of incubation was

shown in Figure 4.4. HepG2 cell viability was 94.70 ± 5.32, 97.16 ± 6.62, 93.04 ± 2.80, 79.38 ± 2.20, 74.47 ± 4.84 % for 6, 13, 25, 50, 100 µg/mL of ethyl acetate fraction after 48 hours of exposure to the varying concentrations of fraction.

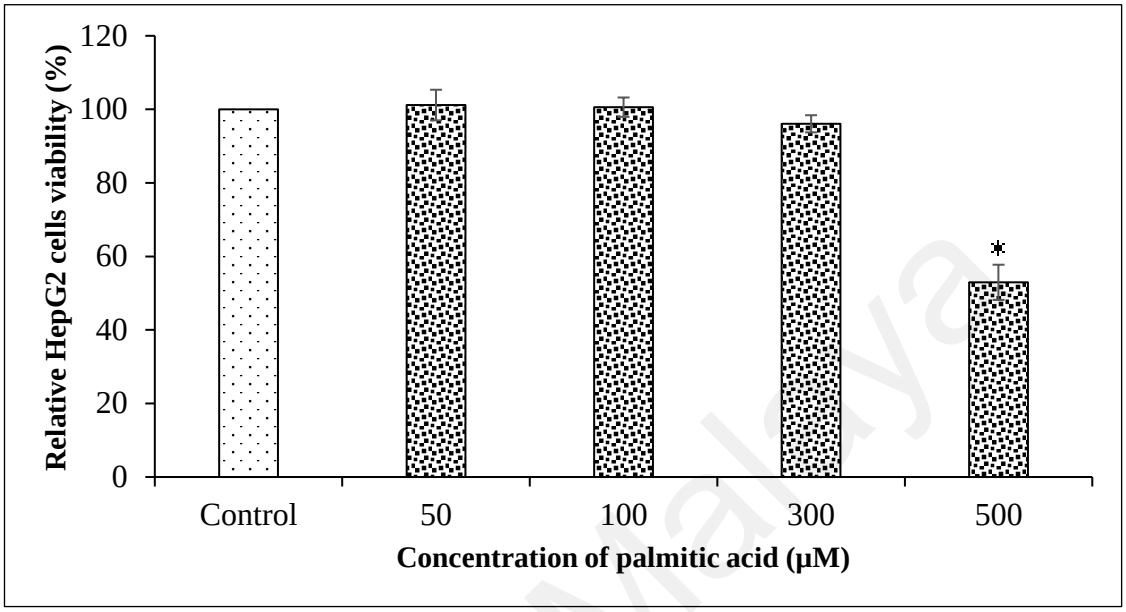

Figure 4.5: Cytotoxicity of palmitic acid.

Various concentration of palmitic acid including 50, 100, 300 and 300 µM were tested for cytotoxicity on HepG2 cell line for 24 hours followed by MTT assay for cell viability. The mean absorbance obtained from wells contained only medium with HepG2 cells (untreated control) was designated as 100 % viable and readings of experimental well were expressed as percentage of untreated control. Data expressed were as mean ± standard deviation of three independent experiments (n=3) in triplicate. *p<0.05 significantly different from untreated control.

4.4.2 Cytotoxic effect of palmitic acid on HepG2 cells

The aim of this assay was to determine the optimal concentration of palmitic acid that would cause minimal cytotoxicity to the cells in order to be able to study the effect of treatment on the palmitic acid induced NAFLD. The effect of palmitic acid on HepG2 cells was determined at concentrations of 50, 100, 300, and 500 µM for 24 hours are presented in Figure 4.5. In the presence of 50 and 100 µM of palmitic acid, cell viability was 101.17 ± 4.16 and 99.69 ± 2.64 % respectively, Palmitic acid at concentration of 300µM led to slight decrease but not significant cytotoxicity towards HepG2 cells viability (96.07 ± 2.33 %). Compared to the lower concentrations of palmtic acid, the survival rate of HepG2 cells drastically decreased upon exposure to 500 µM of pamitic

acid, which caused a significant reduction in cell viability, it led to HepG2 cells viability decreased to 52.95 ± 4.79 %.

4.5 Establishment of palmitic acid-induced hepatic steatotic HepG2 cell model

Lipid accumulation is a hallmark of NAFLD. Hence, based on the MTT assay result, non-cytotoxic concentrations of palmitic acid of 50, 100, 300 µM were exposed to HepG2 cells for 24 hours in order to induce cells to steatotic condition. In order to observe lipid content in HepG2 cells, the cells that had been infused with palmitic acid after 24 hours were stained by Oil Red O (ORO) staining. After ORO staining, lipid accumulated in the HepG2 cells were observed under inverted microscope.

Fusion of lipid droplets in cells indicated that the HepG2 cells were successfully loaded with lipid. Figure 4.6 demonstrated the results of ORO staining for HepG2 cells by observing under bright field of inverted microscope. Evidently, a large amount of lipid droplet was found in HepG2 cells after 24 hours of exposure to 300 µM of palmitic acid, as compared to HepG2 cells treated with BSA only, which indicated successful establishment of *in vitro* hepatic steatosis model. Hence, 300 µM of palmitic acid was used to establish HepG2 cell model of palmitic acid-induced hepatic steatosis for subsequent assays. On the other hand, HepG2 cells exposed to 50 µM of palmitic acid clearly showed absence of intracellular lipid accumulation. Lipid accumulated in HepG2 cells exposed to 100 µM was less than cells cultured in medium with the presence of 300 µM of palmitic acid. Based on viability assay, 500 µM of palmitic acid was cytotoxic towards the cells, hence, it was not evaluated in this assay. Quantitative measurement obtained from 510 nm was consistent with the pictographic analysis. Accumulation of lipid in HepG2 cells exhibited a dose dependent pattern compared to non-induced cells as shown in Figure 4.6.

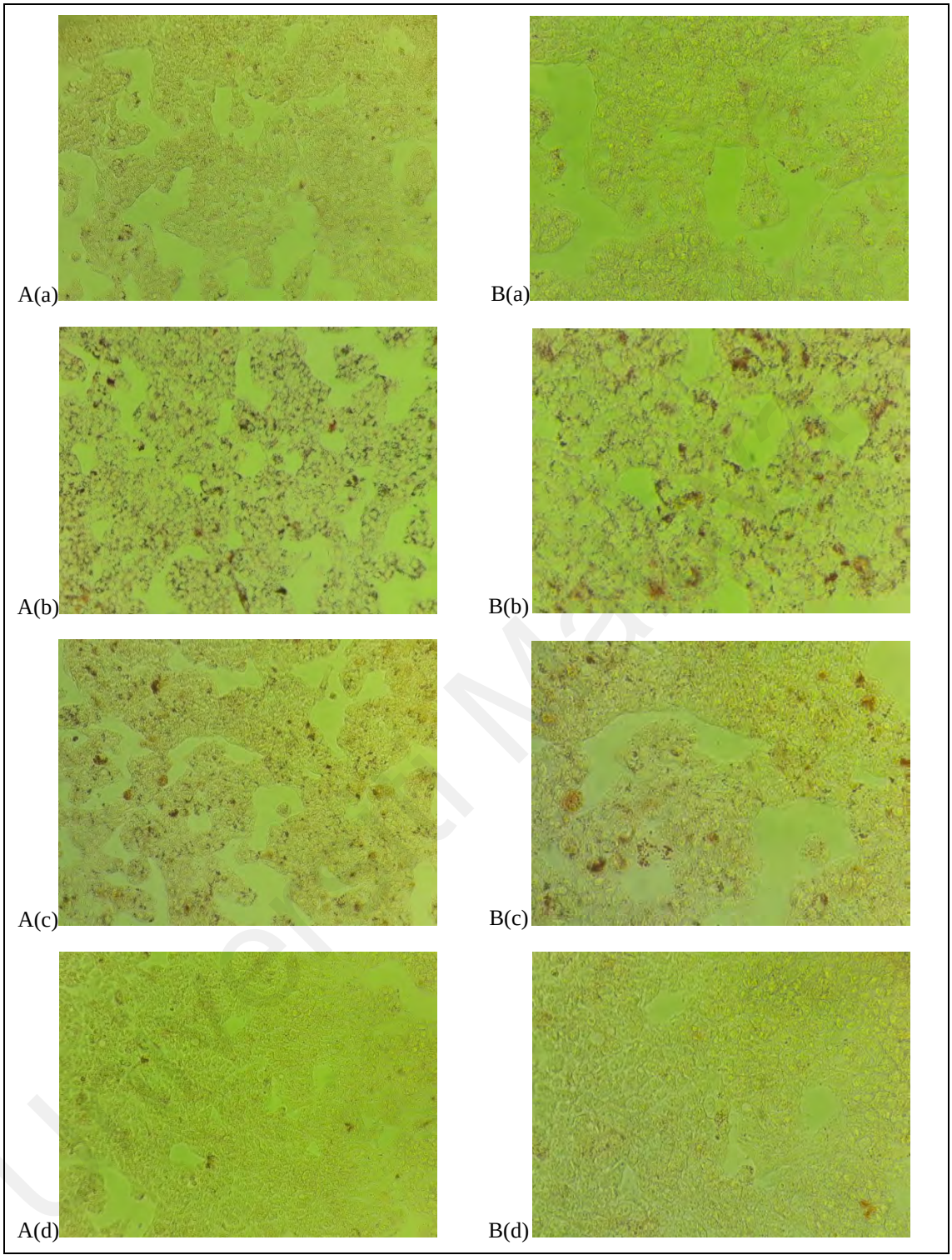

Figure 4.6 Palmitic acid induced lipid accumulation in HepG2 cells.
Lipid accumulation in HepG2 cells was observed by Oil-Red O staining after treating cells with different concentrations of palmitic acid (50, 100 and 300 µM) for 24 hours. (A) Representative photomicrographs of HepG2 cells (magnification × 200). A(a) control cell; A(b) cells induced with 300 µM of palmitic acid; A(c) cells induced with 100 µM of palmitic acid; A(d) cells induced with 50 µM of palmitic acid.
(B) Representative photomicrographs of HepG2 cells (magnification × 400). B(a) control cell; B(b) cells induced with 300 µM of palmitic acid; B(c) cells induced with 100 µM of palmitic acid; B(d) cells induced with 50 µM of palmitic acid.

A quantitative measurement for lipid accumulation showed significant increased in 300 µM palmitic acid treated cells, at absorbance value of 0.251 ± 0.021, when

compared to BSA treated cells (0.134 ± 0.03) as shown in Figure 4.7. In comparison to the control cells, 100 µM of palmitic acid showed non-significant ($p<0.05$) lipid droplet accumulation in cells, at absorbance value of 0.157 ± 0.009. Conversely, cells treated with 50 µM did not show obvious increase of lipid (0.137 ± 0.009), relative to the control cells.

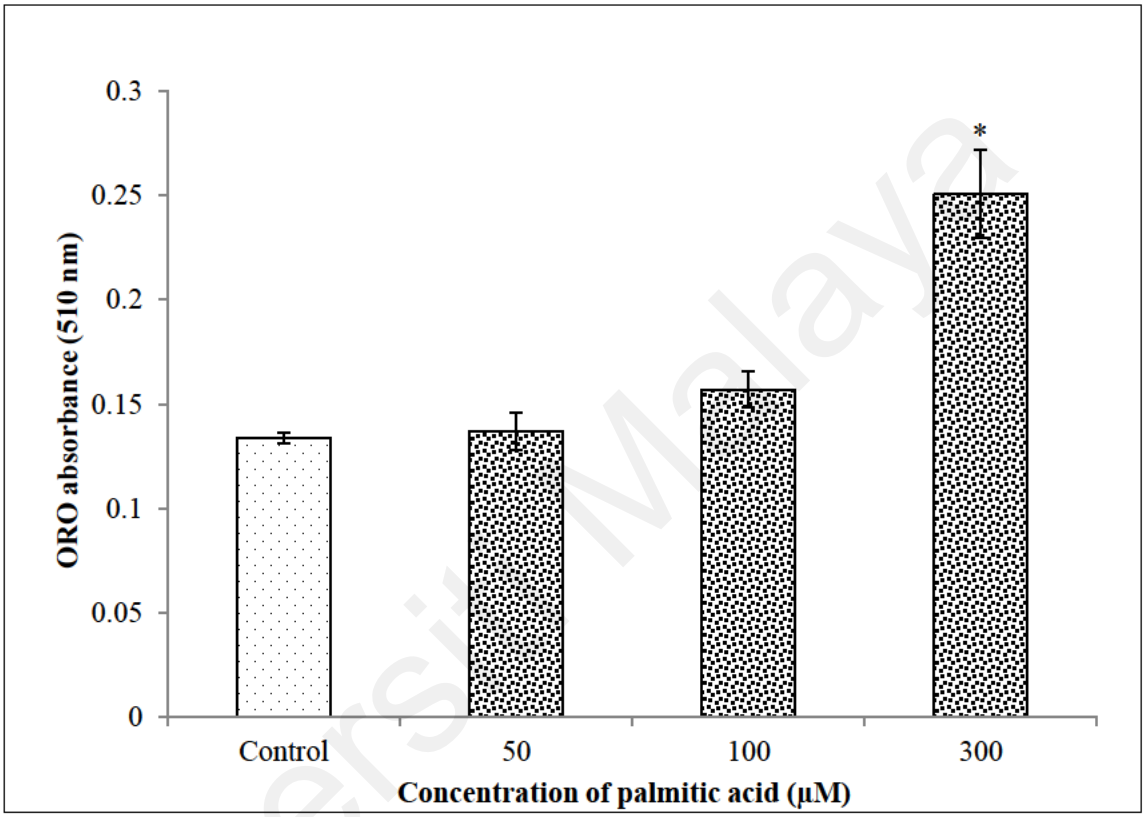

Figure 4.7 Palmitic acid-induced intracellular lipid accumulation in HepG2 cells.
Effect of different concentrations of palmitic acid on lipid accumulation in HepG2 in order to establish induced hepatic steatosis. The optimal palmitic acid concentrations that induce HepG2 cells to become steatotic model was determined by Oil Red O staining. Oil Red O - based colorimetric assay was spectrophotometrically read at 510 nm. Results were expressed as means ± standard deviation of triplicate measurement (n=3) in three independent experiments. Data were analysed by one-way ANOVA followed by Duncan test. The differences between means were significant at * ($p<0.05$), compared with the control (medium with BSA only).

4.6 Anti-hepatosteatotic and intracellular ROS scavenging effects of water and ethyl acetate fractions from *A. nigricans*

4.6.1 Anti-hepatic steatotic effect

To determine the inhibitory effect of water and ethyl acetate fractions on hepatic lipid accumulation, cells were co-treated with 300 µM of palmitic acid and fractions at different concentrations. HepG2 cells were cultured in medium in the presence of BSA, without sample was used as a control, whereas samples were treated to cells at non-cytotoxic concentration of 100, 50, 25, 13, 6 µg/mL for 24 hours. However, the highest non-cytotoxic concentrations of ethyl acetate fraction used in this assay was 50 µg/mL. Similar to section 4.6, after 24 hours of co-treatment in HepG2 cells, cells were stained with Oil Red O, then images were captured by microscope at a magnification of 200X and 400X before a quantitative measurement for lipid accumulation was taken at 510 nm.

Microscopic results showed that induced HepG2 cells with 300 µM of palmitic acid for 24 hours resulted in steatosis, which elicited an abundance of intracellular lipid in the cells. In contrast, little lipid droplet was found in the non-induced cells. Induced-cells co-treated with water fraction from *A. nigricans* did not show obvious decrease of lipid accumulation in the cell at the tested concentrations, as shown in Figure 4.8. Observation from the Oil Red O staining experiment was confirmed by the quantitative measurement, as showed in Figure 4.9. At 510 nm, palmitic acid-induced HepG2 cells gave an absorbance of.0.238 ± 0.012, whereas water fraction treated cells showed no significant ($p<0.05$) different from the palmitic acid induced cells by giving absorbencies of 0.237 ± 0.006, 0.227 ± 0013, 0.223 ± 0.006, 0.222 ± 0.006, 0.223 ± 0.006 for tested concentrations 6, 13, 25, 50 and 100µg/mL, respectively.

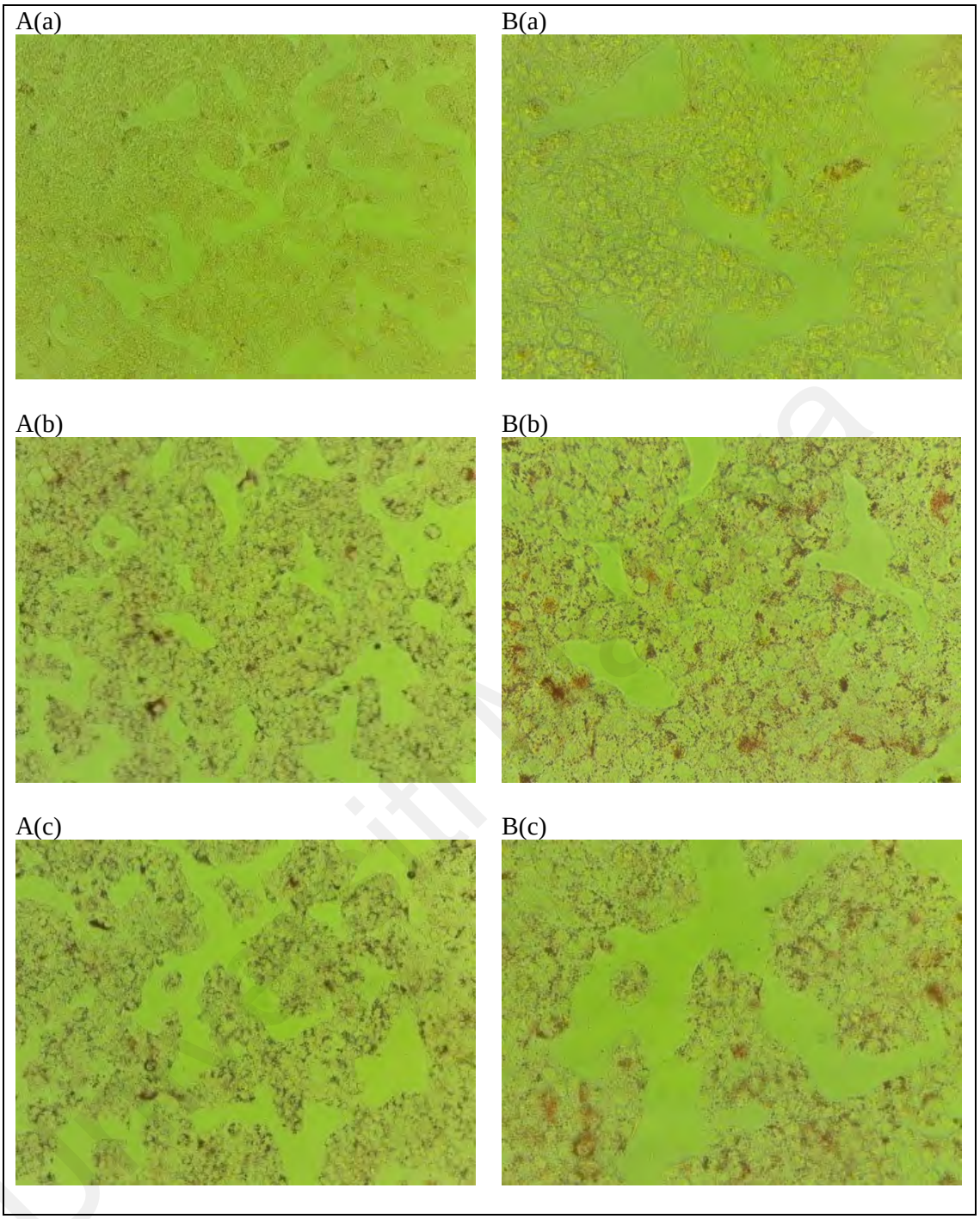

Figure 4.8: Water fraction treatment towards palmitic acid-induced lipid accumulation in HepG2 cells.

Lipid accumulation in HepG2 cells was observed by Oil-Red O staining after co-treating the cells with 300 µM of palmitic acid and various concentrations of water fraction for 24 hours. (A) Representative photomicrographs of HepG2 cells (magnification × 200). (a) control cell; (b) cells induced with 300 µM of palmitic acid; (c) cells co-treated with palmitic acid and 6 µg/mL water fraction; (d) cells co-treated with palmitic acid and 13 µg/mL water fraction. (e) cells co-treated with palmitic acid and 25 µg/mL water fraction; (f) cells co-treated with palmitic acid and 50 µg/mL water fraction; (g) cells co-treated with palmitic acid and 100 µg/mL water fraction. (B) Representative photomicrographs of HepG2 cells (magnification × 400). (B) (a) control cell; (b) cells induced with 300 µM of palmitic acid; (c) cells co-treated with palmitic acid and 6 µg/mL water fraction; (d) cells co-treated with palmitic acid and 13 µg/mL water fraction. (e) cells co-treated with palmitic acid and 25 µg/mL water fraction; (f) cells co-treated with palmitic acid and 50 µg/mL water fraction; (g) cells co-treated with palmitic acid and 100 µg/mL water fraction.

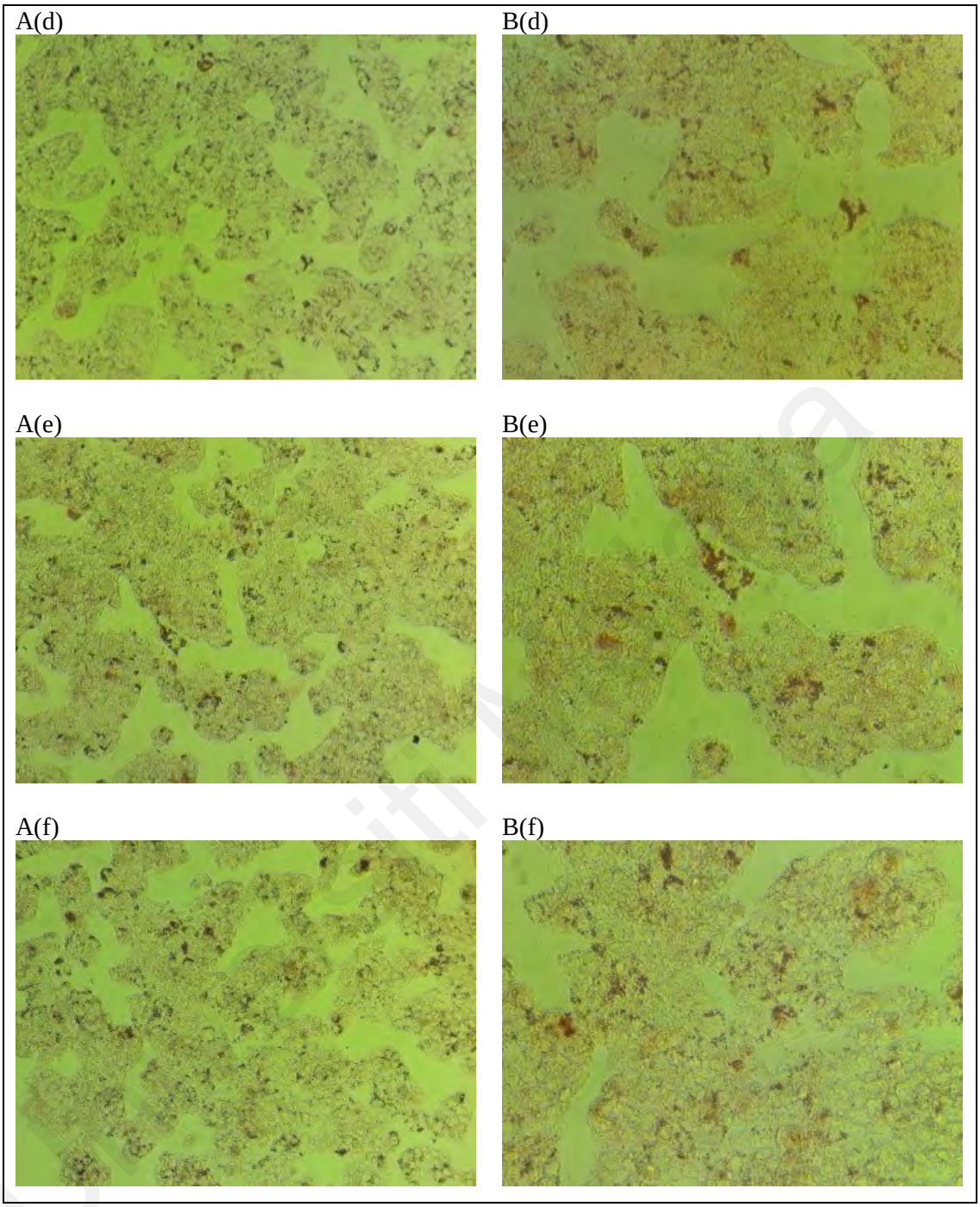

Figure 4.8, Continued: Water fraction treatment towards palmitic acid-induced lipid accumulation in HepG2 cells.

Lipid accumulation in HepG2 cells was observed by Oil-Red O staining after co-treating the cells with 300 µM of palmitic acid and various concentrations of water fraction for 24 hours. (A) Representative photomicrographs of HepG2 cells (magnification × 200). (a) control cell; (b) cells induced with 300 µM of palmitic acid; (c) cells co-treated with palmitic acid and 6 µg/mL water fraction; (d) cells co-treated with palmitic acid and 13 µg/mL water fraction. (e) cells co-treated with palmitic acid and 25 µg/mL water fraction; (f) cells co-treated with palmitic acid and 50 µg/mL water fraction; (g) cells co-treated with palmitic acid and 100 µg/mL water fraction. (B) Representative photomicrographs of HepG2 cells (magnification × 400). (B) (a) control cell; (b) cells induced with 300 µM of palmitic acid; (c) cells co-treated with palmitic acid and 6 µg/mL water fraction; (d) cells co-treated with palmitic acid and 13 µg/mL water fraction. (e) cells co-treated with palmitic acid and 25 µg/mL water fraction; (f) cells co-treated with palmitic acid and 50 µg/mL water fraction; (g) cells co-treated with palmitic acid and 100 µg/mL water fraction.

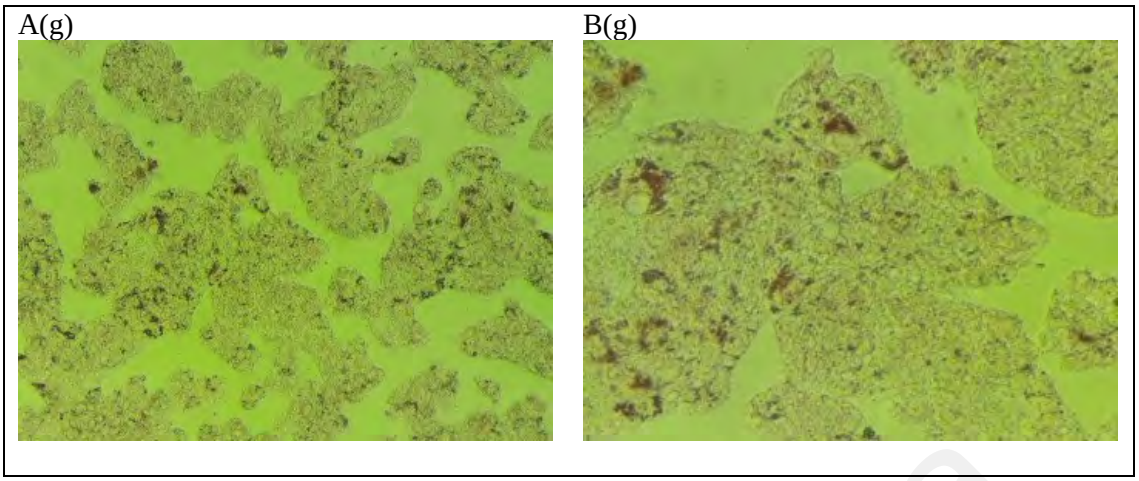

Figure 4.8, Continued: Water fraction treatment towards palmitic acid-induced lipid accumulation in HepG2 cells.

Lipid accumulation in HepG2 cells was observed by Oil-Red O staining after co-treating the cells with 300 µM of palmitic acid and various concentrations of water fraction for 24 hours. (A) Representative photomicrographs of HepG2 cells (magnification × 200). (a) control cell; (b) cells induced with 300 µM of palmitic acid; (c) cells co-treated with palmitic acid and 6 µg/mL water fraction; (d) cells co-treated with palmitic acid and 13 µg/mL water fraction. (e) cells co-treated with palmitic acid and 25 µg/mL water fraction; (f) cells co-treated with palmitic acid and 50 µg/mL water fraction; (g) cells co-treated with palmitic acid and 100 µg/mL water fraction. (B) Representative photomicrographs of HepG2 cells (magnification × 400). (B) (a) control cell; (b) cells induced with 300 µM of palmitic acid; (c) cells co-treated with palmitic acid and 6 µg/mL water fraction; (d) cells co-treated with palmitic acid and 13 µg/mL water fraction. (e) cells co-treated with palmitic acid and 25 µg/mL water fraction; (f) cells co-treated with palmitic acid and 50 µg/mL water fraction; (g) cells co-treated with palmitic acid and 100 µg/mL water fraction.

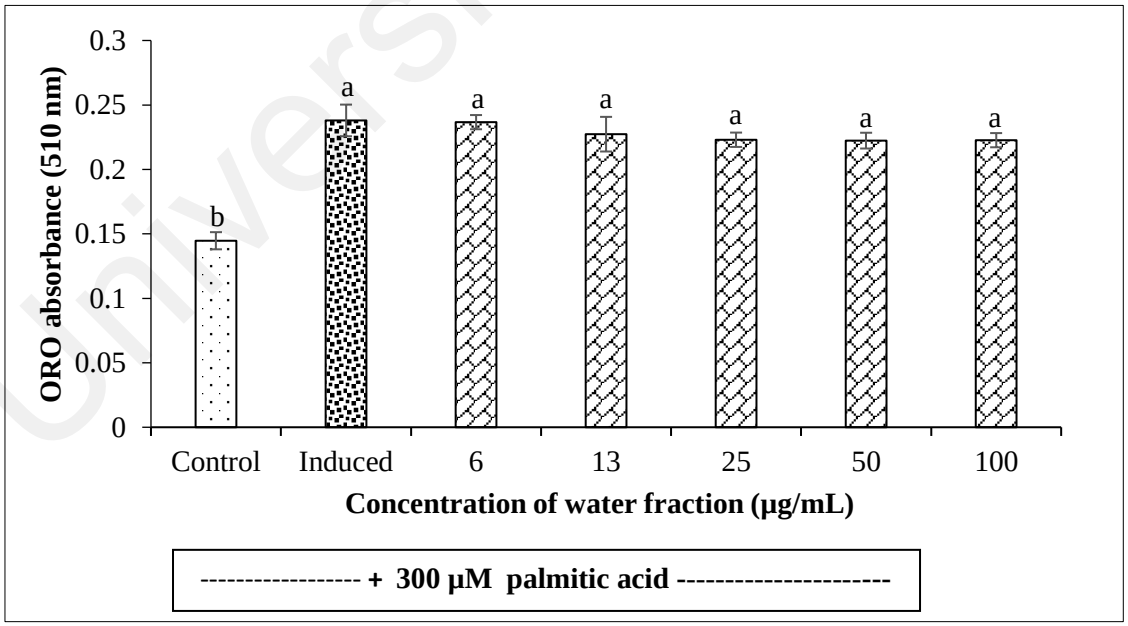

Figure 4.9: Effect of water fraction treatment on intracellular lipid HepG2 cells.

Effect of different concentration of water fraction on lipid accumulation in HepG2. Oil Red O-based colorimetric assay was spectrophotometrically read at 510 nm. Results were expressed as means ± standard deviation of triplicate measurement (n=3) in three independent experiments. Data were analysed by one-way ANOVA followed by Duncan test. The different letters represent means with significant different ($p<0.05$), compared with the control (medium with BSA only).

The influence of ethyl acetate fraction from *A. nigricans* on inhibition of lipid accumulation in HepG2 cells are presented in Figures 4.10 and 4.11. Ethyl acetate fraction exhibited a potential to inhibit lipid droplet accumulation in HepG2 cells when visually observed cells under microscope. Photomicrographic analysis demonstrated that more lipid accumulated in the cells co-treated with 6 µg/mL of ethyl acetate fraction and palmitic acid for 24 hours. Conversely, less intracellular lipid was observed when cells were treated with 50 µg/mL of ethyl acetate fraction. Consistently, result from photomicrographic analysis were in agreement with the quantitative measurement at 510 nm. Ethyl acetate fraction suppressed the intracellular lipid level in HepG2 cells. At all the tested concentractions, ethyl acetate fraction treatment effectively declined the intracelluar lipid level in HepG2 cells. 50 µg/mL of ethyl acetate fraction caused a decline to absorbance to 0.195 ± 0.015, which was approximately 18.1 % reduction in intracellular lipid as compared to the palmitic acid-induced cells (0.238 ± 0.015). The intracellular lipid level in HepG2 cells was 0.205 ± 0.011, 0.215 ± 0.015, 0.215 ± 0.013, or 13.7 %, 9.7 %, 9.7 % individually corresponding to 25, 13, 6 µg/mL of ethyl acetate fraction treatment (Figure 4.11).

Surprisingly, 6, 13, 25 and 50 µg/mL of ethyl acetate fraction showed no significant different in the capability in reducing the accumulation of lipid in HepG2 cells. Both photomicographic analysis and quantitative measurement results suggested that ethyl acetate fraction had significant protective effect against intracellular lipid accumulation in HepG2 cells.

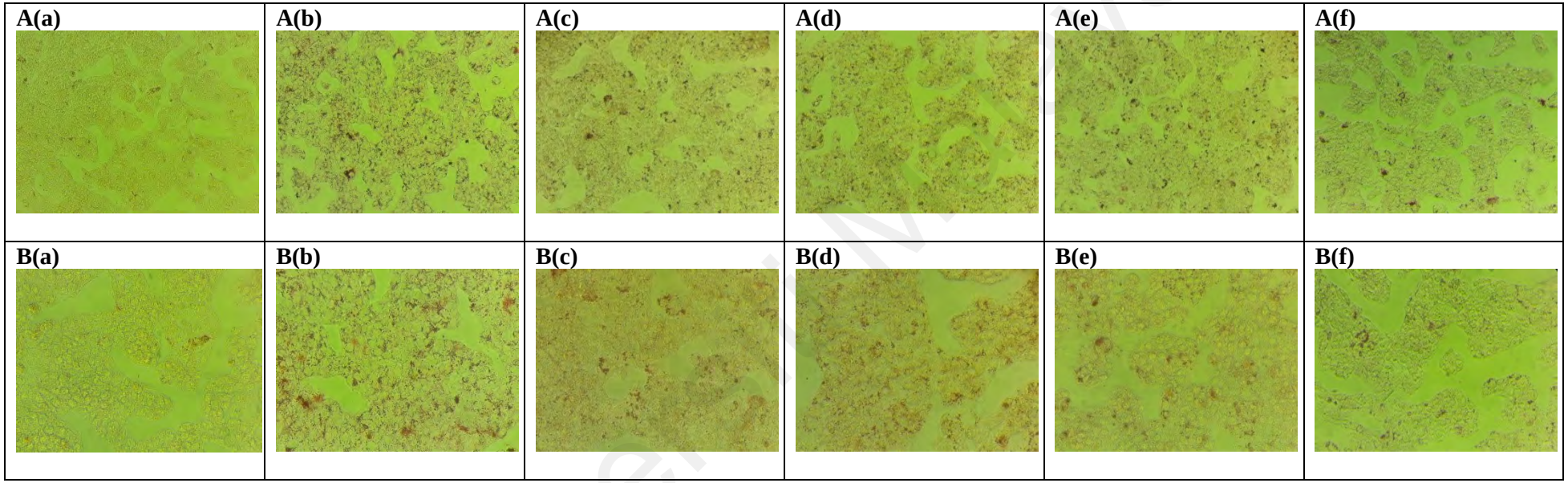

Figure 4.10: Ethyl acetate fraction treatment towards palmitic acid-induced lipid accumulation in HepG2 cells.

Lipid accumulation in HepG2 cells was observed by Oil-Red O staining after co-treating the cells with 300 µM of palmitic acid and various concentrations of ethyl acetate fraction for 24 hours. (A) Representative photomicrographs of HepG2 cells (magnification × 200). A(a) control cell; (b) cells induced with 300 µM of palmitic acid; (c) cells co-treated with palmitic acid and 6 µg/mL ethyl acetate fraction; (d) cells co-treated with palmitic acid and 13 µg/mL ethyl acetate fraction. (e) cells co-treated with palmitic acid and 25 µg/mL ethyl acetate fraction; (f) cells co-treated with palmitic acid and 50 µg/mL ethyl acetate fraction. (B) Representative photomicrographs of HepG2 cells (magnification × 400). (B) (a) control cell; (b) cells induced with 300 µM of palmitic acid; (c) cells co-treated with palmitic acid and 6 µg/mL ethyl acetate fraction; (d) cells co-treated with palmitic acid and 13 µg/mL ethyl acetate fraction. (e) cells co-treated with palmitic acid and 25 µg/mL ethyl acetate fraction; (f) cells co-treated with palmitic acid and 50 µg/mL ethyl acetate fraction.

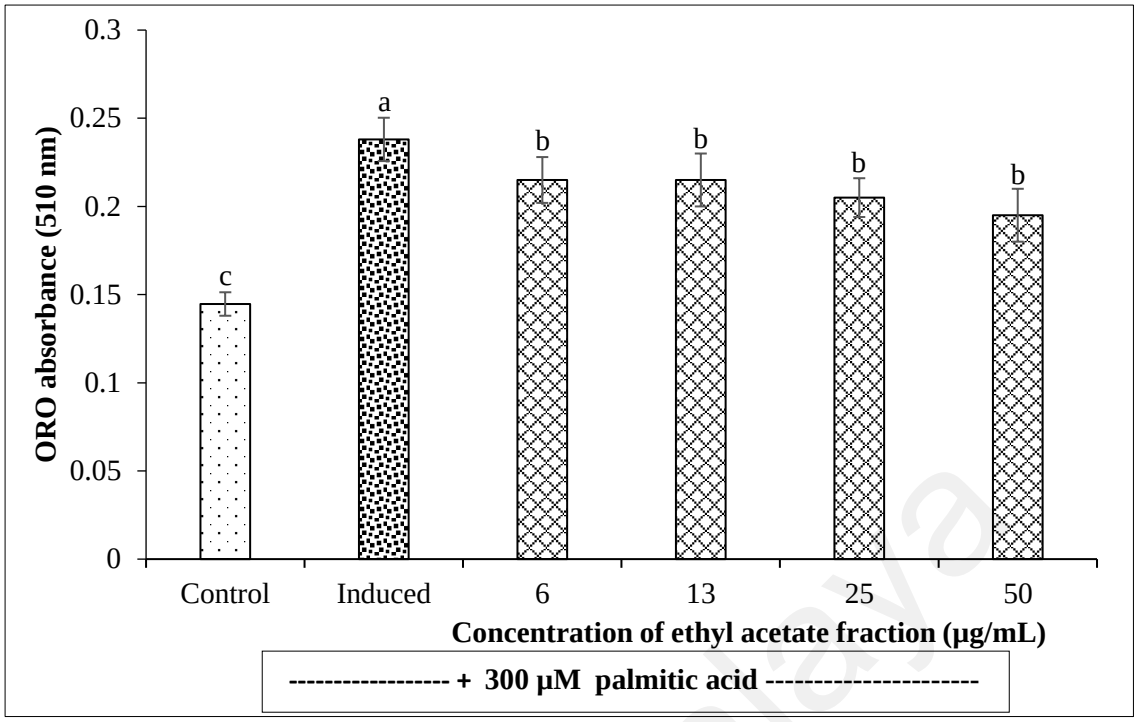

Figure 4.11: Effect of ethyl acetate fraction treatment on intracellular lipid HepG2 cells.

Effect of different concentrations of ethyl acetate fraction on lipid accumulation in HepG2. Oil Red O-based colorimetric assay was spectrophotometrically read at 510 nm. Results were expressed as means ± standard deviation of triplicate measurement (n=3) in two independent experiments. Data were analysed by one-way ANOVA followed by Duncan test. The different letters represent means with significant different ($p<0.05$), compared with the control blank (medium with BSA only).

4.6.2 Scavenging effect of *A. nigricans* towards intracellular ROS effect

To determine the protective effect of water and ethyl acetate fractions of *A. nigricans* against oxidative stress, HepG2 cells were co-treated with sample and 300 µM of palmitic acid for 24 hours. Reactive oxygen species-sensitive fluorescent probe (DCFH2-DA) was employed to monitor cellular oxidative stress, and the DCF fluorescence intensity was used to compare the intracellular ROS level of HepG2 cells among the study group.

Figures 4.12(A) and 4.13 (A), illustrates that after 24 hours of induction, palmitic acid-induced HepG2 considerably increased ROS production in HepG2 cells as compared to the blank control cells. Quantitative measurement analysis indicated that palmitic acid-induced HepG2 cells elevated the ROS level of approximately 5 fold than blank control cells ($p<0.05$) (Figure 4.12 (B) and Figure 4.13 (B)).

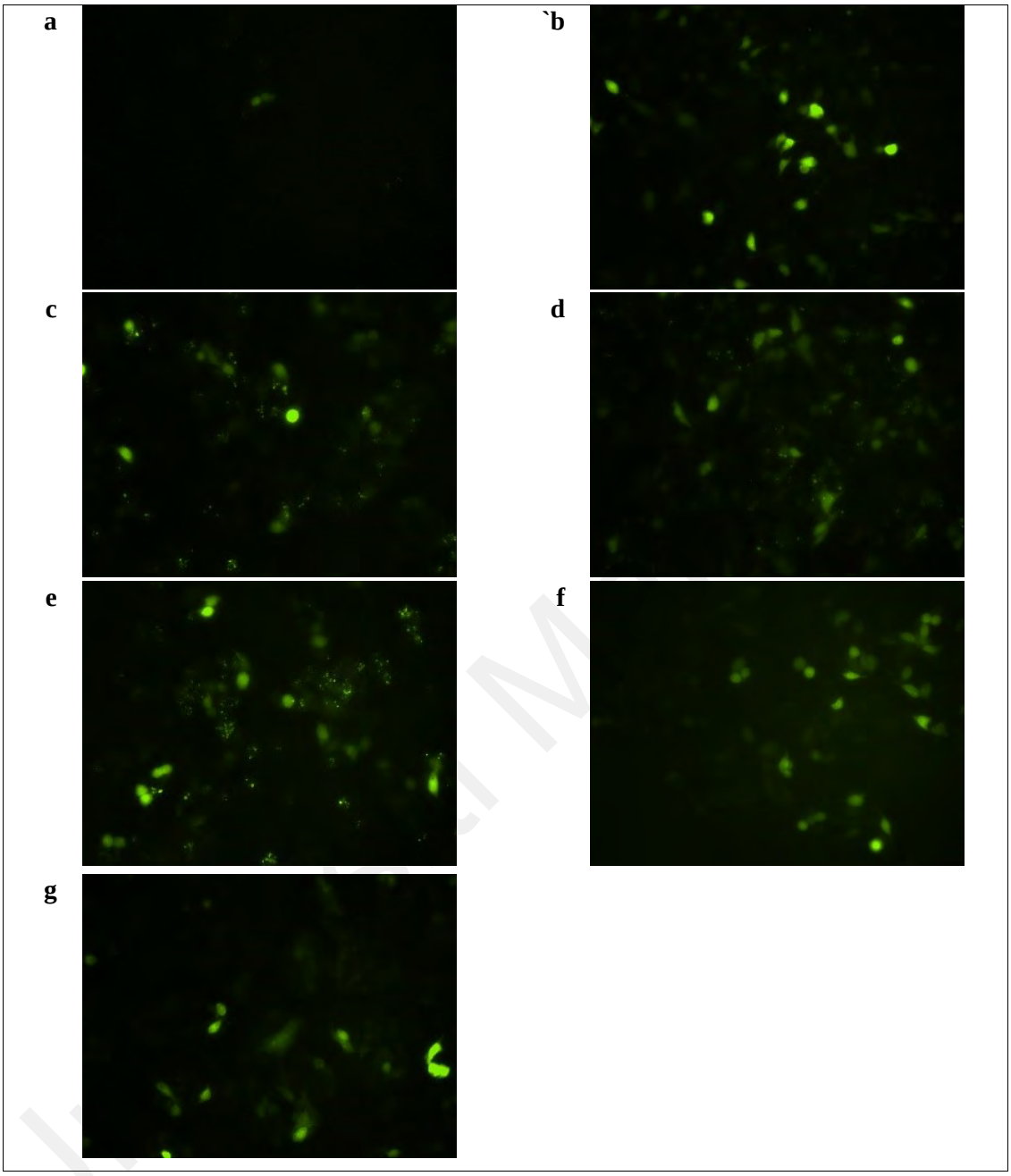

Figure 4.12 (A) Palmitic acid-induced oxidative stress and the effects of water fraction from *A. nigricans* on palmitic-acid-induced intracellular ROS production in HepG2 cells.

Representative immunofluorescence images of ROS in HepG2 cells (magnification x 200). HepG2 cells were induced with 300 µmol/L palmitic acid for 24 hours with or without co-incubation of water fraction from *A. nigricans*, (a) HepG2 cells were incubated in the absence of palmitic acid. (b) HepG2 cells were induced with 300 µM of palmitic acid for 24 hours. (c) Induced-HepG2 cells were co-treated with 6 µg/ml of water fraction. (d) Induced-HepG2 cells were co-treated with 13µg/ml of water fraction. (e) Induced-HepG2 cells were co-treated with 25 µg/ml of water fraction (f) Induced-HepG2 cells were co-treated with 50 µg/ml of water fraction. (g) Induced-HepG2 cells were co-treated with 100 µg/ml of water fraction.

In the co-incubation of water fraction and palmitic acid group, the presence of water fraction led to non significant reversal in the increased ROS level caused by the presence of palmitic acid ($p<0.05$) (as shown in Figure 4.12 (B)). The ROS level was detected at absorbance values of 528.333 ± 10.116, 526.333 ± 7.024, 521.667 ± 3.786, 518.333 ± 8.083, 517.667 ± 5.508 a.u. for concentrations of 6, 13, 25, 50, 100 µg/mL, respectively.

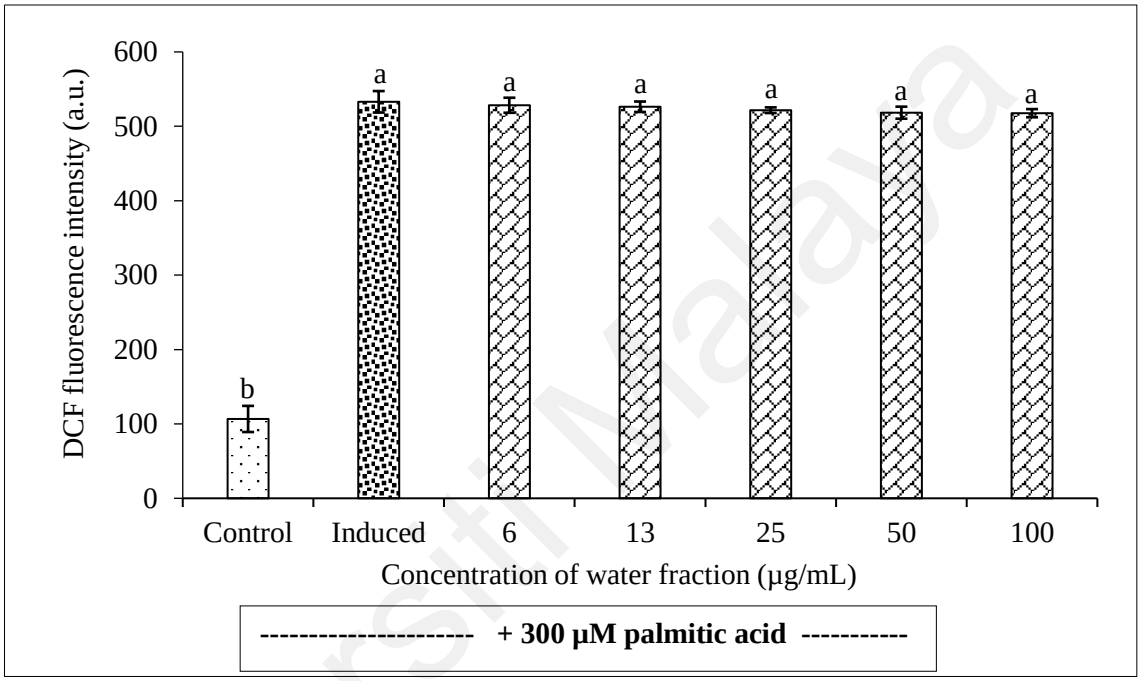

Figure 4.12 (B) Palmitic acid-induced oxidative stress and the effects of water fraction from *A. nigricans* on palmitic-acid-induced intracellular ROS production in HepG2 cells.

HepG2 cells were induced with 300 µM of palmitic acid without or with water fraction of *A. nigricans* for 24 hours. HepG2 cells incubated without palmitic acid and sample was served as control blank. Comparison of intracellular ROS was measured using dichlorofluorescein (DCF) fluorescence intensity, in different groups. Results were presented as means ± standard deviation of triplicate measurements (n = 3) in three independent experiments. Data were analysed by one-way ANOVA followed by Duncan test. The different letters represent means with significant different ($p<0.05$), compared with the control blank (medium with BSA only).

Treatment with ethyl acetate fraction from *A. polytricha* attenuated the palmitic acid-induced increase in ROS production (as shown in Figure 4.13 (A and B)), although the ROS level was nevertheless significantly higher than that of the blank control. The ROS level decreased in concentration dependent manner when palmitic acid-induced HepG2 cells were treated with ethyl acetate fraction at concentration of 6, 13, 25, and 50 µg/mL. The detected ROS level in ethyl acetate fraction treated cells were 522.333 ±

4.163, 519.333 ± 8.145, 489.333 ± 7.638, 474.667 ± 12.583 a.u., for 6, 13, 25, and 50 µg/mL, respectively. Significant decrease ($p<0.05$) was detected at 50 µg/mL, 25 µg/mL, which was approximately 1.12 fold and 1.08-fold decrease when compared to palmitic acid-induced cells.

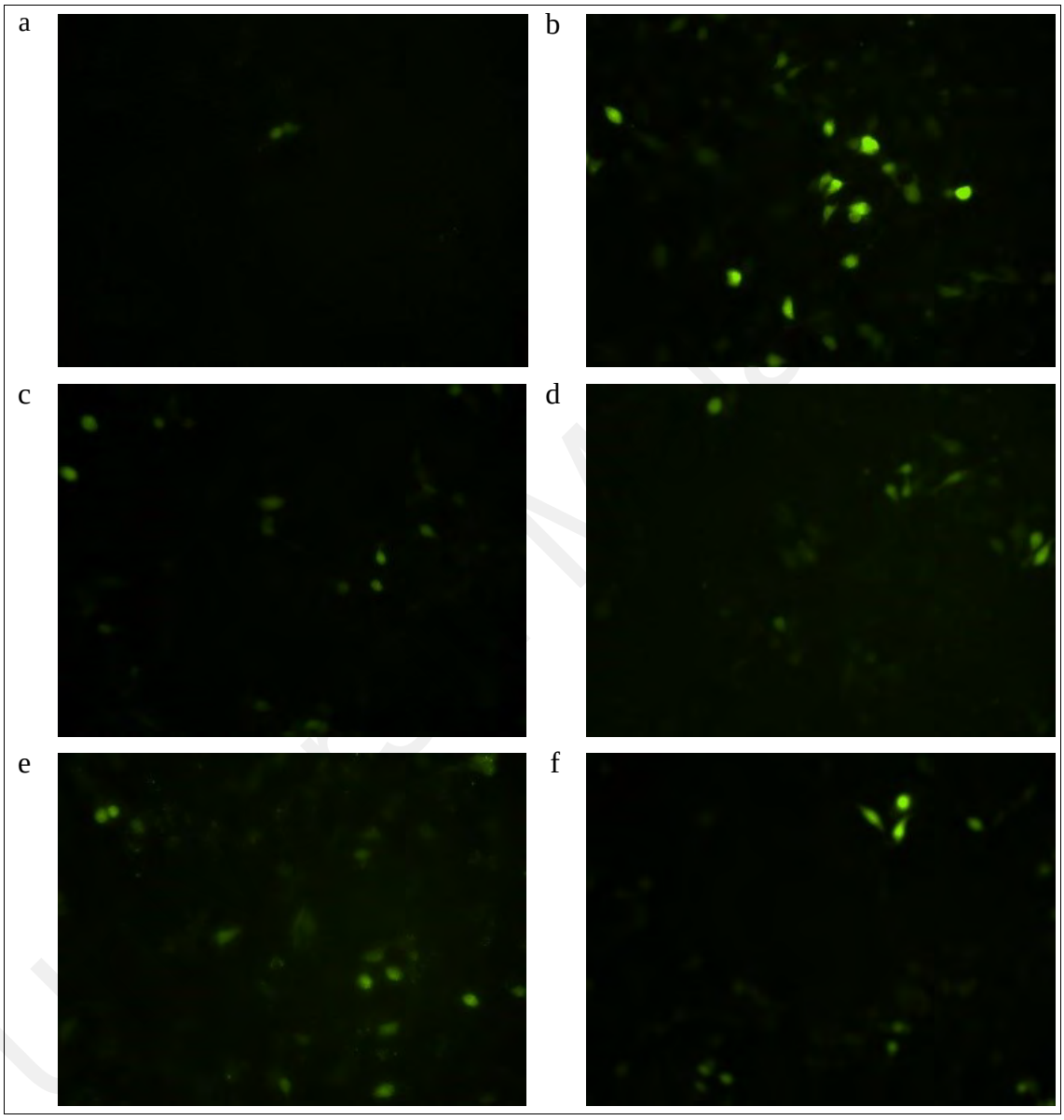

Figure 4.13 (A) Palmitic acid-induced oxidative stress and the effects of ethyl acetate fraction from A. nigricans on palmitic-acid-induced intracellular ROS production in HepG2 cells.

Representative immunofluorescence images of ROS in HepG2 cells (magnification x 200). HepG2 cells were induced with 300 µM palmitic acid for 24 hours with or without co-incubation of ethyl acetate fraction from *A. nigricans*, (a) HepG2 cells were incubated in the absence of palmitic acid. (b) HepG2 cells were induced with 300 µM of palmitic acid for 24 hours. (c) Induced-HepG2 cells were co-treated with 50 µg/mL of ethyl acetate fraction. (d) Induced-HepG2 cells were co-treated with 25 µg/mL of ethyl acetate fraction. (e) Induced-HepG2 cells were co-treated with 13 µg/mL of ethyl acetate fraction. (f) Induced-HepG2 cells were co-treated with 6 µg/mL of ethyl acetate fraction.

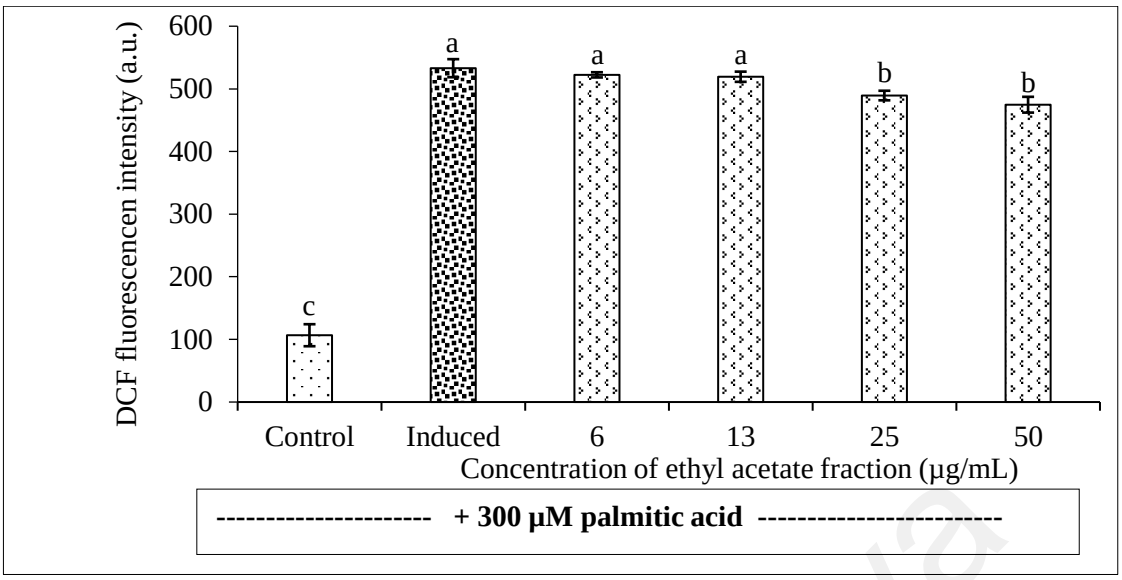

Figure 4.13: (B) Palmitic acid-induced oxidative stress and the effects of ethyl acetate fraction from *A. nigricans* on palmitic-acid-induced intracellular ROS production in HepG2 cells.

HepG2 cells were induced with 300 µM of palmitic acid without or with ethyl acetate fraction of *A. nigricans* for 24 hours. HepG2 cells incubated without palmitic acid and sample was served as control blank. Comparison of intracellular ROS was measured using dichlorofluorescein (DCF) fluorescence intensity, in different groups. Results were presented as means ± standard deviation of triplicate measurements (n=3) in three independent experiments. Data were analysed by one-way ANOVA followed by Duncan test. The different letters represent means with significant different ($p<0.05$), compared with the control blank (medium with BSA only).

4.7 Anti-inflammatory effect of ethyl acetate fraction

4.7.1 Secretion of pro-inflammatory cytokines

The effects of palmitic acid on the production of tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), interleukin 8 (IL-8) and monocytic chemotactic protein 1 (MCP-1) in HepG2 cells were examined. Figure 4.14 showed that palmitic acid exposure for 24 hours significantly increased the production of inflammatory cytokines TNF-α, IL-6, IL-8 and MCP-1 compared to the control blank cells ($p<0.05$). Palmitic acid increased TNF-α, IL-6, IL-8 and MCP-1 to 196.667 ± 19.696, 106.340 ± 10.635, 221.143 ± 12.454, 1108.909 ± 25.985 pg/mL relative to control blank of each cytokine, 87.381 ± 8.371, 19.000 ± 0.957, 82.095 ± 13.727, 417.091 ± 14.966 pg/mL, respectively. To determine the effect of ethyl acetate fraction from *A. nigricans* on the secretion of inflammatory cytokines, HepG2 cells were treated with ethyl acetate fraction in the presence of palmitic acid. Treatment with ethyl acetate fraction for 24 hours significantly alleviated palmitic

acid-induced overproduction of TNF-α, IL-6, IL-8 and MCP-1($p<0.05$). Treatment with 25 µg/mL of ethyl acetate fraction led to reduction cytokine levels to140.476 ± 8.123, 91.163 ± 2.399, 184.00 ± 22.678, 935.879 ± 39.358 pg/mL for TNF-α, IL-6, IL-8 and MCP-1, respectively. Capacity of 50 µg/mL of ethyl acetate fraction in alleviation of inflammatory circumstance was not significantly different from 25 µg/mL. Treatment with 50 µg/mL of ethyl acetate fraction lowered the cytokine levels to 143.095 ± 12.296, 90.986 ± 5.316, 167.810 ± 21.060 and 865.879 ± 58.609 pg/mL for TNF-α, IL-6, IL-8 and MCP-1, respectively.

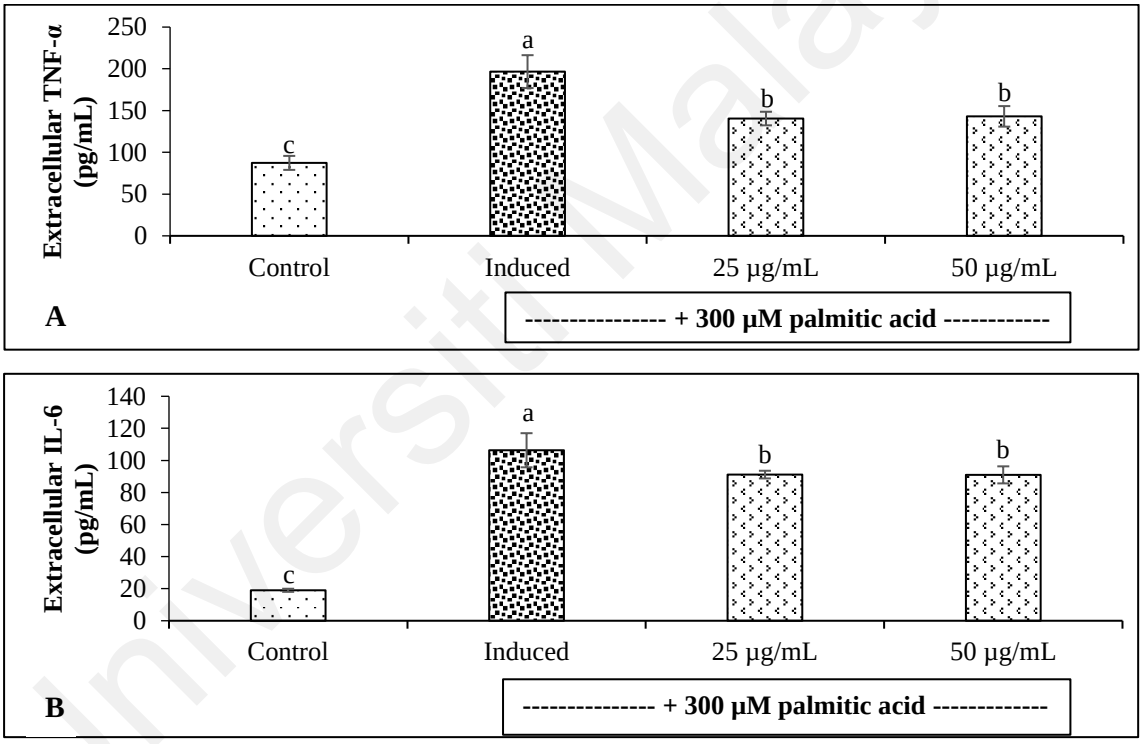

Figure 4.14: Effect of ethyl acetate fraction from *A. nigricans* on inflammatory cytokine and chemokines in palmitic acid-induced HepG2 cells.

HepG2 cells induced with 300 µM of palmitic acid with or without ethyl acetate fraction (25 and 50 µg/mL) for 24 hours. Cells without palmitic acid and ethyl acetate fraction served as control blank. Culture supernatant for detection of extracellular level of (A) TNF-α, (B) IL-6, (C) IL-8 and (D) MCP-1 were collected at 24 hours of palmitic acid induction. Secretion of TNF-α, IL-6, IL-8 and MCP-1 in culture supernatant were assayed by commercial kits. Results were presented as mean ± standard deviation (n=3) in three independent experiments. Data were analysed by one-way ANOVA followed by Duncan test. The different letters represent means with significant different ($p<0.05$), compared with the control blank (medium with BSA only).

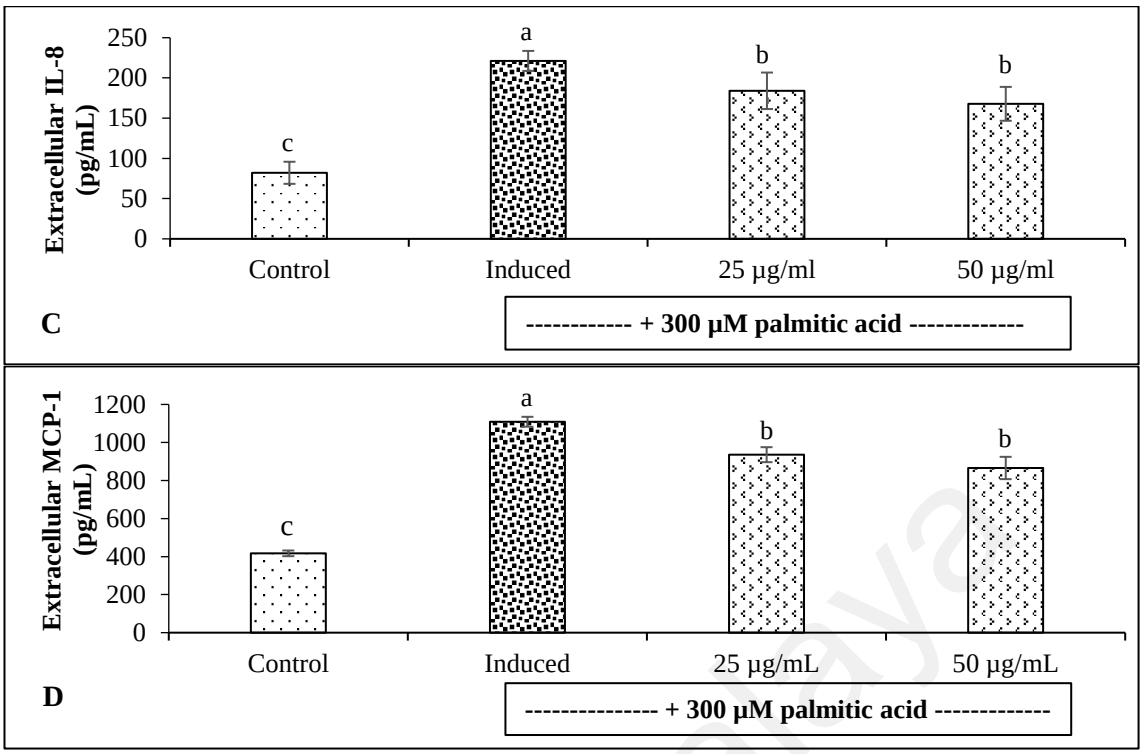

Figure 4.14, Continued: Effect of ethyl acetate fraction from *A. nigricans* on inflammatory cytokine and chemokines in palmitic acid-induced HepG2 cells.
HepG2 cells induced with 300 µM of palmitic acid with or without ethyl acetate fraction (25 and 50 µg/mL) for 24 hours. Cells without palmitic acid and ethyl acetate fraction served as control blank. Culture supernatant for detection of extracellular level of (A) TNF-α, (B) IL-6, (C) IL-8 and (D) MCP-1 were collected at 24 hours of palmitic acid induction. Secretion of TNF-α, IL-6, IL-8 and MCP-1 in culture supernatant were assayed by commercial kits. Results were presented as mean ± standard deviation (n=3) in three independent experiments. Data were analysed by one-way ANOVA followed by Duncan test. The different letters represent means with significant different ($p<0.05$), compared with the control blank (medium with BSA only).

4.7.2 Anti-inflammatory effect of *A. nigricans* towards transduction signalling pathway

To explore the mechanism of the anti-inflammatory effect of ethyl acetate fraction from *A. polytrica* on palmitic acid-induced HepG2 cells, investigation was carried out on several important proteins including NF-κB p65, phospho-NF-κB p65 (Ser536), phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), phospho-Stat3 (Tyr705) and phospho-IκB-α (Ser32) that are involved in NAFLD signalling pathways and important in inflammatory response. Based on the ELISA analysis measured at 450 nm (Table 4.7), palmitic acid significantly activated inflammatory response of HepG2 cells through higher expression of phosphorylated NF-κB p65 (0.336 ± 0.051) and

phosphorylated IκBα (0.416 ± 0.039) as compared to non-induced HepG2 cells, (0.152 ± 0.016) and (0.218 ± 0.020) respectively ($p<0.05$). Similarly, the expression levels of phosphorylated p38 MAPK (1.191 ± 0.061) and phosphorylated STAT3 (1.203 ± 0.098) as well as phosphorylated SAPK/JNK (1.369 ± 0.153) in palmitic acid-induced cells group were significantly higher than those in blank control cells, 0.403 ± 0.047, 0.551 ± 0.055 and 0.645 ± 0.063 for p38 MAPK, STAT3 and SAPK/JNK, respectively ($p < 0.05$).

In contrast, co-incubation with ethyl acetate fraction significantly decreased ($p < 0.05$) the palmitic acid-induced protein expression of p-NF-κB p65 (0.271 ± 0.004) and p-IκBα (0.340 ± 0.023). Even so, ethyl acetate fraction treatment did not bring down the phosphorylated protein levels back to the basal level. Apart from NF-κB p65 and p-IκBα, the presence of ethyl acetate fraction also significantly ($p<0.05$) downregulated the expression of phosphorylated SAPK/JNK (1.064 ± 0.046) and phosphorylated p38 MAPK (0.808 ± 0.078) as well as phosphorylated STAT3 (0.929 ± 0.053) as compared to palmitic acid-induced cells, however, proteins levels were still higher than that of blank control.

Table 4.7: The inhibition of p-IκBα, p-NF-κB, p-SAPK/JNK, p-p38 MAPK, p-STAT3 protein expression in palmitic acid-induced HepG2 cells by ethyl acetate fraction from *A. nigricans*.

Protein	Absorbance values at 450 nm		
	Control	Palmitic acid-induced	Treated
NF-κB p65	2.745 ± 0.049	2.720 ± 0.044	2.722 ± 0.038
Phosphorylated NF-κB p65	0.152 ± 0.016^{c}	0.336 ± 0.051^{a}	0.271 ± 0.004^{b}
Phosphorylated SAPK/JNK	0.645 ± 0.063^{c}	1.361 ± 0.153^{a}	1.064 ± 0.046^{b}
Phosphorylated p38	0.430 ± 0.047^{c}	1.191 ± 0.061^{a}	0.808 ± 0.078^{b}
Phosphorylated IκB-α	0.218 ± 0.020^{c}	0.416 ± 0.039^{a}	0.340 ± 0.023^{b}
Phosphorylated STAT3	0.551 ± 0.055^{c}	1.203 ± 0.098^{a}	0.929 ± 0.053^{b}

HepG2 cells induced with 300 µM palmitic acid were incubated for 24 hours without (Induced control) or with ethyl acetate fraction of *A. nigricans*. Protein extracts for the detection of NF-κB, phosphorylated NF-κB p65, phosphorylated IκBα, phosphorylated SAPK/JNK, phosphorylated p38 MAPK and phosphorylated STAT3 expression were collected at 24 hours. Cells in only DMEM medium served as blank control. Phosphorylated proteins levels were measured at 450 nm. All results were expressed as mean ± standard deviation of triplicate measurements (n=3) in three independent experiments. Data were analysed by one-way ANOVA followed by Duncan test. Different superscripted letter in each row indicate significant difference at $p < 0.05$.

CHAPTER 5: DISCUSSION

5.1 Antioxidant capacity of *A. nigricans*

5.1.1 Total phenolic content

In regards to assortment of structures and uses, natural bioactive compounds became a source for the production of nutraceuticals, functional foods, and food additives (Gil-Chávez *et al.*, 2013). Hence, food and pharmceutical industries put a lot of efforts to isolate bioactive compounds from natural sources. Frequently, solvent extraction is the most common technique applied in the isolation of antioxidant compounds (Sultana *et al.*, 2009). Solvents such as methanol, ethanol, acetone and ethyl acetate were widely used in extracting phenolic compounds from plants (Złotek *et al.*, 2015). In fact, antioxidant compounds from plants and plant-based foods, for instance, plum, strawberry, pomegranate, broccoli, rosemary, sage, sumac, rice bran, wheat grain and bran, mango seed kernel, citrus peel, and many other fruit peels were commonly extracted by methanol and ethanol (Sultana *et al.*, 2009). In the current study, ethanol was selected to be used in the extraction of bioactive compounds from *A. nigricans*. The yield of ethanolic crude of *A. nigricans* was low, 2 %, w/w. However, this is comparable with the yield of *A. auricula-judae* methanolic extraction (2.3 %, w/w) obtained by Oke & Aslim (2011). The slight difference between the two yields may be due to the different alcohol used for extraction. Instead of ethanol, Oke & Aslim (2011) used methanol as an extraction solvent in their study. Do *et al.* (2014) used various concentrations of methanol, ethanol and acetone (50 %, 75 %, and 100 %) to extract a medicinal herb and proved that pure methanol produced a higher extraction yield than pure ethanol. In the current study, ethanol was selected as extracting solvent because it is safe for human consumption (Do *et al.*, 2014) and it is more environmentally friendly than methanol (Yi & Wetzstein, 2011).

The ethanolic crude was then further partitioned with different polarity of solvents. The dried ethanolic crude was suspended in distilled water, then partitioned with non-polar hexane, followed by solvent with intermediate polarity, ethyl acetate. Among the solvents used, water and hexane fractions gave high yield, which indicated that most polar and least polar compounds were abundantly presented. On the contrary, ethyl acetate fraction revealed that the intermediate polarity compounds were lesser in *A. nigricans*.

Mushrooms are widely recognized to possess proteins, minerals, vitamins, crude fiber, carbohydrate, and low fat and oil content (Egwin *et al.*, 2011). It also known to have abundant of secondary metabolites including phenolic compounds, polyketides, terpenes, and steroids, which are of importance to mankind by exhibiting a comprehensive beneficial property, such as antibacterial, antiviral, along with pharmaceutical activities and less toxic effects (Dasgupta *et al.*, 2014).

In the current study, the TPC values of extracts (in descending order) were ethyl acetate fraction > water fraction > ethanolic crude > hexane fraction. Ethanolic extract of *A. nigricans* in this study, produced 11.38 ± 0.47 mg GAE/g of TPC. The highest TPC value of ethyl acetate fraction, compared to other extracts, indicated that phytoconstituents in *A. nigricans* were readily soluble in semi-polar solvent. There was a slight difference to the methanolic extract of *A. auricula-judae* carried out by Oke & Aslim (2011), which gave 10.54 ± 0.53 mg GAE/g of TPC. Among the fractions, ethyl acetate fraction was the least yield from the total ethanolic crude extract of *A. nigricans*, however, it gave the highest TPC value. On the other hand, hexane fraction was the highest yield from the total ethanolic crude, but, the TPC yield was the least. In other words, ethyl acetate fraction may constitute more phenolic compounds than hexane fraction. Nakamura *et al.* (2017) reported that ethyl acetate fraction of *Sasa quelpaertensis* Nakai leaf extract showed the highest TPC, which contributed to its high antioxidant capacity. In addition, polyphenol may have hepatoprotective effects by

modulation of fatty acid oxidation, insulin resistance, oxidative stress and inflammation, which are the main pathogenetic factors associated to the progression from NAFLD to NASH (Abenavoli *et al.*, 2017).

The Folin-Ciocalteu was the most general applicable method to determine the total phenolics in the substrate as it is rapid, convenient, simple and reproducible (Huang *et al.*, 2005). Folin-Ciocalteu reagent, is a mixture of phosphotungstic and phosphomolybdic acids. Gallic acid was used as a standard compound and the total phenols were expressed as mg/g gallic acid equivalent. From the results obtained, ethyl acetate fraction exerted the highest capacity in chemically reducing molybdenum component in the phosphotungstic-phosphomolybdic complexing reagent, which resulted in the colour of Folin-Ciocalteau reagent, changes from yellow to blue (*Asadu et al.*, 2015) However, it is worthy to note that non phenolic compounds, such as ascorbic acid, organic acids, sugars, aromatic amines can also react with Folin-Ciocalteu reagent (Ghafar *et al.*, 2010; Albano *et al.*, 2015). Hence, results of this assay reflect the total reducing capacity of the extracts.

Oxygen is essential to maintain normal cellular metabolism in order to generate energy for cell functions. Simultaneously, free radicals are produced as a natural byproduct in cellular metabolism of oxygen (Pham-Huy *et al.*, 2008). Imbalance between production and elimination of free radical from the body as a result of free radicals overwhelmed by nonenzymatic (e.g., glutathione, α-tocopherol, ascorbic acid, carotenoids) and enzymatic (e.g., catalase, superoxide dismutase and glutathione peroxidase) antioxidants within the cell (Barzegar & Moosavi-Movahedi, 2011) lead to oxidative stress (Katalinic *et al.*, 2006). Oxidative stress has been implicated in the etiology and progression of several human diseases such as cardiovascular diseases (Pham-Huy *et al.*, 2008), and NAFLD (Polimeni *et al.*, 2015; Spahis *et al.*, 2017).

Current research is directed towards finding naturally-occurring antioxidants of natural origin. An antioxidant is a substance, at concentration lower than an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate (Young & Woodside, 2001) and subsequently form a product having low or no toxicity (Magalhaes *et al.*, 2008). In addition to traditional vitamins, numerous dietary antioxidants collectively known as phytonutrients or phytochemicals are being more and more appreciated for their antioxidant activity, (Crosby *et al.*, 2008; Kumar *et al.*, 2015). Dietary antioxidants are essential because it able to enhance cellular defenses and help to prevent oxidative damage to cellular components when body's antioxidant mechanism does not act optimally (Rahman, 2007). Likewise, many health organizations extensively recommended ingestion of naturally-occurring antioxidants rich-food, including nutrient (such as vitamins) and phytochemicals (such as polyphenols) (Bouayed & Bohn, 2010). Therefore, determining the radicals scavenging capacity of antioxidant in *A. nigricans* is important and encouraging.

Analysis of mushrooms extract antioxidant property and the content of antioxidant compounds have been intensively studied (Mujic *et al.*, 2010; Kozarski *et al.*, 2015). Performing biochemical assays to assess the antioxidant capacity has appeared to be one of the most reliable as well as readily available methods (Bektasoglu *et al.*, 2006; Bhakta & Siva, 2012). Owing to a particular antioxidant produce differential response in diverse testing systems, thus, it is essential to employ different antioxidant assays to understand the mode of action of the bioactive principle involved (Bhakta & Siva, 2012). In general, antioxidants react with free radicals through two mechanisms: hydrogen atom transfer (HAT) and electron transfer (ET).

In HAT, the free radical removes a hydrogen atom from the antioxidant (AH) that itself becomes a radical:

$$\mathbf{R^{\cdot} + AH \longrightarrow RH + A^{\cdot}}$$

A higher stability of the radical $A^{\cdot}$ is parallel to a better efficiency of the antioxidant AH, thus, it has no tendency to react with the substrate. In general, hydrogen bonds, conjugation, and resonance, make it a nonreactive radical. In this mechanism, the antioxidant capacity evaluation is on the bond dissociation enthalpy (BDE) of the O-H bonds, because the weaker the O-H bond the easier will be the reaction of inactivation of free radical (Leopoldini *et al.*, 2011). Assays that involved HAT mechanism are oxygen radical absorbance capacity (ORAC) (Huang *et al.*, 2005), scavenging of superoxide radical formation (Shalaby & Shanab, 2013), scavenging hydroxyl radical (Liang & Kitts, 2014) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Vladimir-Knezevic *et al.*, 2011). In ET, the antioxidant can give an electron to the free radical and generate a radical cation:

$$\mathbf{R^{\cdot} + AH \longrightarrow R^{-} + AH^{\cdot +}}$$

Likely, the radical cation produced from the electron transfer must be stable, so that it does not react with substrate molecules. In this mechanism, the antioxidant activity evaluation is based on the ionization potential (IP) values. The lower the ionization potential, the easier is the electron abstraction (Bendary *et al.*, 2013; Liang & Kitts, 2014). For instance, ferric reducing antioxidant power (FRAP), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Huang *et al.*, 2005; Vladimir-Knezevic *et al.*, 2011; Liang & Kitts, 2014) are grouped in ET mechanism.

Samples obtained in this study were screened for their antioxidant characteristics via assays including DDPH radical scavenging activity, ferric reducing antioxidant (FRAP), oxygen radical absorbance capacity (ORAC), scavenging hydroxyl radical, and scavenging of superoxide anion radical ($O_2^{\cdot -}$).

5.1.2 Scavenging of DPPH radical

DPPH assay is an easy, economic, accurate and short time scale method to evaluate the antioxidant radical scavenging capacity (Marinova & Batchvarov, 2011;

Liang & Kitt, 2014). It is broadly used to evaluate antioxidant capacity of natural sources (Marinova & Batchvarov, 2011). In this assay, DPPH was used as the free radical source (Moreno *et al.*, 2006). It is a stable nitrogen centered free radical (Meng *et al.*, 2016) that accepts an electron or hydrogen atom to become a stable diamagnetic molecule (Mujic *et al.*, 2010). DPPH solution exhibits a deep purple colour with absorption maximum at 517 nm.

Antioxidant quenches the stable DPPH radical via hydrogen atom transfer or electron donation (Bigdoli *et al.*, 2013; Liang & Kitt, 2014), which cause fading or disappearing of the purple coloured DPPH radical solution, resulting in a decrease in absorbance at 517nm (Kedare & Singh, 2011; Genwali *et al.*, 2013). The level of diminishing purplish coloured complex revealed the scavenging potential of antioxidant. An antioxidant, either acts as a HAT or ET, is influenced by the nature of solvent and/or the redox potentials of the species involved (Vladimir-Knezevic *et al.*, 2011).

In general, DPPH radical abstract hydrogen atom from antioxidant occurs in non-polar solvent; on the other hand, electron transfer mechanism takes part in polar solvent such as, ethanol and methanol because a hydrogen bond is formed between antioxidant molecule and solvent (Banerjee *et al.*, 2012). DPPH• undergoes a HAT mechanism with antioxidant compounds according to the following reaction (Craft *et al.*, 2012):

$DPPH^{\cdot}$ (purple colour) + ArOH → DPPH-H (faded purple colour) + $ArO^{\cdot}$ (HAT)

$DPPH^{\cdot}$ undergoes a ET mechanism between $DPPH^{\cdot}$ and a phenolic antioxidant (Craft *et al.*, 2012):

$DPPH^{\cdot}$ (purple colour) + ArOH → $DPPH^{-}$ (faded purple colour) + $[ArOH]^{\bullet+}$(ET)

In the present study, ethyl acetate fraction of *A. nigricans* appeared to be the major contributor for antioxidant capacity of *A. nigricans* in DPPH assay because DPPH scavenging activity of ethyl acetate fraction was significantly higher than those of other extracts ($p<0.05$). However, it is not as good as trolox. It was found that the DPPH-

scavenging effect of *A. nigricans* extracts increased as their concentrations increased. The descending order of DPPH scavenging capacity of *A. nigricans* was ethyl acetate > ethanolic crude > water > hexane. Results showed that ethyl acetate fraction has significant capacity in reduction of DPPH radicals, compared to ethanolic crude and fractions from *A. nigricans*.

In comparison to Zeng *et al.* (2012) study, antioxidant capacity of ethanolic crude and all fractions of *A. nigricans* from current study were prominently more efficacious than polysaccharide of *A. auricula judae* that was extracted using microwave, which IC_{50} was 3.29 ± 0.41 mg/mL. In the study conducted on *A. auricula judae*, methanolic extract of *A. auricula judae* gave IC_{50} of 0.86 ± 0.06 mg/mL (Oke & Aslim., 2011), whereas IC_{50} of ethanolic crude in this study was 0.96 ± 0.02 mg/mL, which showed that the methanolic extract of *A. auricular judae* is slightly more potent in scavenging of DPPH radical, compared to ethanolic crude.

5.1.3 Ferric reducing antioxidant power (FRAP)

Originally, ferric reducing antioxidant power (FRAP) used 2, 4, 6-Tripyridyl-s-triazine (TPTZ) as the iron-ligand, however, potassium ferricyanide has been widely used lately (Zhong & Shahidi, 2015). This assay was used to assess the capacity of antioxidant compounds to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) via electron transferring (Bajpai *et al.*, 2013; Palvai *et al.*, 2014). Extract with reducing capacity react with ferricyanide (Fe^{3+}) results in formation of ferrocyanides (Fe^{2+}), which react with ferric chloride and later leads to development of Perl's Prussian blue colour because of formation of ferric ferrous complex (Jothy *et al.*, 2012). An increase in the absorbance indicated an increase in the antioxidant activity. Furthermore, reducing power is associated with the presence of reductones which exert antioxidant action by breaking the free radical chain via reduction of the oxidized intermediates of lipid peroxidation processes, so they can act as primary

and secondary antioxidants (Yen & Chen, 1995; Kuda & Ikemori, 2009; Saranya *et al.*, 2014).

The present study showed that the ferric reducing antixodant power of *A. nigricans* extracts in descending order was ethyl acetate > hexane > water > ethanolic crude. Results showed that ethyl acetate fraction has significant capacity in reduction of ferric ion, compared to ethanolic crude and fractions of *A. nigricans*. This observation indicated that ethyl acetate fraction of *A. nigricans* probably contain antioxidative phytoconstituents that have ET capacity.

Taken together, these indicate that extracts from *A. nigricans* may contain antioxidative phytoconstituents which exert ET mechanism to scavenge DPPH radicals and to trigger reduction of ferric ion. Among the extracts from *A. nigricans*, ethyl acetate fraction exerted the strongest antioxidant capacity. In other words, ethyl acetate fraction could possess antioxidative phytoconstituents that have lower ionization, hence, free radical can simply draw out an electron from antioxidative phytoconstituents of ethyl acetate fraction in ET mechanism (Bendary *et al.*, 2013; Liang & Kitts, 2014), compared to other extracts.

5.1.4 Scavenging of superoxide anion radical ($O_2^{\cdot -}$)

In an *in*-vitro assay, superoxide anion ($O_2^{\cdot -}$) was derived from dissolved oxygen system PMS-NADH system, as shown in below reaction. The generated $O_2^{\cdot -}$ diminish the yellow chromogen (NBT^{2+}) to form blue formazan (Reibeiro *et al.*, 2007; Bajpai *et al.*, 2013).

$$\mathbf{NADH + H^+ + PMS \longrightarrow NAD^+ + PMSH_2}$$

$$\mathbf{PMSH_2 + 2O_2 \longrightarrow PMS + 2O_2^{\cdot -} + 2H^+}$$

$O_2 \cdot^-$ is a predominant cellular free radical (Zorov *et al.*, 2014). $O_2 \cdot^-$ is a reduced form of molecular oxygen as a result of receiving one electron (Kuate *et al.*, 2011). $O_2 \cdot^-$ is chemically weak reactive (Chien *et al.*, 2013). It is unable to cross biological membrane freely but it can exist long before reacting with other free radicals or with specific clusters of iron–sulfur in target proteins (Newsholme *et al.*, 2012). However, its impact can be intensified because $O_2 \cdot^-$ is known as primary ROS and it is the precursor for other aggressive free radicals (Valko *et al.*, 2006). In general, $O_2 \cdot^-$ is converted to H_2O_2 in the presence of superoxide dismutase (Valko *et al.*, 2006; Mohora *et al.*, 2009). Other ROS derived from $O_2 \cdot^-$ include hydroxyl ($OH\cdot$), hydroperoxyl ($OOH\cdot$), peroxyl ($ROO\cdot$) and alkoxyl ($RO\cdot$) (Umamaheswari & Chatterjee, 2008). Therefore, scavenging it will prevent the formation of other reactive ROS. $O_2 \cdot^-$ scavenging capacity of water fraction and trolox showed the proton donating capacity and thereby acting as antioxidant. In other words, water fraction from *A. nigricans* is capable to hinder formation of more deleterious ROS, such as hydroxyl radical, which is very harmful to membrane lipid, phospholipids, cholesterol and DNA (Heo *et al.*, 2005; Vladimir-Knezevic *et al.*, 2011).

Surprisingly, ethanolic crude, ethyl acetate fraction and hexane fraction were unable to scavenge $O_2 \cdot^-$. Water fraction was the only fraction that possessed the capacity of scavenging $O_2 \cdot^-$, however, its capacity was not comparable to trolox.

5.1.5 Scavenging of hydroxyl radical ($OH^{\cdot}$)

In living organisms, $OH\cdot$ is produced from H_2O_2 in the presence of Fe^{2+} via Fenton reaction (Chen & Schopfer, 1999; Valko *et al.*, 2006; Lipinski, 2011):

$$\mathbf{Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH\cdot}$$

The presence of $O_2^{\cdot -}$ in the body encourages the conversion of Fe^{3+} to Fe^{2+}:

$$\mathbf{Fe^{3+} + O_2^{\cdot -} \longrightarrow Fe^{2+} + O_2\cdot}$$

Combination of both steps is known as Haber-Weiss reaction, which is catalyzed by iron:

$$H_2O_2 + O_2^{\cdot -} \longrightarrow OH^{\cdot} + OH^{-} + O_2$$

In vitro, system of ascorbic acid/ferric chloride anhydrous-ethylenediaminetetraacetic acid /H_2O_2 (Fe^{3+}-EDTA/ascorbate and H_2O_2), also known as deoxyribose, is performed according to Fenton reaction, which produce $OH^{\cdot}$ in body. In this antioxidant assay, ascorbic acid is needed to initiate the reaction of transforming Fe^{3+} to Fe^{2+}, as shown in Equation 1 (Eq 1) to accelerate the formation of $OH^{\cdot}$ (Ribeiro *et al.*, 2007; Stoilova *et al.*, 2008). Then Fe^{2+} reacts with H_2O_2 to produced $OH^{\cdot}$ (Eq 2). The generated $OH^{\cdot}$ successively attacks deoxyribose, which later degraded to malondialdehyde (MDA) (Eq 3). Subsequently, MDA reacts with thiobarbituric acid (TBA) and this resulted in a series of reaction forming thiobarbituric acid-reactive substance (TBAR) that presents pink chromogen (Eq 4) (Ribeiro *et al.*, 2007; Chougule *et al.*, 2012; Fernando & Soysa, 2014).

$$Fe^{3+} + \text{Ascorbic acid} \longrightarrow Fe^{2+} + \text{Ascorbyl radical} \quad \textbf{(Eq 1)}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-} \quad \textbf{(Eq 2)}$$

$$OH^{\cdot} + \text{Deoxyribose} \longrightarrow \text{MDA} \quad \textbf{(Eq 3)}$$

$$\text{MDA} + \text{TBA} \longrightarrow \text{TBAR (pink chromogen)} \quad \textbf{(Eq 4)}$$

In this assay, extract competes with deoxyribose for $OH^{\cdot}$. Hence, efficiency of extract in inhibition of degradation of deoxyribose is based on its capacity of decreasing the pink chromogen (Heo *et al.*, 2005). Among the reactive oxygen species (ROS), $OH^{\cdot}$ is the most reactive radical that it bears the shortest half-life than other radicals (Choagule *et al.*, 2012). Therefore, it can simply react with adjacent cells (Valko *et al.*, 2006). The highly reactive $OH^{\cdot}$ is the most detrimental species because it easily attacks other molecules in the body, such as damaging the DNA which in turn leads to carcinogenesis, mutagenesis and cytotoxicity (Vladimir-Knezevic *et al.*, 2011). Furthermore, it is an

important initiator of peroxidation of membrane lipids, phospholipids, and cholesterol because it is able to abstract a methylene hydrogen atom from polyunsaturated fatty acids, phospholipid in membrane to produce lipid-derived free radicals, which leads to a chain reaction of lipid peroxidation triggering further free radical-mediated peroxidation of polyunsaturated membrane lipids, generating new hydroperoxide products (Heo *et al.*, 2005). Hence, removing $OH^{\cdot}$ is very important for protecting the living system.

In the present study, the scavenging $OH^{\cdot}$ capacity of *A. nigricans* extracts were investigated by assessing the capacity of *A. nigricans* extracts to compete with deoxyribose for $OH^{\cdot}$ generated from Fe^{3+}-EDTA/ascorbate/H_2O_2. Ethyl acetate fraction appeared to be the main contributor for antioxidant capacity of *A. nigricans* in scavenging of $OH^{\cdot}$ because scavenging of $OH^{\cdot}$ capacity of ethyl acetate fraction was significantly highest among the extracts ($p<0.05$). The descending order of $OH^{\cdot}$ scavenging capacity of *A. nigricans* extracts was ethyl acetate > water > ethanolic crude > hexane. The potency of *A. nigricans* extracts in scavenging hydroxyl radical suggested the ability to hinder initiation of lipid peroxidation, therefore, exhibited protective effect towards cells structure (Heo *et al.*, 2005; Vladimir-Knezevic *et al.*, 2011). The potential scavenging ability of ethanolic crude and fractions of *A. nigricans* might be due to the active hydrogen-donating ability of antioxidant compounds presented in samples.

5.1.6 Oxygen radical absorbance capacity (ORAC)

Peroxyl radical are capable of rapidly attacking biomolecule with high electron density such as unsaturated fatty acid in foods and human body, DNA, protein due to it's an extremely unstable and electrophilic character (Infante *et al.*, 2016). In addition, it is a predominant free radical found in lipid oxidation in foods and biological systems (Güçlu *et al.*, 2014).

ROS such as OH· initiate peroxidation by extracting a hydrogen atom from an allelic ethylene group of unsaturated fatty acid (as shown in the below reaction chain), X· is the initiating free radicals, such as OH·

1. Initiation: $X\cdot + LH \rightarrow L\cdot + XH$
2. Propagation: $L\cdot + O_2 \rightarrow LOO\cdot$
3. $LOO\cdot + LH \rightarrow L\cdot + LOOH$
4. Termination: $LO\cdot + LO\cdot \rightarrow$ Nonradical polymers
5. $LOO\cdot + LOO\cdot \rightarrow$ Nonradical polymers
6. $L\cdot + L\cdot \rightarrow$ Nonradical polymers
7. $LOO\cdot + L\cdot \rightarrow$ Nonradical polymers

Generated L· from step (1) react directly with oxygen to yield peroxyl radical (LOO·) (step 2). The LOO·propagates the reaction by abstracting a hydrogen atom from a neighboring lipid (LH) resulted in formation of lipid hydroperoxide (LOOH) and another L· (step 3). The reaction carries on until the chain is terminated, either by the combination of two radicals to form a non-radical product, or by termination of propagation reaction if a hydrogen or electron donor are present (Yoshikawa & Naito, 2002; Jones & Kubow, 2006).

In vitro assay, peroxyl radical is general through thermal decomposition of 2, 2'-azobis(2-amidino-propane) dihydrochloride (AAPH) (Wada & Ou, 2002; Atmani *et al.*, 2011). Principle of ORAC is to measure the efficacy of antioxidant to shield fluorescein from destruction by peroxyl radical (Davalos *et al.*, 2003; Atmani *et al.*, 2011). Therefore, the presence of chain-breaking antioxidant in an extract is able to retard the decay of fluorescence (Magalhaes *et al.*, 2008). Antioxidant breaks the radical chain by donating hydrogen atoms to peroxyl radical (Hogg & Kalyanaraman, 1999; Kim *et al.*, 2015).

Samples with higher ORAC values indicated that they possessed better antioxidant capability (Alam *et al.*, 2013). Similar to scavenging of hydroxyl radical assay,

ethyl acetate fraction displayed as the best free radical scavenger among the fractions and ethanolic crude because it had the highest neutralizing capacity of peroxyl radical, whereas capacity of water fraction was the weakest, compared to others. In other words, extracts derived from *A. nigricans* are capable to break the chain reaction of lipid peroxidation at vary extends. Kettawan *et al.* (2011) reported that ORAC values for raw and boiled *Auricularia auricular judae* was 2.15 ± 0.49 and 0.76 ± 0.23 µmole TE/g, respectively. Obviously, ethanolic crude and fractions of *A. nigricans* in our finding is markedly higher than the former. However, it may due to different ORAC protocol applied in each study, such as sample preparation and concentration of peroxyl radical generator AAPH used.

In general, ethyl acetate fraction from *A. nigricans* may contained antioxidative phytoconstituents that have the weakest O-H bonds. In consequence, free radical can easily remove a hydrogen atom from the antioxidative phytoconstituents in HAT mechanism (Leopoldini *et al.*, 2011). This property made ethyl aetate fraction to be the strongest peroxyl radical scavenger, compared to other extracts from *A. nigricans*.

Consistent to its high phenolic content, ethyl acetate showed the strongest scavenging radicals effects among the four extracts tested, except for scavenging of $O_2 \cdot^-$. On the other hand, water fraction was the only extract that was capable of scavenging all the radicals tested, even though its phenolic content was less than ethyl acetate fraction, which possibly owing to both fractions contained different type of phenolic. This suggest that ethyl acetate and water fraction as good sources of antioxidant through HAT and ET mechanisms. Water fraction acts better as an electron donor because it scavenged $O_2 \cdot^-$ This combined results suggest that both fractions may have potential for treating NAFLD and NASH because oxidative stress has been implicated in NAFLD and NASH (Park et al., 2015). Hence, ethyl acetate and water fractions were chosen for profiling of bioactive compounds using GCMS and LCMS-MS.

5.2 Profiling of phytoconstituents by GCMS and LCMS/MS

In the current study, ethyl acetate fraction of *A. nigricans* was profiled with the aid of GCMS and LCMS/MS, phytoconstituents namely, linoleic acid ethyl ester; butyl 9,12-octadecadienoate; 9,12-Octadecadienoic acid; Ergosta-5,7,22-trien-3-ol; 2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzene propanoic acid; 3, 30-di-O-methyl ellagic acid were present. Phytoconstituents including oxooctadecanoic acid; 2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid and 2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid isomer were identified in the water fractions. The phytoconstituents detected in *A. nigricans* were distinct from that of *A. auricula-judae*. In the study carried out by Oke & Aslim (2011), eleven phytoconstituents were identified using high performance liquid chromatography, namely gallic acid, catechin, p-hydroxybenzoic acid, caffeic acid, syringic acid, vanillin, p-coumaric, sinapinic acid, rosmarinic acid and cinnamic acid, luteolin in methanolic crude of *A. auricula-judae*. In general, methanol has been recognized to be particularly efficient solvent in extraction of lower molecular weight polyphenols (Dai & Mumper, 2010). Furthermore, Do *et al.* (2014) reported that increasing polarity of solvent promoted the extraction yield. In his study on impact of different extraction solvent on *Limnphila aromatica* showed that the extraction yield of pure methanol (26.06 %) is higher than that of pure ethanol (17.03 %) and pure acetone (12.33 %). In agreement to Do *et al.* (2014), Boeing *et al.* (2014) also proved that methanol was the most efficient solvent for extraction of antioxidant compounds from berries, followed by water, ethanol and acetone. In the study carried out by Dailey & Vuong (2015) on the effect of different solvents on the recovery of total phenolic content, flavonoids, proanthocyanidins and antioxidant properties of the macadamia skin also revealed that absolute methanol not only was more efficient in recovery of bioactive compounds yield, but also extracted bioactive compounds with more potent antioxidant properties than absolute ethanol; however, combination of

organic solvent such as methanol, ethanol, acetonitrile and acetone with water had the highest recovery yield of TPC, flavonoids and proanthocyanidins and exhibited greatest antioxidant properties. The presence of ethyl radical in chemical structure of ethanol may play a role in its low solvation of antioxidant molecules, compared to methanol (Boeing *et al.*, 2014).

Lipids are one of the important nutrient for mankind, which are made of fatty acids hydrocarbon chains with a carboxyl group at one end and a methyl group at the other (Patterson *et al.*, 2012; Orsavova *et al.*, 2015). Fatty acids are categorized into saturated and unsaturated fatty acids based on the presence or absence of double bonds (Park *et al.*, 2014; Orsavova *et al.*, 2015). Fatty acids without double bonds within the hydrocarbon chain is recognized as saturated fatty acids, whereas fatty acids which contain at least one double bond is known as unsaturated fatty acids, with two or more double bonds found in the hydrocarbon chain is called as polyunsaturated fatty acids (PUFA) (Patterson *et al.*, 2012). PUFAs can be further subdivided into omega-3 (n-3) and omega-6 (n-6) according to the position of the last double bond from the terminal methyl end of the molecule. The fundamental difference between these two PUFAs is that omega-6 (n-6) is linoleic acid (LA) whereas α-linolenic acid (ALA) is the main member of omega-3 (n-3) (Patterson *et al.*, 2012; Orsavova *et al.*, 2015; He *et al.*, 2016). In the current study, linoleic acid ethyl ester and 9, 12-Octadecadienoic acid were detected in the bioactive ethyl acetate fraction of *A. nigricans*. 9, 12-Octadecadienoic acid is also known as linoleic acid (Pubmed Open Chemistry Database). Both linolenic acid and linoleic acid belong to essential fatty acids because body cannot synthesize them and must be obtained through diet (Patterson *et al.*, 2012; Orsavova *et al.*, 2015).

Numerous *in vitro* study have reported the antioxidative capacity of compounds derived from natural origin in scavenging free radicals. Ergosta-5,7,22-trien-3-ol, which is more commonly called as ergosterol is also found in the bioactive ethyl acetate fraction.

Ergosterol is the main sterol in fungi (Urbain *et al.*, 2011) and is easily extracted because it is a part of the cytoplasmic membrane (Nowak *et al.*, 2016). In general, sterol is well recognized as the third lipid class, which is important in maintaining biological function and strengthen the structure of membranes (Dufourc, 2008). Ergosterol in mushrooms is a precursor to vitamin D_2 (Nowak *et al.*, 2016), it is converted to vitamin when mushroom is exposed to UV light (Banlangsawan & Sanoamuang, 2016). The presence of ergosterol in ethyl acetate fraction may play certain role in antioxidant capacity of the fraction in view of the capability of white and brown button mushrooms in scavenging DPPH radical was positively tally with free ergosterol content in the mushrooms (Shao *et al.*, 2010).

This study also identified 3, 30-di-O-methyl ellagic acid in bioactive ethyl acetate fraction. 3, 30-di-O-methyl ellagic acid is a derivative of ellagic acid. Ellagic acid is stable at high temperature because its melting point is 350 ^{o}C. It is made up by four rings characterizing the lipophilic domain, four phenolic groups and two lactones characterizing hydrophilic part (Sepúlveda *et al.*, 2011). Guo *et al.* (2011) found that acetone extract of *Euphorbia hylonoma* contained few ellagic acid derivatives, including 3,3', 4- tri -O-methylellagic acid, 3, 3'-di-O-methyl ellagic acid, 3-O-methylellagic acid and 3,3'-di-O-methylellagic acid-4'-O-β-dxylopyranosid, were active in scavenging DPPH radical, superoxide anion and hydroxyl radical. However, ethyl acetate fraction in this study was inactive in scavenging superoxide anion, which may due to less of variety of ellagic acid derivatives compared to acetone extract of *Euphorbia hylonoma*. The competence of ethyl acetate fraction from *A. nigricans* in scavenging free radicals could be due to the synergistic effect of compounds that were identified in the fraction. A study reported that synergistic effect of 3,30-di-O-methyl ellagic acid and other compounds in betel quid contributed to its effectiveness in scavenging DPPH radical, capable in reducing ferric ion and exhibited better inhibition of lipid peroxidation than α-tocopherol (Sazwi *et al.*, 2013). Besides catechin, tryptophan, ellagic acid, ellagic acid hexoside, and

3, 30-di-O-methyl ellagic acid were identified in pomegranate juice, which showed better antioxidant capacity than pomegranate seed against DPPH radical (Rouhi *et al.*, 2017).

In the current study, butyl 9,12-octadecadienoate was identified in bioactive ethyl acetate fraction. This phytoconstituent was also identified in *Agaricus bisporus* (J.E. Lange) Imbach (Mohamed, 2012), root and rhizome of *Zingiber niveum* Mood and Theilade from Laos (Theanphong *et al.*, 2015), riceberry bran from Thai (Suttiarporn *et al.*, 2016), leaves of *Abutilon indicum* (Radhakrishnan *et al.*, 2017), and *Asparagopsis taxiformis* alga (Mellouk *et al.*, 2017). Butyl 9,12-octadecadienoate is a derivative of octadecadienoate, which is an unsaturated fatty acid (Mellouk *et al.*, 2017). In the study carried out by Radhakrishnan *et al.* (2016), both *Abutilon indicum* leaves and seeds exerted significant scavenging DPPH radical capacity. Butyl 9,12-octadecadienoate is one of the phytoconstituent identified in the leaves, which indicate that the compound may work synergistically with other compounds in the extract to evoke its antioxidant activity. Other derivative of octadecadienoate, such as ethyl(9E,12E)-9,12-octadecadienoate was found as one of the phytoconstituents among hexadecanoic acid, ethyl palmitate, linoleic acid, (z,z,z)-9,12,15-octadecatrienoic acid, (z,z,z)-ethyl ester-9,12,15-octadecatrienoic acid, and ethyl linoleolate in the ethanol extract of *Prunella vugaris* var. *lilacina*, obviously revealed that this extract had good antioxidant capacity when tested on DPPH assay, FRAP, ABTS, and SOD activity, compared to other solvent extracts, including hexane, butanol and chloroform (Hwang *et al.*, 2013). The antioxidative capacity of ethanol extract from *Prunella vugaris* var. *lilacina* could be due to the synergistic effect of the compounds in the extract. Likewise, butyl 9,12-octadecadienoate in ethyl acetate fraction could also have similar synergistic impact on its antioxidative capacity.

One of the phytoconstiuents identified in bioactive water fraction is oxooctadecanoic acid. It is a stearic acid. Rouhi *et al.* (2017) compared antioxidant

capacity between pomegranate fresh juice and pomegranate seed through DPPH radical assay, both fresh juice and seed were active as a radical scavenger, even thought fresh juice exert better antioxidant capacity. Oxooctadecanoic acid was identified among other phytoconstituents in pomegranate seed including 3, 30-di-O-methyl ellagic acid, 15, 16-dihydroxy-9Z,12Z-octadecadienoic acid, methyl 2-[-cyclohex-2-en-1-yl(hydroxy)methyl]-3-hdroxy-4-(2-hydroxyethyl)-3-methyl-5-oxoprolinate and p-hydroxybenzoic acid (Rouhi *et al.*, 2017).

2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid and its isomers were also identified in bioactive water fraction. 2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid was also identified in ethyl acetate fraction. They are derivative of benzenepropanoic acid. Benzenepropanoic acid is reported to be used for flavoring, such as flavoring for ice cream, bakery and confectionary, sweeteners for food as well as emulsifier to separate oil and water mixture; it also is used as a preservative to maintain the original aroma quality of frozen foods (Grover & Patni, 2013). In addition, it acts as antioxidant to prolong shelf life of food, and commonly used in cosmetic products, soap, liquid and powder detergents, fabric softeners to make the products emit floral scent (Grover & Patni, 2013). Ravi *et al.* (2015) reported that Neerkovai treated sinusitis via exerting antioxidant as well as antibacterial activities, and these bioactivities were caused by the presence of various phytoconstituents. Furthermore, benzenepropanoic acid was found to be one of the major phytoconstituents that may contribute to Neerkovai's bioactivities.

Therefore, this study suggested that ergosta-5,7,22-trien-3-ol; 3,30-di-O-methyl ellagic acid; butyl 9,12-octadecadienoate; 2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid may partially be responsible for antoxidant capacity of ethyl acetate fraction. Oxooctadecanoic acid and 2(3,4-Dihydroxyphenyl)-7-hydroxy-5-

benzenepropanoic acid acid possibly mediate water fraction in scavenging of free radical as shown by the *in vitro* antioxidant activities in this study.

The "two-hit hypothesis" stated that fatty degeneration of hepatocytes led to oxidative stress, which mediate progression of NAFLD to NASH that involve inflammation (Liang *et al.,* 2015). Continuous consumption of a high-fat, high-fructose diet was suggested to be culprit factor for oxidative stress, liver damage, and increase the risk and progression of NAFLD in mice (Jarukamjorn *et al.* ,2016). This is because high-fat, high-fructose diet caused an increase in the production of superoxide anion and hydrogen peroxide, increase in MDA level and increase in the oxidized GSSG content while the total GSH and the reduced GSH were decreased, resulting in the increase in the GSH/GSSG ratio in both livers and brains of the high-fat, high-fructose diet fed mice (Jarukamjorn *et al.*, 2016). In contrast, Jensen *et al.* (2018) reported that dietary fat and cholesterol, but not dietary sugar, are responsible for the development of NAFLD. Furthermore, Kumar *et al*. (2013) reported that NAFLD patients showed higher oxidative stress compared to chronic viral hepatitis patients because the former had significantly higher MDA, higher GPx activity, lower GSH level and decreased CAT activity. Hence, when the first line defence antioxidants mechanisms (SOD, CAT, GPx) come to failure, exogenous antioxidant (intake of antioxidant rich foods or antioxidant supplements) as a second line defence antioxidants is needed to minimize the deleterious effect caused by ROS (Ighodaro & Akinloye, 2018). Second line defence antioxidants act as a radical scavenger to inhibit chain initiation, break chain propagation reactions by donating electron to them, and in the process become free radicals themselves but of lesser damaging effects and easily neutralized (Ighodaro & Akinloye, 2018).

Therefore, the antioxidant effect of ethyl acetate and water fractions may mediate the hepatoprotective effects against NAFLD and NASH. Phytoconstituents in ethyl acetate and water fractions may have vary potential healthcare effects (biochemical

mechanisms underlying the hepatoprotective roles). Thus, more effort is needed to investigate the potential effects to combat oxidative stress and inflammatory process by using *in vitro* cell line model in order to alleviate NAFLD and NASH.

5.3 Cytotoxicity assay of water, ethyl acetate fractions and palmitic acid

The common method to study the cytotoxicity effect of an extract is through conventional MTT cytotoxicity study against a cell line. In this study, the cytotoxicity effects of bioactive fractions on HepG2 cells were evaluated prior to cell based assays. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was carried out to ascertain the safe concentration of samples to be used in present study. Besides that, cytotoxicity of palmitic acid was determined in order to obtain a safe concentration for establishment of an *in vitro* cell culture model of hepatic stetaosis in present study.

MTT is a widely used method to evaluate cell viability (Mueller *et al.*, 2004). MTT readily penetrates viable eukaryotic cells attributed to its positive charged (Riss *et al.*, 2004). The principle of MTT is based on the conversion of MTT into formazan crystals by living cells through dehydrogenase mitochondria activities in living cells (Meerloo *et al.*, 2011; Mutua *et al.*, 2013). Dead cells on the other hand lose the ability to convert MTT into formazan, hence, colour development serves as a useful and convenient marker of only the viable cells (Riss *et al.*, 2004). Capability of cells of reducing MTT determined mitochondrial integrity and activity (Beena *et al.*, 2011). Total mitochondrial activity is proportional to the number of viable cell in most of the cells population, thus, MTT crystal formazan is directly proportional to the number of living cells (Meerloo *et al.*, 2011; Mutua *et al.*, 2013).

Water fraction was non-cytotoxic towards HepG2 cells at the tested concentrations used (6-100 µg/mL) after both 24 and 48 hours treatments. Interestingly, water fraction significantly enhanced the growth of cells at all tested concentrations (6-

100 µg/mL), compared to the control group for 24 hours incubation. In addition, a 48 hours treatment of water fraction at all concentrations, except for 100 µg/mL, also significantly promoted HepG2 cells growth.

DMSO (0.2%) was used as a vehicle control to evaluate cytotoxicity effect of ethyl acetate fraction towards HepG2 cells. Likewise, Lee *et al.* (2011) and Bardi *et al.* (2014) reported using the same concentration of DMSO in their study on WRL-68 cells, human prostate cancer cells and HepG2 cells.

In comparison to water fraction, ethyl acetate fraction was cytotoxic to HepG2 cells at the highest tested concentration (100 µg/ml) for 24 hours as well as 48 hours duration of treatment. According to Park *et al.* (2015), samples are considered non-cytotoxic if the percentage of cell viability did not decrease below 90 % of the control level. In agreement, Basak *et al.* (2016) indicated that percentage of viability of cells above 90 % showed a sample is non-cytotoxic and slight cytotoxic when viability cells reach 60-90 %. In contrast, López-García *et al.* (2014) referred to ISO 109935 reported that percentages of cell viability above 80% are considered as non-cytotoxic; within 80-60 % as weakly; 60-40% moderately and below 40% as strongly cytotoxic respectively. As a consequence, ethyl acetate fraction at 100 µg/mL was cytotoxic to the cells for 24 and 48 hours duration of treatments, because cells viabilities were 88.56 ± 1.03 % and 74.47 ± 4.84 %, respectively. On the other hand, 50 µg/mL of ethyl acetate fraction only showed slight or weak cytotoxicity effect towards cells after 48 hours treatment (79.38 ± 2.20 %), but not after 24 hours treatment (97.23 ± 6.00 %). After 24 and 48 hours of incubation, low concentrations of the ethyl acetate fraction (6, 13, 25 µg/mL) exhibited non cytotoxicity to the tested cells.

From this assay, it can be concluded that all fractions were non cytotoxic to HepG2 cells except for the highest concentration of ethyl acetate fraction. Ethyl acetate fraction (50 µg/mL) is save to be used for assays carried out for 24 hours. Therefore, all

tested concentration for fractions were further used in the next assay, however, 100 µg/mL of ethyl acetate fraction was excluded in the present study.

Fatty acids with 16 and 18 carbon atoms, such as palmitic, stearic, oleic and linoleic are abundant in animals and plant tissues (Tvrzicka *et al.*, 2011). Gambino *et al.* (2016) reported that palmitic and oleic acids are the most plentiful circulating fatty acid present in the high serum level of FFA in NAFLD subjects. Due to lipotoxic impact, the abundance of palmitic acid presence in blood stream cause cellular dysfunction and cell death in vary cell types, especially cells from non - adipose tissues (Khan *et al.*, 2012). Serum FFA levels was also reported to be significantly correlated to NAFLD (Zhang *et al.*, 2014). Furthermore, abundant of saturated palmitic acid (C16:0) and monounsaturated oleic acid (C18: 0) are found in abundance in the steatotic liver (Moravcová *et al.*, 2015). In general, saturated fatty acids is well recognized to be responsible for the most parts of lipotoxicity, whereas unsaturated fatty acid commonly plays protective role in against saturated fatty acid - induced hepatotoxicity (Ning *et al.*, 2016). Moreover, palmitic acid is the saturated fatty acid that widespread in animals, plants and microorganism, chronically exposure and overloading of it resulted in cellular dysfunction and apoptotic cell death in various cells from non-adipose tissues (Khan *et al.*, 2012; Park *et al.*, 2014). Therefore, palmitic acid was chosen and exposed to HepG2 cells to establish a cellular hepatic model to study hepatic steatosis *in vitro*.

In this study, HepG2 cells were selected to be used as an *in vitro* model due to their stable features including ease of cultivation (Cao *et al.*, 2016), also they have been widely used as an *in vitro* model to study cytoprotective, genotoxicity and antigenotoxicity of compounds (Vidyashankar *et al.*, 2013). More importantly, HepG2 cells have been used to investigate fatty liver pathogenesis due to the preserved many specialized function of normal human hepatocytes (Vidyashankar *et al.*, 2013), besides,

HepG2 cells also demonstrated the closest resemblance of signalling network patterns to primary hepatocytes (Saez-Rodriguez *et al.*, 2011; Razali *et al.*, 2015).

Addition of FFAs to cells may cause an obvious increase of lipid droplet in the cytoplasma, which lead to toxic manifestations such as apotosis and necrosis if it reached high level (Yao *et al.*, 2011). In the current study, palmitic acid at concentrations of 50, 100 and 300 µM had no cytotoxic effect on HepG2 cells compared to the control as determined by the MTT assay. Conversely, palmitic acid was found significantly toxic at 500 µM because it caused viability rate to decrease as low as 52.95 ± 4.79 % as shown by MTT assay. It was in agreement with Kim *et al.* (2010) as they reported that palmitic acid at concentration of 500 µM led to a decrease in cell viability of HepG2 by 48 %. However, Kim *et. al.* (2010) continued exposure of HepG2 cells to the cytotoxic concentration of palmitic acid with the aim of investigating the direct effect of this fatty acid on apoptosis, endoplasmic reticulum stress and insulin signaling in human HepG2 cells, also the effect of metformin intervening hepatic insulin resistance and cell death. Therefore, palmitic acid at concentrations of 50, 100, 300 µM were further used for testing induction of hepatic steatosis in HepG2 cells. While, the non-cytotoxicity of concentrations of extracts were applied in anti-hepatosteatosis assay and other subsequent assays when *in vitro* palmitic-acid induced cell model was established.

5.4 Establisment of palmitic acid-induced HepG2 cells

The hallmark of NAFLD is characterized by accumulation of excessive fatty acid in liver (Woods *et al.*, 2015), which lead to a condition termed lipotoxicity (Ning *et al.*, 2016). In general, hyperlipidemia, obesity, diabetes mellitus, insulin resistance are the risk factor for primary NAFLD (Marchesini *et al.*, 2003; Ong & Younossi, 2007; Wu *et al.*, 2016). Diet plays an important role in the complex pathophysiology of NAFLD (Sullivan 2010; Marchesini *et al.*, 2016). Dietary fats have crucial impact on health as a

source of energy (Juárez-Hernández *et al.*, 2016). In addition, fatty acids are basic components of lipids and cell membranes in a form of phospholipids (Tvrzicka *et al.*, 2011). Fatty acid is also important as a precursor for ligands that bind to nuclear receptors as well as play key roles in intracellular signalling (Ricchi *et al.*, 2009). However, over-nutrition or inappropriate diet such as high caloric intake leads to NAFLD (Perumpail *et al.*, 2107) because excessive influx of fatty acids to liver is strongly associated with nutritional factors (Juárez-Hernández *et al.*, 2016). Habitually, NAFLD patients are more likely to consume diet containing a higher content of saturated fatty acids, cholesterol and fructose, but lower ingestion of polyunsaturated fatty acids (Dongiovanni & Valenti, 2017).

The safe concentrations of palmitic acid, 50, 100 and 300 µM were tested for induction of hepatic steatosis in HepG2 cells. Only palmitic acid at concentration of 300 µM highly increased intracellular lipid in the cells. In comparison to control, HepG2 cells induced with 300 µM of palmitic acid showed an increase by 1.9 fold. In HepG2 cells exposed to 50 and 100 µM of palmitic acid showed no significance different from the control. Therefore, 300 µM of palmitic acid was chosen as the optimal concentration to induce steatosis in HepG2 cells for the subsequent experiments because 300 µM of palmitic acid is more steatogenic than 50 and 100 µM of palmitic acid ($p<0.05$) and non-cytotoxicity to HepG2 cells.

In consistent with Joshi-Barve *et al.* (2007), HepG2 cells was induced by using palmitic acid at concentration of 300 µM, in order to study the association of palmitic acid and interleukin 8 (IL-8) through nuclear factor kappa B and c-Jun N terminal kinase/activator protein 1. Similarly, Yan *et al.* (2017) constructed an *in vitro* hepatic steatosis model by inducing HepG2 cells with 300 µM of palmitic acid for the purpose of investigating the amelioration lipid accumulation, inhibition of endoplasmic reticulum stress and anti-oxidative stress effects of natural product isolated from the root of

Rehmannia glutinosa on cholesterol homeostasis in non-alcoholic fatty liver disease. In search of way to treat or prevent NAFLD, Heo *et al.* (2018) also constructed an *in vitro* steatosis model by inducing HepG2 cells with 300 µM of palmitic acid with aim to determine whether melatonin can improve hepatic insulin resistance and hepatic steatosis.

Palmitic acid has long been used as an inducer in the study of NAFLD. Various concentrations of palmitic acid were exposed to the cell lines for research purpose. Gao *et al.* (2010) added palmitic acid to HepG2 cells at concentrations of 150, 250 and 350 µM for 24 hours and assessed viability of cells by MTT assay to exclude side effect of palmitic acid, for instance apoptosis. Their study showed that 250 µM of palmitic acid had no cytotoxic effect towards HepG2 cells. Furthermore, Park *et al.* (2014) also added 250 µM to Chang liver cells in the study of pathways involved in palmitic acid-induced toxicity such as protein related to apoptosis, endoplasmic reticulum stress, caspases, p53, necroptosis, and autophagy. Some researchers would even use palmitic acid at high concentration. Han *et al.* (2012) induced lipid accumulation in HepG2 cells with palmitic acid but at a higher concentration, 500 µM, in their study of *Alisma orientale* extract in treatment of palmitic aid-induced cellular injury, to determine the properties of the extract in inhibition of apoptosis, capability of suppressing intracellular ROS and lipid peroxidation, also c-Jun NH_2-terminal kinase (JNK), induced by palmitic acid.

5.5 Anti-hepatic steatotic and scavenging of intracellular ROS effects of water and ethyl acetate fractions

NAFLD is well known as a condition of liver disease begins from simple steatosis, then progress to non-alcoholic steatohepatitis (NASH), hepatic fibrosis/cirrhosis to hepatocellular carcinoma (Ferolla *et al.*, 2015). Lifestyle habits has an impact on the occurrence of NAFLD with diet ~~is~~ contributing as the most important determinant of lifestyle responsible in the development of NAFLD pathogenesis (Wit *et al.*, 2012; Liu *et*

al., 2016). Westernized foods that peoples habitually consume is well-known to have high fat and high sugar contents and these are associated to the development of NAFLD (Chen *et al.*, 2016; Tessitore *et al.*, 2107). Furthermore, long term high-fat diet consumption increased the risk of developing NAFLD (Zhou & Xie, 2015). Jensen *et al.* (2018) carried out an experiment on investigation of the effect of a high-fat diet, a high-fructose diet and a combination diet with added cholesterol on the development of NAFLD in rats. Based on the experiment, dietary fat and cholesterol are the key drivers of NAFLD development and progression in rats by elevating hepatic triglyceride content, elevating cholesterol and also led to hepatic dysfunction and inflammation. Rats that fed on high-fructose diet, however, only showed higher level of plasma triglyceride compared to the other groups. Several lines of evidence also documented that diet with a high intake of fat, especially saturated fatty acids, have a tendency to promote the development of NASH (Musso *et al.*, 2003; Gentile *et al.*, 2008; Luo *et al.*, 2012). For that reason, palmitic acid was used as an inducer in the current study. In general, saturated fat is more likely to stimulate hepatic lipid accumulation and progression into NASH (Wit *et al.*, 2012). Several lines of evidence reported that dietary palmitic acid and cholesterol have been related to liver disease progresses from simple steatosis to NASH (Li *et al.*, 2009; Charlton *et al.*, 2011; Ioannou *et al.*, 2013). It has been reported that feeding mice with westernized diet that contained moderately high saturated and trans-fat, cholesterol and sucrose successfully developed a NASH phenotype in mice that possessed many of clinical features of human NASH such as hepatic injury, inflammation, oxidative stress and fibrosis (Jump *et al.*, 2016).

Since diet is the important contributor to the development and progression of NAFLD and NASH, so lifestyle modification remains as the basis of prevention and treatment of NAFLD and NASH (Barb *et al.*, 2016). Furthermore, there is no evidence-based pharmocological treatment for NAFLD up till now (De Wier *et al.*, 2017). The

primary therapy of lifestyle changes is through weight reduction, consisting of diet, physical activity and behavioral change, or a combination of all three (Hardy *et al.*, 2015; Ferolla *et al.*, 2016). Acquiring a healthy dietary pattern alone is also able to reduce body weight, which has been shown to contribute to histological improvement in fatty liver; therefore, nutrition therapy become a cornerstone of treatment for NAFLD (Papandreou & Andreou, 2015). Hence, it is important to investigate anti-hepatic steatotic effect of ethyl acetate and water fraction of *A. nigricans* because it is one of the highly consumed commercial edible mushroom.

One "first" hit of the "two-hit theory" is the accumulation of lipid in hepatocytes. Based on the theory, this so-called "first" hit is an important target for prevention and treatment for NAFLD. Hence, this study aims to investigate the effect of water and ethyl acetate fractions on palmitic acid-induced intracellular lipid accumulation in HepG2 cells. The role of water and ethyl acetate fractions of *A. nigricans* in eliminating lipid accumulation in hepatocytes was evaluated by detecting the lipid droplet in hepatocytes via Oil Red O staining. The present results showed that no significant reduction in intracellular lipid was observed when palmitic-acid induced-HepG2 cells were treated with water fraction of *A. nigricans* at concentrations of 100, 50, 25, 13 and 6 µg/ml. Benzenepropanoic acid also known as hydrocinnamic acid (NIST Chemistry WebBook, SRD 69) was identified in water sample as its derivative 2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid. In this study, it may mildly exert certain extend in inhibition of lipid accumulation because caffeic acid phenethyl ester, a derivative of hydrocinnamic acid, was reported to ameliorate hepatic steatosis in mice fed with high-fat diet (Bezerra *et al.*, 2012; Alam *et al.*, 2016).

Oxooctadecanoic acid in water fraction is a stearic acid, which is major long chain dietary fatty acid (Shen *et al.*, 2014) that found in animals and fungi and a minor component in most plants (Rustan & Drevon, 2005). From the Oil Red O stain results, it

was observed that the presence of stearic acid did not cause an increase in lipid accumulation in HepG2 cells. It may either, due to stearic acid was taken up slowly by cells and was poorly incorporated into both cellular and secreted triglyceride (Bruce & Salter, 1996), or owing to the synergistic effect of compounds in water fraction.

From the results, it showed that palmitic acid-induced HepG2 cells treated with increasing concentration (6-50 µg/mL) of ethyl acetate fraction of *A. nigricans* marked a significant ($p<0.05$) attenuated the intracellular lipid accumulation. 50 and 25 µg/mL of ethyl acetate fraction contributed to 18.07 and 13.86 %, respectively, decreased in hepatic lipid accumulation compared to palmitic acid-induced HepG2 cells. While 13 and 6 µg/mL of ethyl acetate fraction caused 9.7 % reduction in lipid accumulation. Park *et al.* (2015) reported that oligonol (10 µg/mL), a low molecular weight form of polyphenol polymer derived from lychee fruits, caused approximately 29.4 % decrease in lipid accumulation of palmitic acid (250 µg/mL)-induced HepG2 cells. The current results discrepancy from Park *et al.* (2015) probably due to their used of pure polyphenol compound in the experiment. Kim *et al.* (2018) reported that ginseng oil at concentration of 25 and 50 µg/mL contributed approximately 33.33 and 41.67 %, respectively, reduction of lipid accumulation in mixture of palmitic acid and oleic acid-induced HepG2 cells. Interestingly, the presence of linoleic acid ethyl ester and 9, 12-Octadecadienoic acid (also known as linoleic acid) in ethyl acetate fraction did not promote accumulation of lipid in HepG2 cells. The current results were consistent with the study carried out by Maruyama *et al.* (2014). Based on their results, lipid accumulation showed no significant different between palmitate-treated cells, linoleate-treated cells, and palmitate and linoleate-treated cells. Linoleic acid in ethyl acetate fraction could possibly interacted with the inducer in this study, palmitic acid, thus, exhibited synergistic effect that led to attenuation of the intracellular lipid accumulation in ginseng seed oil - oleic (73.12 %) and linoleic acids (17.33 %), palmitic acid (2.01 %) and palmitoleic acid (0.25 %). This

caused a significant drop in lipid accumulation in HepG2 cells that were induced by 1 mM of a mixture of free fatty acids (FFA; oleic acid: palmitic acid = 2:1) (Kim *et al.*, 2018). An evidence indicated that the intake of linoleic acid higher than 4.5% of energy resulted in suppression of hypercholeterolemic effect of palmitic acid (French *et al.*, 2002). Additionally, linoleic acid has long been well recognized in cholesterol-lowering effect through human trial by reducing of coronary heart disease risk when saturated fatty acid was replaced by linoleic acid (Harris *et al.*, 2009). Based on the observation, linoleic acid in ethyl acetate fraction may work synergistically with the inducer, palmitic acid, give rise to hepatoprotective effect during the co-treatment that carried out on the cells.

Polyphenols present abundantly in our daily diet and contribute beneficial effects on human health by modulating metabolism in order to attenuate hyperglicemia, dyslipidemia, insulin resistance, and alleviate oxidative stress, hence prevent several chronic diseases such as cardiovascular disease (Lin *et al.*, 2016). A phenolic compound, 3, 30-di-O-methyl ellagic acid, was found in ethyl acetate sample. This is a derivative of ellagic acid. Ellagic acid capability to reduce lipid accumulation have been reported in a number of studies. A study comparing the lipid-lowering effects of pomegranate peel polyphenol, punicalagin, ellagic acid, gallic acid, phlorizin and epigallocatechin gallate on an *in vitro* HL7702 steatosis hepatic cell model indicated that all single monomer components and pomegranate peel polyphenol possessed significant alleviation of lipid accumulation in cells, with better outcome was observed in prevention group than in treatment group. However, pomegranate peel polyphenols (mixture of gallic acid, punicalagin, catechin, chlorogenic acid, caffeic acid, epicatechin, rutin and ellagic acid) had stronger capacity in alleviation of intracellular lipid than the single compositing mononer components (Zhao *et al.*, 2014). Besides, Lu *et al.* (2016) also proved that *Phyllanthus emblica* L. with ellagic acid as a major compound in water extract, significantly decreased fat accumulation in HepG2 cells when authors performed an in

vitro hepatic steatosis. Hence, 3, 30-di-O-methyl ellagic acid may possessed the same attenuation of lipid accumulation effect. Hydrocinnamic acid derivative, 2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid that present in water fraction was also identified in ethyl acetate fraction. Taken together, it could be suggested that the reduction of intracellular lipid accumulation effect of ethyl acetate fraction may partially due to the interaction of compounds in the fraction.

Fisetin, a flavonoid, was reported to be capable of suppressing lipid accumulation in obese mice through activation of sirt1/AMPK and enhanced of lipolysis and β-oxidation (Liou *et al.,* 2018). Low fatty acid oxidation (Suzuki-Kemuriyama *et al.,* 2016; De Wier *et al.,* 2017) and mitochondrial dysfunction causes inhibition on β-oxidation of lipids (Ucar *et al.,* 2013) are the factors that cause lipid accumulation in liver. Impairment of β-oxidation hinders removal of lipids in the cells, which result in an increase in toxic intermediate lipid product (De Wier *et al.,* 2017). Therefore, enhanced β-oxidation can prevent progression of lipid accumulation in hepatocytes. Ginseng seed oil was reported to have anti-hepatosteatosis activity through increase expression of genes associated with β-oxidation (Ppara, Ppargc1a, Sirt1 and Cpt1a) and decrease expression of lipogenic genes (Srebf1 and M1xipl) in high-fat fed mice (Kim *et al.,* 2017). Similarly, DHA inhibits lipid accumulation by suppressing lipid synthesis in the liver through impairing the proteolytic release of SREBP-1c and/or by suppressing SREBP-1c gene expression and upregulating fatty acid oxidation by serving as an *in vivo* activator of peroxisome proliferator-activated receptor alpha (PPAR-α) (Luo et al., 2012). Hence, inhibiting the proteolytic cleavage of SREBPs suppressed lipogenesis and alleviated intrahepatic lipid accumulation condition. *De novo* lipogenesis contributes less than 5 % of fatty acid incorporated into secreted VLDL in normal subjects, whereas the rate of *de novo* lipogenesis highly increased and contributed approximately 20 % of lipid accumulation in hepatocytes of NAFLD subjects (Chen *et al.,* 2014). Decreased in lipid accumulation

in HepG2 cells by ethyl acetate fraction observed in this study could be due to enhanced β-oxidation and/or inhibition of lipogenesis. The lipid accumulation in hepatocytes are determined by inhibition or activation of intrahepatic fatty acid oxidation and lipogenesis. Therefore, it is important to determine the effect of ethyl acetate and water fractions on β-oxidation and lipogenesis in further study.

High-fat diet contributes to oxidative stress through promoting steatosis, active expression levels and activity of enzymes involved in β-oxidation thus, led to increased mitochondrial hydrogen peroxide production (Kakimoto & Kowaltowski, 2016). Oxidation of fatty acids mainly occurs in the mitochondria. Despite this, excess fatty acid accumulate in hepatocytes can lead to β-oxidation in peroxisomes and β-oxidation in microsomes (endoplasmic reticulum) (Park *et al.*, 2015). Enhanced mitochondrial and peroxisomal β-oxidation and microsomal β-oxidation further elevation of ROS production (Gudson *et al.*, 2014). Therefore, a decrease in lipid accumulation in HepG2 cells by extracts of *A. nigricans* in this study led to investigation of the effect of extracts on production of ROS in HepG2 cells.

Dichlorodihydrofluorescein diacetate (DCFH2-DA) is a popular technique used to measure ROS due to it is simple to use, particularly sensitive to changes in the redox state of a cell, inexpensive and can be used to follow changes in ROS over time (Eruslanov & Kusmartsev, 2010). DCFH2-DA is a non-polar and non-fluorescent probe that easily cross the cell membrane due to its lipophilic property (Karlsson *et al.*, 2010; Zapolska-Downar *et al.*, 2012). Inside cells, DCFH2-DA is deacetylated by esterase to form non-fluorescent, hydrophilic dihydrochlorofluorescein (DCFH2) (Karlsson *et al.*, 2010; Dwivedi *et al.*, 2014) and trapped in cells (Chavez-Tapia *et al.*, 2012). DCFH2 is converted to highly fluorescent 2',7'-dichlorofluorescein (DCF) upon oxidation by intracellular oxidants, in particular H_2O_2 (Kalsson *et al.*, 2010; Kalyanaraman *et al.*, 2012; Chavez-Tapia *et al.*, 2012; Zapolska-Downar *et al.*, 2012).

Under normal physiological circumstance, hepatocytes release small number of ROS through mitochondrial respiration in the form of by-product, (Schattenberg & Czaja, 2014). Depending on tissues and varies with the redox state, mitochondria commonly generate 0.2-0.5 % ROS from the total of oxygen consumption (Auger *et al.*, 2015). The presence of palmitic acid in cells such as hepatocytes, pancreatic β cells, cardiomyocytes, vascular smooth muscle cells, endothelial cells, skeletal muscle cells, glomerular podocytes and adipocytes greatly enhance ROS product (Ly *et al.*, 2017). In NAFLD, enhanced cytochrome P4502E1 (CYP2E1) expression and electron leakage from the mitochondrial respiratory chain (MRC) seem to be important sources of ROS, which trigger oxidative stress (Aubert *et al.*, 2011).

Oxidative stress is well-known as a core mechanism of hepatocellular injury in NAFLD, furthermore, excessive ROS production contribute to transition of simple steatosis to NASH and fibrosis (Duvnjak *et al.*, 2007; Pacana & Sanyal, 2012; Chen *et al.*, 2015; Yan *et al.*, 2015; Sunny *et al.*, 2017) The damage of liver is resulted from direct interactions with critical cellular macromolecules such as DNA, proteins and lipids (Schattenberg & Czaja, 2014; Anavi *et al.*, 2015) that destroy their function or trigger their degradation, ultimately leading to cell death (Schattenberg & Czaja, 2014).

Accumulation of fatty acid in cells leads to increase in ROS production as indicated by Park *et al.* (2014). ROS was generated when cells were exposed to palmitic acid. Furthermore, the level of ROS elevation is dependent on the concentration of palmitic acid (Park *et al.*, 2014). In consistent with previous study, the present study also showed that ROS was successfully generated by HepG2 cells when exposed to palmitic acid. ROS level was approximately 5 folds increased in relative to control blank. The elevation of ROS production in the cells in present study may be due to enhanced β-oxidation as Nakamura *et al.* (2009) proved that the presence of palmitic acid in cells

accelerated β-oxidation that led to excess electron flux in mitochondrial respiratory chain, as consequence increased ROS generation.

Antioxidants in natural plant extracts revealed that NAFLD can be cured through elimination of oxidative stress. For instance, punicalagin eliminated oxidative stress in high-fat diet induced NAFLD (Zou *et al.*, 2014). In this study, we assessed the effect of water and ethyl acetate fractions from *A. nigricans* in protecting HepG2 cells from excessive ROS production, under the condition of induction of palmitic acid. Based on our results, treatment with concentration of ethyl acetate fraction > 13 µg/mL showed a significant reduction ($p<0.05$) in ROS level. Treatment of ethyl acetate fraction at concentration of 6, 13, 25, 50 µg/mL contributed to 2.00, 2.56, 8.19 and 10.95 % decrease in ROS level in cells. Derivative of ellagic acid found in ethyl acetate fraction could possibly display ROS protective effect by decreasing the ROS level in palmitic acid-induced cells because ellagic acid in water extract of *Phyllanthus emblica* L. proved to protect fat-loaded HepG2 cells from injurious ROS via altering lipogenesis-related gene expression and stimulating AMP-activated protein kinase (AMPK) signaling (Lu *et al.*, 2016). Pepe *et al.* (2015) reported that green lettuce exhibited antioxidant capacity by decreasing ROS that generated in lipopolysaccharide (LPS)-induced macrophage, partly because of the presence of hydrocinnamic acid derivatives. Similarly, we also found hydrocinnamic acid derivative in ethyl acetate fraction, 2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid. Hence, we suggested that antioxidant capacity of ethyl acetate fraction partly due to the presence of hydrocinnamic acid derivative. Based on research done by Pereira *et al.* (2014), ergosta-7,22-dien-3ol alone did not lower the high intracellular ROS level caused by LPS in RAW 264.7. However, combination of palmitic acid, cis 11-eicosenoic acid, cis 11,14-eicosadienoic and ergosta-7,22-dien-3-ol reduced the ROS level by about 50 %. Therefore, we believe that antioxidative activity by ethyl acetate fraction in palmitic acid-induced HepG2 cells may partially be attributed

to interaction among compounds in the sample. Overload of fatty acid in mitochondria resulted in enhanced ROS generation due to increase of fatty acid oxidation, and stimulating an increase electron flux in the electron transport chain (ETC), which contribute to electron leakage. The escaped electrons react to oxygen lead to ROS formation (Masarone *et al.,* 2018). Biochemical, histological and molecular experiments have proven that resveratrol is capable of inhibiting ROS generation in rat model of high-fat diet-induced NAFLD through inhibiting free fatty acid β-oxidation via inhibition of carnitine palmitoyltransferase 1(CPT-1) and mitochondrial uncoupling protein-2 (UCP2) (Khaleel *et al.,* 2018). Alleviation of oxidative stress through promotion of fat oxidation in order to reduce accumulation of fatty acid in hepatocytes by fisetin was reported to suppress lipid accumulation in liver of obese mice through enhancement of β-oxidation via recovering of CPT-1 expression (Liou *et al.,* 2018). Furthermore, fisetin decreased oxidative damage to liver cell of NAFLD mice (Liou *et al.,* 2018). Park *et al.* (2015) also reported that oligonol hepatoprotective effects was through enhancing the β-oxidation for degradation of fatty acid by increasing the mRNA level of carnitine palmitoyltransferase 1a (CPT 1a). Simultaneously, oligonol also reduced the level of cytochrome P450 2E1 (CYP2E1) and CYP4A11, which are increased in palmitic acid-induced HepG2 cellss. Cytochrome P450 enzyme CYP2E1, a potential direct source of ROS (Gracía-Ruiz *et al.,* 2015; Masarone *et al.,* 2018) are involved in ω-oxidation, an alternative pathway of fatty acid metabolism, is activated upon failure of β-oxidation (Park *et al.,* 2015). ω-oxidation becomes crucial during period of increased influx of fatty acid into liver, for instance high-fat diet, obesity and when mitochondrial oxidation is insufficient to metabolize fatty acids (Hoek-van den Hil *et al.,* 2013). ω-oxidation leads to oxidative stress by the ability of cytochrome P450 2E1 and cytochrome P450 4A to partially reduce oxygen (Gudson *et al.,* 2014). In agreement, Jian *et al*. (2018) also reported that total flavonoids isolated from Loquat leave alleviated oxidatives stress in ambient air particulate matter (PM)-

induced NAFLD mice by suppressing the expression of cytochrome P450 2E1 (CYP2E1) and strengthening the anti-oxidative capacity of superoxide dismutase (SOD). PM is a class of heterogenous substances present in polluted air, such as metals, salts, volatile organic compounds, hydrocarbons, and even endotoxins (Jian et al., 2018). In this study, ethyl acetate fraction could be suggested to alleviate oxidative stress by enhancing the activity of antioxidant enzymes and/or suppressing cytochrome P450 enzymes as well as enhancing or suppressing β-oxidation.

Unlike ethyl acetate fraction, water fraction from *A. nigricans* in our study scavenged ROS weakly, it was a non-significant decrease, as compared to palmitic acid-induced-HepG2 cells. However, animal study showed that condition of NAFLD was ameliorated by elevation of antioxidant activities of superoxide dismutase, glutathione peroxidase and glutathione reductase as well as decreased level of lipid peroxidation when rats fed with high-fat diet were treated with water extract of *A. polytricha* (Chiu *et al.*, 2014). This outcome suggested that ROS generated by high-fat diet were removed. Our result discrepancy from Chiu *et al.* (2014) may due to different method used during sample preparation, which give variation in yield and number of extracted compounds (Bimakr *et al.*, 2011; Sun *et al.*, 2015).

Generation of ROS caused lipid peroxidation that contribute to cell injury. ROS that lead to lipid peroxidation of mitochondrial membrane resulted in impairment of mitochondrial and perpetuate ROS generation (Kakimoto & Kowaltowski, 2016). Furthermore, oxidative stress also triggers production of inflammatory cytokines and lead to inflammatory response (Ucar *et al.*, 2013; Kakimoto & Kowaltowski, 2016) as a result of activation of the major transcription factor linked with inflammation (Kesh *et al.*, 2016). Hence, ethyl acetate fraction was chosen over water fraction of *A. nigricans* for investigation of anti-inflammatory capacity and inflammatory signaling transduction pathways because the former showed significant positive capacity in reducing of ROS

generation in HepG2 cells. In the current finding, increased concentration of 50 µg/mL ethyl acetate fraction did not further reduce the ROS level, for this reason, 25 µg/mL was established for further anti-inflammatory assay in view of the optimal activity in scavenging of ROS. Antioxidant activity for the ethyl acetate fraction had reached it plateau phase for 25 µg/mL, this can be shown in the experiment of different concentrations of extract for ROS activity.

5.6 Anti-inflammatory capacity of ethyl acetate fraction

Inflammatory response is known to be a rapid host immune defense response against internal and external damage, such as lipotoxicity (Chávez-Tapia *et al.*, 2014). NAFLD is closely associated with chronic inflammation that involve abnormal cytokine production (Hijona *et al.*, 2010; Zhao *et al.*, 2015). In addition, chronic inflammation is a key factor in the progression of NAFLD to NASH pathogenesis (Lee *et al.*, 2015). Studies suggested that inflammation not only occur at the process of second hit but similar to lipid, as an important risk factor for hepatic steatosis (Chen *et al.*, 2014). Furthermore, inflammation may modify hepatic lipid homeostasis result in enhancing lipid accumulation in the liver (Chen *et al.*, 2014). Free fatty acid such as palmitic acid has been suggested, to be the source of chronic low grade inflammation, a fundamental of metabolic syndrome and its complications (Chávez-Tapia *et al.*, 2014). The inflammatory response is induced as a result of up-regulation of inflammatory cytokines in hepatocytes (Choi *et al.*, 2011; Chávez-Tapia *et al.*, 2014; Liang *et al.*, 2015). In consistent with previous studies, our results demonstrated that exposure of HepG2 cells to palmitic acid resulted in overproduction of inflammatory cytokines in culture supernatant, compared to control blank. In addition, our results are in agreement with Joshi-Barve *et al.* (2007), HepG2 cells exhibited significant elevation of IL-8 in response of 300 µM of palmitic acid. In the presence of palmitic acid, therefore, TNF-α, IL-6, IL-8 and MCP-1 were

probably activated in the *in vitro* NAFLD model constructed in this study. TNF-α and IL-6 are crucial in the progression of hepatic steatosis to a more advanced stage of liver damage (Choi & Diehl, 2005; Chen *et al.*, 2015). For this reason, it is crucial to determine the capacity of ethyl acetate fraction from *A. nigricans* in retarding the progression of the disease through lowering the level of pro-inflammatory cytokines. It would be a good sign if ethyl acetate fraction demonstrates a positive outcome from this assays because it is essential that the disease can be controllable.

The potential roles of TNF-α in NAFLD are induction of inflammation, insulin resistance, promotion of necrosis and fibrosis and participation in the loop of liver steatosis mediated by inflammation. TNF-α contributes in the development of liver injury in NASH and an increase in serum level of TNF-α have been noticed in NASH rat model as well as in NASH patients (Kawaratani *et al.*, 2013). IL-8 plays important role in recruitment of neutrophils whereas elevation of IL-6 is associated with disease severity, activation of immune cells, hepatocytes and hematopoietic stem cells as well as mediating the synthesis of several acute phase proteins (Chávez-Tapia *et al.*, 2014).

To determine the anti-inflammatory effect of ethyl acetate fraction from *A. nigricans,* HepG2 cells were treated with ethyl acetate fraction in the presence of palmitic acid. Based on current results, ethyl acetate fraction treatment at the tested concentrations decreased inflammatory cytokines secretion, including TNF-α, IL-6, IL-8 and MCP-1 by 28.57, 13.83, 16.80 and 15.60 %, respectively, compared to palmitic acid-induced cells. In the study carried out by Maruyama *et al.* (2014), treatment of palmitic acid in HepG2 cells and Huh7 cells led to significant elevation of IL-8, which is in agreement with the results of this study. However, co-treatment of linoleic acid and palmitic acid surprisingly suppressed IL-8 production lower that with palmitic acid alone in both cells. Therefore, linoleic acid may synergistically interact with the inducer, palmitic acid, to subdue the increased pro-inflammatory cytokines caused by palmitic acid during co-treatment. In

contrast, linoleic acid also was reported to be competent of exhibiting anti-inflammatory effect by itself, without synergistically interact with other compounds. Linoleic acid extracted from *Agaricus brasiliensis* protected RAW 264.7 from inflammation by suppressing the expression of pro-inflammatory cytokine including TNF-α, IL-6, IL-1β, when it was exposed to lipopolysaccharide (Saiki *et al.*, 2017). Besides, Zhang *et al.* (2012) reported that apoptosis was not observed when cells were co-treated with combination of palmitic acid and low concentration of linoleic acid. This suggests that linoleic acid in ethyl acetate fraction may also possess similar anti-inflammatory activity when cells were co-treated with ethyl acetate fraction and inducer, palmitic acid, in the present study. Hence, linoleic acid in ethyl acetate fraction could either act alone, or work synergistically with palmitic acid in displaying its anti-inflammatory effect. Lipophilic extract of *Marthasterias glacialis* contained main compounds including palmitic acid, *cis* 11-eicosenoic acid, *cis* 11,14-eicosadienoic acid and ergosta-7,22-dien-3-ol showed its anti-inflammatory capacity by decreasing IL-6 level in culture media when RAW 264.7 cells pre-treated with the lipophilic extract prior to LPS induction (Pereira *et al.*, 2014). It implied that ergosta-5,7,22-trien-3-ol in ethyl acetate fraction of *A. nigricans* may also work synergistically with the inducer in the current study, palmitic acid, in protecting HepG2 cells from inflammation. In a study performed by Nagao *et al.* (2010), NAFLD mice with characteristic of obesity, dyslipidemia, diabetes showed a decrease in MCP-1 level after four weeks of feeding with mukitake mushroom (*Panelluls serotinus*).

Polyphenols found abundantly in our diet have frequently been studied in model of NAFLD. For instance, quercetin exerted anti-inflammatory effect through decreasing levels of TNF-α and IL-8 in the oleic acid induced-HepG2 cells (Vidyashankar *et al.*, 2013). Human randomised controlled trials reported that consumption of bayberry juice and resveratrol showed anti-inflammatory effect, with a reduction in serum cytokines, particularly TNF-α, IL-6, IL-8, and increased serum level of adiponectin (Abenavoli *et*

al., 2017). Besides, Rafiei *et al.* (2017) reported that polyphenols (resveratrol, quercetin, catechin, cyanidin, kuromanin, and berberine) and phenolic degradation products (caffeic acid, protocatechuic acid, and 2,4,6-trihydroxybenzaldehyde) suppressed TNF-α expression in steatosis of HepG2 cells. Based on study carried out by Chiu *et al.* (2014), a four weeks' treatment with water extract of *A. polytricha* resulted in a decrease in serum level of TNF-α and IL-6 of the high-fat diet fed rats. According to Chiu *et al.* (2014), biological activities of *A. polytricha* such as anti-inflammatory, anti-oxidative, lipid-lowering and glucose-homeostatic are due to the synergistic effect of polysaccharide and abundant polyphenol, including gallic acid, tannic acid, protocatechuic acid and tannins. Hence, we suggested that part of anti-inflammatory effect of ethyl acetate fraction on palmitic acid-induced HepG2 cells may be due, at least partially, to the presence of the two polyphenols found in the fraction, which are 30-di-O-methyl ellagic acid and 2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid. Based on this assay, we suggested that anti-inflammatory effect of ethyl acetate fraction from *A. nigricans* may either, due to interaction among the compounds in the fraction and /or, the compounds interacted with palmitic acid, an inducer used in this study.

Pro-inflammatory cytokines have been known associated to pathogenesis of progressive NAFLD such as apoptosis of hepatocyte, and play a role in the hepatic and systemic insulin resistance linked to NASH (Cortez-Pinto *et al.*, 2006), such as IL-6 (Hijona *et al.*, 2010) and TNF-α (Xirouchakis *et al.*, 2009). Furthermore, TNF-α is also reponsible for stimulation of VLDL production and reduction of HDL, which contribute to pro-atherogenic circumstance (Das & Balakrishnan, 2011). In addition, normalization of the levels of pro-inflammatory has been shown to blunt the progression of NAFLD (Gudson *et al.*, 2014). Therefore, lowering of pro-inflammatory cytokines after treatment of ethyl acetate fraction of *A. nigricans* probably obstruct the development and progression of NAFLD and reduce the risk of extra-hepatic chronic complications, such

as type 2 diabetes mellitus, hypertension and cadiovascular disease (Targher et al., 2011). Production of pro-inflammatory cytokines are due to activation of transcription factors by fatty acid and ROS. Hence, further experiment was conducted to investigate the effect of ethyl acetate fraction of *A. nigricans* on the important transcription factors of inflammatory cytokine in NAFLD and NASH.

NF-κB is a protein complex that controls the transcription of DNA and regulate cellular stress in all cell types in the liver by playing important role in regulating the immune response to infection and in both acute and chronic inflammation (Kawaratani *et al.,* 2013). NF-κB is a nuclear transcription factor found in various cells that is closely associated with inflammation by promoting the expression of inflammatory cytokine. In turn, inflammatory cytokine such as TNF-α can further worsen the inflammation via further activation of NF-κB. (Zeng *et al.*, 2014). There are evidence that NF-κB activity was up-regulated in NAFLD patients (De Wier *et al.*, 2017) and was markedly increased in NAFLD rats compared to control rats (Leclercq *et al.*, 2004; Tipoe *et al.*, 2009). IκB kinase (IKK) complex is responsive to many inflammatory stimuli (Chen *et al.,* 2014). Activation of NF-κB can be initiated by FFA and ROS that resulted in production of pro-inflammatory such as TNF-α, IL-6 and IL-8 (Duvnjak *et al.*, 2007; Zeng *et al.,* 2014). Saturated free fatty acids such as palmitic acid activates toll-like receptor (TLR), lead to downstream activation of IκB kinase (IKK) complex. Upon activation, IKK phosphorylates and degradation of IkB-α protein resulted in p65 entering the nucleus and trigger transcriptional expression of multiple pro-inflammatory cytokines and chemokines (Maloney *et al.*, 2010; Baker *et al.*, 2011; Farrell *et al.*, 2012; Estadella *et al.*, 2013; Chen *et al.*, 2015). Similarly, ROS invigorated NF-κB also via activation of IKK complex and phosphorylation of IκB protein (Farrell *et al.,* 2012). In agreement with previous experiments, our study showed that accumulation of palmitic acid in hepatocytes led to higher expression of phosphorylated p65 NF-κB in palmitic acid treated cells and

hence, suggested the induction of NF-κB signaling pathway. Thus, elevation of hepatic level of TNF-α, IL-6, IL-8 and MCP-1 in this study have possible association with increased NF-κB activity.

To explore the mechanism of treatment of ethyl acetate fraction on palmitic acid-induced HepG2 cells, several proteins which play important roles in the signal transduction of NF-κB signaling pathway were determined in this study. Based on present results, the protective roles of ethyl acetate fraction on NAFLD was mediated through the inhibition of IκB-α phosphorylation and NF-κB p65 subunit nuclear translocation. These results showed that the inhibitory effect of ethyl acetate fraction from *A. nigricans* towards the production of pro-inflammatory cytokines may be associated with the inhibition of NF-κB expression in palmitic acid-induced HepG2 cells.

Linoleic acid in ethyl acetate fraction could also displayed anti-inflammatory capacity either individually or synergistically with palmitic acid to inhibit the reduction of IκB-α expression caused by palmitic acid treatment. Our result is in agreement with western blot analysis carried out by Maruyama *et al.* (2014). Their study showed that cells treated with linoleic acid alone as well as co-incubated with palmitic acid and linoleic acid significantly inhibited the reduction of IκBα expression, as compared to palmitic acid treated cells. This phenomenon indicated that linoleic acid possessed anti-inflammatory effect that protect cells from palmitic acid-induced inflammation. Likewise, LPS caused a decrease in the level of IκBα in the LPS-induced RAW 264.7 macrophage model of inflammation. In contrast, ergosta-7,22-dien-3-ol from *Marthasterias glacialis* reversed the inflammatory circumstance by elevation of IκB-α level when RAW 264.7 cells were pre-incubated with it (Pereira *et al.*, 2014). Ergosterol in ethyl acetate fraction probably possess similar anti-inflammatory property as described in the study of Pereira *et al.* (2014). Furthermore, linoleic acid from *Agaricus brasiliensis* was reported to

suppress the expression of pro-inflammatory cytokine by inhibition of NF-κB subunit p50 expression in lipopolysaccharides-stimulated RAW 264.7 (Saiki *et al.*, 2017).

Phenolic compounds have been extensively studied, and data exist proved that phenolic compounds exerted anti-inflammatory effect via inhibition of NF-κB pathway. For instance, quercetin was reported to decrease IκB-α degradation by inhibiting up-regulating of IKK complex (Dias *et al.*, 2005). Hence, effect of IKK/IκB cascade contribute to inhibition of NF-κB activation in streptozotocin diabetes rats, which hinder the development of the disease (Dias *et al.*, 2005). Likewise, study by Kuo *et al.* (2012) documented that curcumin, a naturally occurring phenolic compound of spice tumeric inhibited lipoapoptosis elicited by high free fatty acid in primary hepatocytes through down-regulation of the NF-κB p65 subunit. In this study, derivatives of ellagic acid identified in ethyl acetate from *A. nigricans* could probably exerted certain extent of anti-inflammatory impact on the cells during co-treatment in view of the fact that polyphenols have been generally subscribed to the inhibition of canonical NF-κB pathway. Panchal *et al.* (2013) performed an animal study by feeding high-carbohydrate, high-fat diet to rats in order to develop oxidative stress and inflammation circumstance. They observed that such rats treated with ellagic acid derived from nuts and fruits such as raspberries and pomegranates subsided the abovementioned circumstance as a result of decreasing in protein level of NF-κB. Animal model study reported that ellagic acid inhibited NF-κB, ameliorating dyslipdemia and diabetic nephropathy in rats. Furthermore, administration of an increasing dose of ellagic acid significantly inhibited NF-κB-p65, transforming growth factor β, fibronectin and improved insulin resistance (Kang *et al.*, 2016). In LPS-stimulated RAW 264.7 experiment showed that 1-*p*-coumaroyl *β*-D-glucoside, a derivative of hydroxycinnamic acid exerted its anti-inflammatory effect through suppression of LPS-induced degradation of IκB (Vo *et al.*, 2104). Recent evidence reported that n-butanol fraction of *Naematoloma sublateritium* (Fr.) P. Karst. (a chestnut

mushroom) may be a potential therapeutic agent against vascular inflammation because it inhibited the expression of TNF-α induced adhesion molecules in addition to regulating the inducible NO synthase/cyclooxygenase (iNOS/Cox-2) pathways through repression the translocation of p65 NF-κB in endothelail cells (Lee *et al.*, 2012). A recent report by Jeong *et al.* (2014) documented that ethanol extract of mushroom *Poria cocos* (Schwein.) F.A. Wolf suppressed the LPS-induced DNA-binding activity of NF-κB as well as the nuclear translocation of NF-κB p65 in RAW 265.7 resulted in significant decreased secretion of IL-1β, TNF-α, inducible NO synthase level and cyclooxygenase (COX-2). Likewise, mukitake mushroom was reported can ameliorate NAFLD through NF-κB pathway, however, the interference occurred at IκB kinase (IKK)-β (Nagao *et al.*, 2010)

Therefore, the present finding suggests that the synergistic effect of fatty acid and polyphenol compounds that were identified in the sample may partially be attributed to the cytoprotection of ethyl acetate fraction from *A. nigricans* against activation of NF-κB through inhibition of IκB-α phosphorylation and NF-κB p65 subunit nucleus translocation. Inactivation of NF-κB may contribute to a decrease in secretion of pro-inflammatory cytokines in this study.

c-Jun N-terminal protein kinase (JNK) is also recognized as stress-activated MAP kinase (SAPK) (Cargnello & Roux, 2012). Inflammatory cytokine such as TNF-α, FFAs, oxidative stress, hepatocyte fat accumulation, free cholesterol, lysophosphatidylcholine can activate JNK in NAFLD pathogenesis resulted in activation of mitochondrial cell death pathway, generation of pro-inflammatory molecules through activation of activator protein-1 (AP-1), and trigger insulin resistance (Farrell *et al.*, 2012; Zeng *et al.*, 2014). In general, p38 MAPK are activated in response to inflammatory cytokines such as IL-1, TNF-α, IL-6, and by ROS and other stimuli, such as hormones, ligand for G protein-coupled receptor as well as stresses such as heat shock, osmotic shock (Son *et al.*, 2011; Zeng *et al.*, 2014). In addition, activated MAPK can also lead to expression of

inflammatory cytokines. For instance, p38 signaling pathways is activated during stress condition and are involved in the cell inflammatory response and apoptosis process, which are associated with the release of various inflammatory cytokines (such as IL-1, TNF-α and IL-6, etc.) (Zeng *et al.*, 2014). Cohen et al. (2009) reported that exposure of mice to ethanol caused activation of MAPK that led to an increase in TNF-α expression.

A study exploring the association of FFAs and JNK proved that JNK were activated when palmitic acid or oleic acid added to hepatocytes, however, JNK activation were greater during exposure to saturated compared to monounsaturated FFA. (Malhi *et al.*, 2006). In addition, Bae & Park (2009) showed that palmitic acid played a role in activation of JNK and p38 MAPK in rat hepatocytes resulted in apoptosis through oxidative stress. Notably, Joshi-Barve *et al.* (2007) reported that JNK/AP-1 pathway was successfully activated by when HepG2 cells were exposed to 300 µM of palmitic acid. Animal model study analyzed the phosphorylation of JNK and p38 level in high-fat diet induced NAFLD proved that both phospho-JNK and phosphop38 MAPK level were significantly higher than the normal control group (Sinha-Hikim *et al.*, 2011). Consistent with previous studies, the present study showed higher expression of phosphorylated SAPK/JNK, p38MAPK in palmitic acid-induced HepG2 compared to non-induced HepG2 cells suggested the induction of MAPK signaling pathway.

Silymarin is a polyphenolic flavonoid derived from milk thistle (*Silybum marianum*) possessed anti-inflammatory, cytoprotective, anti-carcinogenic (Manna *et al.*, 1999), antioxidative, chemopreventive, and hepatoprotective effects (Xiao *et al.*, 2013) and silymarin exerted its anti-inflammatory effect of protecting steatohepatitis through MAPK (Xiao *et al.*, 2013). Manna *et al.* (1999) reported that silymarin exhibits its beneficial effects through inhibition of mitogen-activated protein kinase kinase (MEK) and JNK and the apoptosis induced by TNF. Therefore, we believe that derivative of ellagic acid, a phenolic compound, identified in ethyl acetate fraction from *A. nigricans*

could probably inhibit activation of MAPK pathway through similar way. Natural products have long been used since ancient times and in tradition for the treatment of many disease and illness (Dias *et al.*, 2012) A recent report by Yang *et al.* (2014) documented that Traditional Chinese Medicine of Chaihu-Shugan-San and Shen-ling-bai-zhu-San moderate NASH progression through inhibition TLR4 protein expression, p38 MAPK phosphorylation. A report suggested that dioscin, a natural steroid found in various herbs used in traditional Chinese medicines, to be a new candidate for obesity and NAFLD prevention because it down-regulated phosphorylation level of p-p38, p-ERK and p-JNK of high-fat fed mice upon treatment (Liu *et al.*, 2015). *A. nigricans* is not only an edible but also a medicinal mushroom that has been long used by folks are known to have property of attenuation of inflammatory response (Liang *et al.*, 2016), for that reason, in our opinion ethyl acetate fraction from *A. nigricans* could possibly down-regulated the MAPK pathways as abovementioned natural products did.

The increased phosphorylation of JNK and p38 MAPK was alleviated by the presence of ethyl acetate fraction (Table 4.7). The present results revealed that the inhibition of pro-inflammatory cytokine and chemokines production by ethyl acetate fraction may be mediated through down-regulation of JNK/p38 MAPK pathway. These results suggested that protective effect of ethyl acetate fraction from *A. nigricans* may be mediated by affecting MAPK phosphorylation level. This finding suggested that the hepatoprotective way of ethyl acetate fraction through alleviation inflammatory reponse in palmitic acid-induced HepG2 cells by down-regulation of JNK/p38 MAPK pathway may be due, at least partially, to the synergistic effect of the compounds in ethyl acetate fraction.

The current finding showed that the expression of phosphorylated STAT3 protein in palmitic acid-induced was significantly up-regulated, as compared with non-induced HepG2 cells. In other words, the presence of palmitic acid induced the phosphorylation

of STAT3. The present results are consistent with Park *et al.* (2015), the level of phospho-STAT3 increased when HepG2 cells were induced with palmitic acid. IL-6 is a major STAT3 activator in the liver, it is well-known for its role in the liver acute phase response, exerts many of its function through activation of STAT3 (He & Karin., 2011). Furthermore, IL-6 involved in induction of insulin resistance in the liver through activation of signal transducer and activator of transcription 3 (Kim *et al.*, 2007). Min *et al.* (2015) concluded that STAT3 pathways is activated in NAFLD by reporting that both human subjects with NAFLD and NASH showed significantly higher circulating IL-6 and gp130 expression compared with obese controls and lean control as well as higher level of phosphorylated STAT3 were detected in NAFLD and NASH compared with controls. Furthermore, high-fat diet fed wild type mice demonstrated an increase in the total STAT3 protein, but the total STAT3 in IL-6 deficient mice fed with high-fat diet remain unchanged (Vida *et al.*, 2015). Besides that, the basal level of hepatic STAT3 protein in standard-fed wild type mice were barely phosphorylated, and no phosphorylation was observed in standard-fed IL-6 deficient mice. On the other hand, high-fat fed wild type mice demonstrated that STAT3 proteins were phosphorylated. For that reason, the up-regulation of STAT3 protein expression and p-STAT3 status were reported to be dependent on IL-6 levels (Vida *et al.*, 2015). Therefore, the elevated IL-6 level caused by palmitic acid is believed to mediate in phosphorylation in STAT3 in present study. In addition, a recent report by Handa *et al.* (2017) documented an increased of hepatic STAT3 gene expression in NASH patients relative to NAFLD patients. Phosphorylated STAT3 can also worsen insulin resistance (Min *et al.*, 2015). Hence, ethyl acetate fraction of *A. nigricans* suppressed production of IL-6 may partly mediated inhibition of phosphorylation of STAT3. This finding suggested that inhibition of phopshorylation STAT3 by ethyl acetate fraction may alleviate NAFLD, NASH and insulin resistance.

The present results revealed that ethyl acetate fraction from *A. nigricans* suppressed phosphorylation of STAT3. Therefore, ethyl acetate fraction may display its anti-inflammatory effect through inhibition of phosphorylation of STAT3. Oligonol, a long chain polyphenol generally found in various fruits, suppressed mRNA of STAT3 and phosphorylated STAT3 expression in palmitic acid-induced HepG2 cells (Park *et al.*, 2015). A report by Kessoku *et al.* (2016) documented that resveratrol did not suppress steatosis in a high-fat diet-induced model of NAFLD mice, but, dramatically inhibited inflammation and fibrosis in a high fat-diet fed in combination with low-dose LPS-induced animal model of NASH through inhibition of phosphorylated STAT3 in the liver. Besides that, Kessoku *et al.* (2016) also performed an *in vitro* experiment using RAW 264.7, by adding murine recombinant leptin to induce activation of the STAT3 pathway. Their study showed that resveratrol inhibited the phosphorylated-STAT3 level and lowered the p-STAT3: STAT ratio. Therefore, this study indicated that the presence of polyphenol compounds in ethyl acetate fraction could partially be responsible for alleviation of the activation of STAT3.

In general, these findings suggested that ethyl acetate fraction from *A. nigricans* may suppress the NF-κB pathway by inhibiting the phosphorylation of IκBα and NF-κB p65, as shown Figure 5.1. Besides that, these finding also proposed that ethyl acetate fraction may inhibit palmitic acid-induced inflammatory response through inhibition of phosphorylated SAPK/JNK and phosphorylated p38 MAPK as well as phosphorylation STAT3.

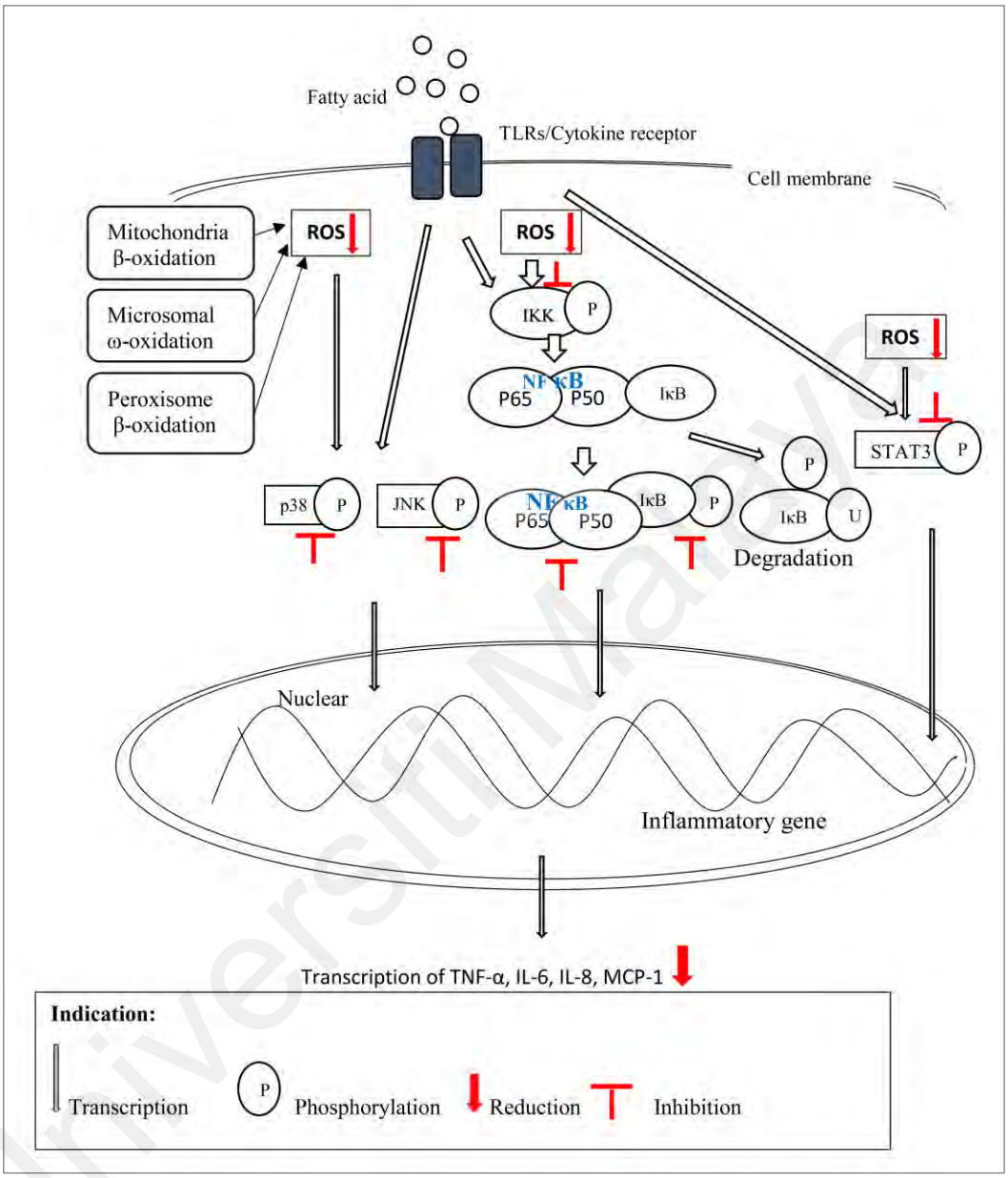

Figure 5.1 Hypothetic mechanism of anti-inflammatory activity of ethyl acetate fraction in palmitic acid-induced signaling pathways.

Based on the present findings, the phosphorylated NFκB, p38 MAPK, JNK and STAT3 signaling pathways were inhibited by ethyl acetate fraction of *A. nigricans*. Simultaneously, ethyl acetate fraction of *A. nigricans* reduced ROS production that also mediated the inhibition of the mentioned signaling pathways.

CHAPTER 6: CONCLUSION

6.1 Conclusion

In the present study, the hepatoprotective effect of solvent-fraction from *A. nigricans* was investigated *in vitro* by looking into anti-oxidation, anti-hepatic steatotic and anti-inflammatory capacity**.** Based on current observations, semi-polar ethyl acetate fraction from *A. nigricans* showed high efficiency as antioxidant agent amongst all other samples based on free radical scavenging capability with exception towards superoxide. Water fraction from *A. nigricans* showed good antioxidant potentials via free radical scavenging across all kinds of radical group. Hence, both were further studied for their properties by cell-based assay in order to obtain more biological relevant data. Both fractions were non-cytotoxic, however, ethyl acetate fraction was cytotoxic at high concentration (100 µg/mL) to HepG2 cells. Based on data obtained, ethyl acetate fraction showed significant capacity ($p<0.05$) in amelioration of lipid accumulation in palmitic acid-induced hepatocytes, whereas water fraction gave a non-significant reduction in hepatocytes lipid loading. Retrospectively speaking, ethyl acetate fraction is an anti-hepatic steatosis agent, but, not water fraction. Further investigation on the scavenging of intracellular ROS in palmitic acid-induced HepG2 cells showed that ethyl acetate fraction showed greater antioxidant capacity than water fraction by exhibiting significant reduction ($p<0.05$) in ROS generation. In other words, ethyl acetate fraction is a strong antioxidative agent and demonstrated significant capability in ameliorating oxidative stress in palmitic acid-induced HepG2 cells. Data obtained from scavenging of intracellular ROS showed that cell-based ROS scavenging assay is consistent with data collected from biochemical assay. Oxidative stress is associated to development and progression of hepatic steatosis to a form of steatosis with inflammation by producing pro-inflammatory cytokines. ELISA assay demonstrated that ethyl acetate fraction significantly repressed ($p<0.05$) the

palmitic acid-induced cytokines and chemokines including TNF-α, IL-6, IL-8 and MCP-1. Further investigation on the transcription factors that actively involved in inflammatory response of NAFLD and NASH showed ethyl acetate fraction led to a significant decrease in the increased phosphorylated IκB-α, phosphorylated NF-κB p65, phosphorylated SAPK/JNK, phosphorylated p38 MAPK, phosphorylated STAT3 that were induced by palmitic acid. Therefore, the NF-κB, SAPK/JNK, p38 MAPK and STAT3 signaling pathways were suppressed. In conclusion, our study demonstrated the protective effects of ethyl acetate fraction from *A. nigricans* through reversal of NAFLD symptoms by decreasing lipid load in HepG2 cells, intracellular ROS level, pro-inflammatory cytokine and chemokine secretion. Ethyl acetate fraction may also prevent NAFLD through regulating NF-κB, SAPK/JNK, p38 MAPK and STAT3 signaling pathways. These data suggested that ethyl acetate fraction of *A. nigricans* exerts protective effect on NAFLD and NASH not only at the "first" hit but also the "second" hit. Hence, ethyl acetate fraction has a direct, high-intensity intervention effect for NAFLD and NASH.

Future investigations:

The present study may be the first report on *the in vitro* anti-antioxidant, anti-hepatosteatosis and anti-inflammatory effects and the underlying mechanisms of ethyl acetate fraction of *A. nigricans*. The combined results from this study indicated that ethyl acetate fraction displayed effective antioxidant, anti-inflammatory and anti-hepatosteatosis capacities. Therefore, further *in vivo* analyses on antioxidant, anti-inflammatory and anti-hepatosteatosis capacities of ethyl acetate fraction is required to be confirm using high-fat fed animal model. Besides that, an exploration of mechanism of action for ethyl acetate sample is necessary to be carried out *in vivo* experiments in order to obtain a solid knowledge of hepatoprotective effect of the sample.

Furthermore, evaluation on the safety aspects of ethyl acetate fraction consumption is essential to be carried out because it is crucial for the development of *A. nigricans* as a dietary supplement. The *in vivo* evaluation on liver and kidney condition is principle to ensure no severe complication occur to important organs after a longer duration of treatment.

Previous study reported that polysaccharide from *A. nigricans* revealed anti-hypercholesterolemic effects in rats. Therefore, considering the current results and previous report, it is likely to suggest that future research focusing on a dietary intervention that include whole mushroom *A. nigricans* to combat the effects of NAFLD and to prevent its progression toward NASH because dietary intervention provides a more practical approach toward synergistic beneficial effects of nutrient combinations. In addition, lifestyle modification including diet remain the first line approach of in the prevention and treatment of NAFLD.

NAFLD is strongly associated with insulin resistance, hence, it is worthy to study insulin pathway, such as insulin receptor substrate-1(IRs-1, responsible for the transduction of insulin signaling), protein kinase B (Akt, a crucial component of insulin signaling cascade) and AMPK (critical enzyme for regulating hepatic lipogenesis and insulin resistance). Furthermore, exploring on lipid metabolism, such as fatty acid β-oxidation signaling pathway and and transcription factors such as peroxisome proliferator-activated receptor alpha (PPARα) (regulation genes involved of uptaking, transporting and metabolism fatty acids) and sterol regulator element binding protein (SREBPs: a family of transcription factors involved in the biogenesis of cholesterol, fattys and triglycerides) on palmitic acid-induced cells model and/or high-fat fed animal model, is recommended. Besides that, it is essential to perform study for the effect of ethyl acetate fraction of *A. nigricans* or whole mushroom of *A. nigricans* on antioxidant enzymes, including superoxide dismutase, catalase and glutathione peroxidease.

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LIST OF PUBLICATIONS AND PAPER PRESENTED

PUBLICATIONS

1. **Teoh, H. L**., Ahmad, I. S., Johari, N. M. K., Aminudin, N., & Abdullah, N. (2018). Antioxidant properties and yield of wood ear mushroom, *Auricularia polytricha* (Agaricomycetes), cultivated on rubberwood sawdust. *International Journal of Medicinal Mushrooms*, 20(4), 369-380.

2. **Teoh, H. L**., Aminudin, N., & Abdullah, N. (2021). *In vitro* antioxidation, antihepatic steatosis, and anti-inflammatory effects of ethyl acetate fraction from *Auricularia nigricans* (Agaricomycetes). *International Journal of Medicinal Mushrooms*, 23(2), 43-56.

PAPER PRESENTED

1. Teoh, H. L., Aminudin, N., & Abdullah, N. (2014). Effect of *Auricularia polytricha* on atherosclerosis, paper presented at the 19th Annual Biological Science Graduate Congress, 12th- 14th December 2014. Singapore