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The effects of full-spectrum recreational/medical cannabis formulations on inflammation and autophagy in Alcoholic Liver Disease ALD and Non-alcoholic fatty liver disease NAFLD

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ABSTRACT

With recent legalizations, Canada has seen a dramatic rise in the number of recreational cannabis users. Cannabis has been used over centuries illegally in most countries for medical and recreational purposes. Recent epidemiological reports suggest that cannabis use can alleviate alcoholic and non-alcoholic fatty liver diseases. Other reports suggest that some active ingredients in cannabis can adversely exacerbate ALD and NAFLD outcomes. The reasons behind the differential impact of cannabis in promoting or alleviating disease outcomes remain ill-defined. Our study revealed that full-spectrum Cannabis (with high CBD and low THC contents) treatment significantly reduced LPS induced TNF-α production in macrophages using the ELISA method. Also, it reduced LPS induced TLR4 mediated NF-κB activation as revealed by decreased the p65 nuclear translocation which we analyzed by fluorescent microscopy. Conversely, full-spectrum cannabis (with high THC and low CBD contents) treatment did not impact the cellular release of TNFα in macrophages following LPS stimulation. Moreover, we revealed that the two full-spectrum cannabis formulations assessed could both induce the autophagy process as revealed by increased p62 and cellular accumulation of LC3II. We also found that the cannabis (with high THC and low CBD contents) treatment insignificantly increased lysosomal gene expression and possibly induced better degradation of autophagosomes as revealed by LAMP1 and LC3II/I ratio using western blotting analysis. Altogether, we revealed that full-spectrum cannabis formulations with varying contents of THC and CBD can differentially impact key cellular processes that regulate ALD and NAFLD. High CBD and low THC cannabis suppressed inflammation while high THC: low CBD enhanced more complete autophagy. Taken together, this might account for observed clinical differences in patient outcomes amongst individuals with liver ALD and NAFLD who additionally use cannabis.

Keywords: Cannabis, Cannabinoids, ALD and NAFLD, Inflammation, Autophagy.

RÉSUMÉ

La légalisation récente de l'usage récréatif de cannabis au Canada a entrainé une augmentation du nombre d'usagers au cours des dernières années. Le cannabis a été utilisé illégalement pendant des siècles dans la plupart des pays à des fins médicales et récréatives. Des rapports épidémiologiques récents suggèrent que la consommation de cannabis peut atténuer les effets de la maladie dite du foie gras alcooliques et non alcooliques. D'autres rapports suggèrent que certains composés actifs du cannabis pourraient aggraver les symptômes de ces maladies métaboliques du foie. Les causes entrainant des impacts différentiels du cannabis, sur la promotion ou l'atténuation de ces maladies restent mal définies. Notre étude a révélé que le traitement au cannabis à spectre complet (avec une teneur élevée en CBD et une teneur en THC faible) réduit considérablement la production de TNF-α, mesurée par la méthode ELISA, induite par le LPS dans les macrophages. En outre, il a réduit l'activation de NF-κB (TLR4 dépendante) induite par le LPS, comme le révèle la diminution de la translocation nucléaire de la protéine p65 que nous avons analysée par microscopie à fluorescence. À l'inverse, le traitement au cannabis à spectre complet (teneur élevée en THC et teneur en CBD faible) n'a pas eu d'impact sur la libération cellulaire de TNF-α par les macrophages après stimulation par le LPS. De plus, nous avons révélé que les deux formulations du cannabis à spectre complet testées dans notre étude pouvaient induire le processus d'autophagie comme le révèle l'augmentation de la protéine p62 ainsi qu'une accumulation cellulaire de LC3II. De plus, nous avons constaté que le traitement au cannabis (haute teneur en THC et faible teneur en CBD) augmente légèrement l'expression des gènes lysosomaux et pourrait ainsi induire une meilleure dégradation des autophagosomes, comme le révèlent le niveau d'expression de LAMP1 et le ratio LC3II/I mesurés par la technique de Western-blot. Pour conclure, nous avons révélé que les traitements au cannabis à spectre complet (teneurs variables en THC et CBD) peuvent avoir un impact différentiel sur les processus cellulaires clés qui régulent les maladies métaboliques du foie. Le cannabis à teneur en CBD élevé (et teneur en THC faible) supprime l'inflammation, tandis que le cannabis à teneur en THC élevé (et teneur en CBD faible) améliore le processus autophagique. L'ensemble de ces résultats pourrait expliquer les différences cliniques observées parmi les personnes atteintes de maladies métaboliques du foie consommant en plus du cannabis.

Mots clés : Cannabis, ALD et NAFLD, Inflammation, Autophagie.

SOMMAIRE RÉCAPITULATIF

Contexte : La légalisation récente du cannabis, au Canada, a entrainé une augmentation spectaculaire du nombre d'usagers au cours des dernières années. Le cannabis a été utilisé illégalement pendant des siècles dans la plupart des pays à des fins médicales et récréatives. Les effets psychoactifs négatifs du cannabis ont été bien documentés. Malgré ces révélations, de nouvelles recherches révèlent maintenant que le cannabis pourrait offrir des avantages thérapeutiques pour des maladies spécifiques. La légalisation du cannabis a également vu son utilisation combinée avec d'autres drogues légales en particulier l'alcool. L'usage malsain d'alcool a été associé au développement de maladies hépatiques progressives caractérisées par une stéatose hépatique, de fibrose, de cirrhose et de carcinomes hépatocellulaires chez certains individus. Un rapport récent a suggéré que la consommation de cannabis peut atténuer les maladies dites du foie gras alcooliques et non alcooliques. D'autres rapports suggèrent que certains ingrédients actifs du cannabis pourraient aggraver ces maladies métaboliques du foie. Les raisons des impacts différents du cannabis, aggravation ou atténuation, sur ces maladies restent encore mal définies. Aujourd'hui, les traitements les plus utilisées avec du cannabis à spectre complet sont ceux à forte teneur en tétrahydrocannabinol (THC) et à faible teneur en cannabidiol (CBD) ainsi que ceux à teneur élevée en CBD et à faible teneur en THC. Des études ont révélé que le THC et le CBD pourraient avoir des effets opposés en raison de leur rôle fonctionnel dans l'activation des récepteurs cannabinoïdes endogènes. Bien qu'il ait été démontré que le CBD fournit de probables bénéfices thérapeutiques (anti-inflammatoires), il a principalement été lié à des effets neurologiques indésirables, tel que la psychose. Malgré les connaissances actuelles, on ne sait toujours pas si les traitements utilisant le cannabis récréatif à spectre complet (avec des teneurs variables en CBD et THC) pourraient avoir un impact sur divers processus pathologiques tel que les maladies métaboliques du foie et avec quel mécanisme.

Objectif : Évaluer l'impact de deux traitements utilisant du cannabis récréatif à spectre complet (avec des teneurs variables en THC et CBD) sur les changements mécanistes cellulaires associés à une maladie métabolique du foie. L’emphase sera mis principalement sur la production induite de cytokines pro-inflammatoires et sur le mécanisme d’action du cannabis sur le processus de signalisation, qui entraîne une inflammation via l'activation de NF-kB. De plus, afin d’étudier comment deux traitements au cannabis à spectre complet modulent l’autophagie dérégulée retrouvée dans les maladies métaboliques du foie sera étudiée.

Méthodologie : Afin de simuler l'activation des macrophages hépatiques par des endotoxines dérivées de l'intestin qui sont associées à des fuites intestinales et caractérisent les maladies métaboliques du foie dues à une consommation excessive d'alcool et/ou à un régime hypocalorique malsain, nous avons utilisé des méthodes *in vitro*. En bref, nous avons utilisé la lignée cellulaire de macrophages de souris (cellules RAW264.7) qui a été cultivée dans un milieu de culture contrôle ou contenant 50 mM d'éthanol pendant 72 heures (afin d'imiter l'utilisation d'éthanol chronique). Certaines cellules ont été traitées ou non avec du cannabis à spectre complet (teneurs variables en THC et CBD) pendant 2 h, puis avec 100 ng/mL de lipopolysaccharide (LPS) pendant 6 h. Les surnageants des cellules ont été collectés puis analysés afin de quantifier le TNF-α par ELISA. La régulation mécanistique de la production de TNF-α impliquant le NF-kB, a été évaluée dans des cellules RAW264.7 qui ont été traitées avec du cannabis (CBD élevé et THC faible) suivi d'une stimulation avec 100 ng/mL de LPS pendant 45 minutes. La translocation nucléaire de p65 a ensuite été analysée dans ces cellules par microscopie à fluorescence. Les effets négatifs du cannabis à spectre complet sur l'autophagie qui se produit lors de maladies métaboliques du foie ont également été évalués dans les cellules RAW264.7. Plus précisément, certaines cellules ont été traitées ou non avec un extrait total de cannabis contenant des quantités variables de THC et de CBD suivi d'une stimulation par le LPS pendant 24h. Les niveaux d'expression des protéines p62, LC3I/II, LAMP1, ß-actine ont été mesurés à partir de lysats protéiques totaux par la méthode de Western blot.

Résultats : Nous avons mis en évidence que le traitement au cannabis à spectre complet (haute teneur en CBD et faible teneur en THC) réduit considérablement la production de TNF-α, induite par le LPS, dans les macrophages. En outre, le cannabis à teneur élevée en CBD a abrogé l'activation de NF-κB par le LPS, comme le révèle la diminution de la translocation nucléaire de la protéine p65. À l'inverse, le traitement au cannabis à spectre complet (teneur élevée en THC et teneur en CBD faible) n'a aucun effet sur la libération cellulaire de TNF-α dans les macrophages après stimulation par le LPS. De plus, nous révélons que les deux formulations de cannabis évaluées pourraient à la fois induire le processus d'autophagique, comme le révèle une augmentation de p62 et une accumulation cellulaire de LC3II. Nous avons également constaté que le traitement au cannabis (à haute teneur en THC et à faible teneur en CBD) augmente légèrement l'expression lysosomale et améliore peut-être la dégradation des autophagosomes, comme le révèle les niveaux d'expression LAMP1 et le ratio LC3II/I mesurés par Western blot.

Conclusions : Notre étude a révélé que les traitements de cannabis à spectre complet (teneurs variables en THC et CBD) peuvent avoir un impact différentiel sur les processus cellulaires clés

qui régulent les maladies métaboliques du foie. À teneur en CBD élevé, le cannabis à faible teneur en THC supprime l'inflammation tandis que le cannabis à teneur élevé en THC et à faible teneur en CBD améliore le processus autophagique. Ces deux effets pourraient expliquer les différences cliniques observées chez des patients atteints de maladies métaboliques du foie qui consomment en plus du cannabis.

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LIST OF ABBREVIATIONS

- ALD: Alcohol liver disease.
- NAFLD: Non-alcohol fatty liver diseases.
- CBD: Cannabidiol.
- THC: Tetrahydrocannabinol.
- LPS: Lipopolysaccharides.
- TNF-α: Tumor necrosis factor-alpha.
- ELISA: enzyme-linked immunosorbent assay.
- NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells.
- p65: Nuclear translocation.
- P62: Sequestosome-1.
- LC3: Microtubule-associated protein 1A/1B-light chain 3.
- Lamp-1: Lysosomal-associated membrane protein 1.
- pCBs: Phytocannabinoid.
- 2-AG: 2-arachidonoylglycerol.
- AEA: Ethanolamide.
- CB1&2: Cannabinoid receptors.
- MLD: Metabolic liver disease.
- CLD: Chronic liver diseases.
- HCC: Hepatocellular carcinoma.
- MetS: Metabolic syndrome.
- NASH: Non-alcoholic steatohepatitis.
- KC: Kupffer cells.
- MoMFs: Bone marrow monocyte-derived macrophages.
- PRR: Pattern recognition receptors.
- MAMP: Microbial associated molecular patterns.
- DAMP: Damage-associated molecular patterns.
- TGF-β: Transforming growth factor beta.
- IL-1β: Interleukin 1 beta.
- CCL5: Chemokine ligand 5.
- CCL2: Chemokines ligand 2.
- ASH: Alcoholic steatohepatitis.
- TLRs: Toll-like receptors.

- NLRs: Nod-like receptors.
- M1: Macrophages 1.
- M2: Macrophages 2.
- IL-6: Interleukin 6.
- IL-8: Interleukin 8.
- IL-12: Interleukin 12.
- IFNγ: Interferon gamma.
- TGF-β: Transforming growth factor-beta.
- PDGF: Platelet-derived growth factor.
- EGF: Epidermal growth factor.
- NO: Nitric oxide.
- iNOS: Inducible nitric oxide synthase.
- HSCs: Hematopoietic stem cells.
- NAD+: Oxidized nicotinamide adenine dinucleotide.
- NADH: Reduced nicotinamide adenine dinucleotide.
- ALDH2: Aldehyde dehydrogenase 2.
- ADH: Alcohol dehydrogenase.
- CYP2E1: Cytochrome P450 2E1.
- NADPH: Nicotinamide adenine dinucleotide phosphate.
- ROS: Reactive oxygen species.
- ·OH: Hydroxyl radicals.
- CD14: Cluster of differentiation 14.
- LSECs: Liver sinusoidal endothelial cells.
- MCP-1: Monocyte chemoattractant protein-1.
- ERK1/2: Extracellular signal-regulated kinases.
- IRAK-1: The interleukin-1 (IL-1)-receptor-associated kinase.
- IKK: IκB kinase.
- STAT3: Signal transducer and activator of transcription 3.
- IR: Insulin resistance
- GLP-1: Glucagon-like peptide 1.
- INSL5: Insulin-like peptide 5.
- GIP: Glucose-dependent insulinotropic polypeptide.
- FGF15 and FGF 19: Fibroblast growth factor 15 and 19.
- XBP-1: X-box binding protein-1.

- CYP7A1: Cholesterol 7α-hydroxylase.
- CYP27A1: Sterol-27-hydroxylase.
- p65/P50: Nuclear Transcription factor.
- NEMO: NF-kappa-B essential modulator.
- JNK: c-Jun N-terminal kinase.
- mTORC1: Mammalian target of rapamycin complex 1.
- CMA: Chaperone-mediated autophagy.
- ATG: Autophagy-related protein.
- VPS34: Vacuolar protein sorting 34.
- PIP: Phosphatidylinositol 3-phosphate
- AMPK: AMP-activated protein kinase.
- ULK1: Unc-51-like kinase 1.
- FIP200: Focal adhesion kinase family interacting protein of 200 kDa.
- ATG: Autophagy-related protein.
- PI3P: Phospholipid phosphatidylinositol 3-phosphate.
- TFEB: The transcription factor EB.
- LAMP-2A: Lysosome associated membrane protein type 2A.
- Hsc70: Heat shock cognate 70 kDa protein.
- VLDL: Very-low-density lipoprotein.
- NRLP3: NOD-, LRR- and pyrin domain-containing protein 3.
- 2-AG: 2-arachidonoylglycerol.
- AEA: Arachidonoyl ethanolamide.
- ICAM1: Intercellular adhesion molecule 1.
- VCAM1: Vascular cell adhesion molecule 1.
- GCPR: G protein-coupled receptors.
- PPAR-γ: Peroxisome proliferator-activated receptor gamma.
- Bcl-2: B-cell lymphoma 2.
- ATF4: Activating Transcription Factor 4.
- CHOP: C/EBP homologuons protein.
- TRIB3 : Tribbles homolog

INTRODUCTION

The recent legalization of cannabis for medical and recreational purposes in Canada in 2018 has seen increased utilization amongst the general population (Valleriani *et al.*, 2018). The diverse psychoactive effects of cannabis have been well described (Cohen *et al.*, 2019; Hall, 2015; Hall & Solowij, 1998). Notwithstanding, recent research is now revealing that cannabis might provide some health benefits for specific disease conditions (Kogan & Mechoulam, 2007). Today, individuals have access to an ever-growing cannabis market offering diverse and very potent full-spectrum cannabis formulations. Consumption of cannabis could be alone or with other legal drugs like alcohol. Using cannabis in both conditions has been associated with diverse disease outcomes, both positive and negative (Budney *et al.*, 2007; Volkow *et al.*, 2014a). Reasons for these differences in disease outcomes with cannabis use remain ill-defined. Previous research findings have revealed the potential effects of cannabis on liver diseases (Adejumo *et al.*, 2018b; Marchesini *et al.*, 2016). Liver diseases are mostly caused by excessive unhealthy alcohol (Sharma & Nagalli, 2020) and or high-calorie food consumption (Marchesini *et al.*, 2016). These liver diseases are classified under alcoholic and non-alcoholic fatty liver diseases (ALD and NAFLD) (Kovalic *et al.*, 2019). Currently, there are no clinically approved treatments for progressive stages of chronic ALD and NAFLD with long-term efficacy in patients (Shah *et al.*, 2020). Recently, numerous reports have suggested that the use of cannabis might provide some therapeutic benefits for ALD and NAFLD (Adejumo *et al.*, 2018a; Adejumo *et al.*, 2017). Despite this knowledge, it remains unknown how the various potent full spectrum recreational cannabis formulations with varying CBD and THC contents impact the diverse molecular mechanistic disease processes associated with ALD and NAFLD (National Academies of Sciences & Medicine, 2017; Webb & Webb, 2014).

The cannabis plant extracts contain over 545 bioactive compounds. They can broadly be characterized as terpenes, flavonoids, alkanes, sugars, non-cannabinoid phenols, phenylpropanoids, steroids, fatty acids, and various nitrogenous compounds (Gonçalves *et al.*, 2019; Oláh *et al.*, 2017). Of significant scientific interest have been bioactive phytocannabinoids (pCBs) specifically (−)-trans-Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD), which are the most studied (Oláh *et al.*, 2017). Cannabidiol (CBD) is non-psychotic and has been shown to provide anti-anxiety, and anti-inflammatory effects (Oláh *et al.*, 2017). However, THC has been shown to mediate adverse psychoactive effects, as well as euphoric and relaxing intoxication (Oláh *et al.*, 2017). Strikingly, cannabinoids are also produced naturally by animals, mostly

referred to as endocannabinoids. The most studied endogenous cannabinoids are 2-arachidonoylglycerol (2-AG) and arachidonoyl ethanolamide (AEA) (Oláh *et al.*, 2017). The functional effect in both plants and animals (Cannabinoids) are mediated by interactions with cannabinoid receptors (CBR) (Silver, 2019). Currently, two main cannabinoid receptors have been described. Specifically, CB1 and CB2 receptors. CB1receptors are highly expressed on brain cells with a low but functionally relevant expression on hepatic cells and CB2 receptors are highly expressed on immune and hematopoietic cells with significant liver expression in some hepatic disease (Reggio, 2010). Recently, researchers have shown that normal liver has low expression of cannabinoid receptors (Parfieniuk & Flisiak, 2008). However, many studies have confirmed increasing the expression of CB1 and CB2 receptors in hepatic diseases such as hepatic inflammation, steatosis, and the development of fibrosis, as well as raise levels of endocannabinoids in the liver in the course of chronic progressive liver diseases (Parfieniuk & Flisiak, 2008). It has been shown that stimulation of CB1 receptors causes pro-fibrogenic and pro-inflammatory effects in the liver tissue (Bazwinsky-Wutschke *et al.*, 2019). This has been attributed to CB1 receptors activation on pro-fibrogenic hepatic stellate cells. The activation of CB2 receptors has been shown to inhibit or even reverses characteristic features of chronic liver diseases (Steatohepatitis and fibrosis)(Dai *et al.*, 2017). Contrastingly, the activation of CB1 receptors is associated with the progression of liver steatosis (Bazwinsky-Wutschke *et al.*, 2019). The scientific study of cannabis in humans remained limited especially in the context of chronic liver diseases. On the other hand, a few new studies have shown that using cannabis could decrease the progression of chronic liver disease (Adejumo *et al.*, 2018a; Dai *et al.*, 2017; Dibba *et al.*, 2018). Furthermore, controlling CB1 or CB2 signaling seems to be an attractive target in managing liver diseases (Mallat *et al.*, 2011). Taken together, our current research was aimed at evaluating the impact of two full-spectrum recreational plant cannabis formulations with varying THC and CBD contents on pathomechanistic changes associated with ALD and NAFLD. A prime focus of our current research was to assess the effects of these full-spectrum cannabis formulations on pro-inflammatory cytokine production. Mechanistically, we will focus on the effects of these formulations' sequential signaling events that drive inflammation through NF-kB activation. Also, we will investigate how two full-spectrum cannabis impacts dysregulated autophagy that characterizes most ALD and NAFLD.

CHAPTER 1: LITERATURE REVIEW

1.1. Alcoholic liver diseases ALD and Non-alcoholic fatty liver disease NAFLD

NAFLD and ALD are major causes of significant morbidity and mortality globally (Paik *et al.*, 2020a; Paik *et al.*, 2020b; Younossi & Henry, 2016). Both (NAFLD and ALD) impair normal liver function that can ultimately result in injury (Golabi *et al.*, 2018; Jarvis *et al.*, 2020). Long-lasting injury and uncontrolled hepatic healing characterized by excessive collagen deposition in the liver can cause fibrosis which if not effectively managed can progress to cirrhosis in some individuals. Clinical manifestations of severe hepatic dysfunction and damage may include jaundice (yellowing of the skin), ascites and haptic encephalopathy. Moreover, the untreated of all these situations can result in hepatocellular carcinoma (HCC) and death (Sivakrishnan & Pharm, 2018). Over, the past decade the world has witnessed an increase in both entities (ALD and NAFLD), contributing to the elevation of the disease burden of cirrhosis and HCC and surpassing the figures of viral hepatitis infection as chief etiology of hepatic diseases (Idalsoaga *et al.*, 2020). These trends in the contributions of NAFLD and ALD might due to several factors including rising rates of obesity and type 2 diabetes (T2DM), and the changing patterns of alcohol consumption in the general population. NAFLD and ALD have numerous commonalities in their progressive pathophysiology and may ultimately cohabit in the same individual (Idalsoaga *et al.*, 2020).

ALD and NAFLD have similar progressive disease stages including inflammation, cirrhosis, and HCC. All these disease stages are considered to be global health problems because they can cause significant burdens both to patients and health care systems. Inflammation in the liver is indicative of the immune system's response to foreign substances such as exposure of the liver to toxins like alcohol, excess fat, and viral infection (Dirchwolf & Ruf, 2015). The second stage of liver damage is fibrosis, where the healthy tissue replacement by scar tissue in the inflamed liver (Dirchwolf & Ruf, 2015). The formation of an abnormally large amount of scar tissue in the liver can lead to fibrosis. Fibrosis appears when the liver attempts to repair and replace damaged cells (Ahmad & Ahmad, 2012; Sivakrishnan & Pharm, 2018).

The development of fibrosis occurs when the damage of the liver is repeated or continued. Fibrosis can sometimes be reversed if the cause is identified promptly and corrected. For instance, acute hepatitis commonly repairs itself by making new liver cells and linking them to the connective tissue that is left when liver cells die, here the fibrosis can be reversible (Popper &

Uenfriend, 1970; Schaffner & Klion, 1968). On the opposite, in CLD the injury is repeated or continuous, liver cells attempt to reform the damage, but the attempts result in scar tissue (fibrosis). In this case, fibrosis is irreversible (Bataller & Brenner, 2005). Furthermore, fibrosis can improve more rapidly when it is caused by a blockage in the bile ducts. In this case, a lot of liver cells are replaced by scar tissue which has no function (Engler *et al.*, 2003). Also, scar tissues can limit the blood supply from and to the liver by distorting the liver's internal structure. Respectively, the blood pressure increases in the portal vein that carries blood from the intestine to the liver. This case is called portal hypertension (Schuppan & Afdhal, 2008). Actually, after months or years of repeated or continuous damage, fibrosis becomes widespread and permanent, which causes severe scarring called cirrhosis (Schuppan & Afdhal, 2008). Cirrhosis is defined as liver histological damage that develops by serious and extensive injury or scarring in hepatic cells and hepatic sinusoids (Sharma & John, 2020). This scarring can cause chronic liver injury which leads to portal hypertension and end-stage liver disease. Cirrhosis is an advanced stage of liver fibrosis that is associated with distortion of the hepatic vasculature that appears when the direct blood supply from the portal vein and arterial into the liver is low (Schuppan & Afdhal, 2008). The abnormalities of the circulatory in cirrhosis include splanchnic vasodilation, vasoconstriction, hypoperfusion of kidneys, water, salt retention, and increased cardiac output. All of these conditions occur with hepatic vascular alterations and the resulting portal hypertension (Schuppan & Afdhal, 2008). The development of portal hypertension and hyperdynamic circulation in cirrhotic patients is the main cause of morbidity and mortality. Also, they can be responsible for more than 1 million deaths annually (Wong & Huang, 2018). Finally, progressive cirrhosis over months, years, or decades, can cause liver failure which is known as end-stage liver disease (Sharma & John, 2020). In this stage, liver functions are disabled or impaired that can lead to ascites, variceal hemorrhage, hepatic encephalopathy, or renal impairment. The only option to treat this case is liver transplantation (Cox-North *et al.*, 2013).

The development and multiplication of unhealthy cells in the liver can cause liver cancer at any stage of liver injury (Mittal *et al.*, 2016). HCC is the most common liver cancer. HCC is a malignant tumor composed of cells resembling hepatocytes. However, the degree of clinical severity of HCC is commonly associated with extensive hepatic cirrhosis (Balogh *et al.*, 2016).

NAFLD is liver disorder that has a global prevalence of about 25% worldwide (Maurice & Manousou, 2018). The increased occurrence of NAFLD is as a result of rising levels of obesity, type 2 diabetes, and metabolic syndrome (Maurice & Manousou, 2018). In the next decade,

researchers predict that NAFLD will become the leading cause of cirrhosis requiring liver transplantation (Maurice & Manousou, 2018). NAFLD is a liver disease. that happens by macrovascular steatosis in ≥ 5% of hepatocytes, in the absence of common causes such as alcohol or drugs. NAFLD is a spectrum of liver diseases including simple fatty liver, NASH, fibrosis, and cirrhosis, with a complex 'multi-hit' pathophysiology (Younossi & Henry, 2016). NAFLD is considered one of the major reasons for chronic liver disease worldwide, but the complete understanding of the pathomechanisms of this disease are incomplete(Ghevariya *et al.*, 2014). Increased accumulation of lipids in hepatocytes can cause hepatic steatosis or fatty liver and this results from increased production or reduced clearance of hepatic triglycerides and or fatty acids (Manne *et al.*, 2018). Hepatic steatosis can develop to NASH in a significant proportion of subjects. NASH is a necro inflammatory liver disease governed by multiple pathways that are not completely elucidated. NASH is the more serious stage of NAFLD which is characterized by steatosis, hepatic inflammation, and hepatocellular ballooning and may include varying degrees of fibrosis (Cohen *et al.*, 2011; Manne *et al.*, 2018). The understanding of the pathophysiology of NASH has improved substantially from the original 2-hit hypothesis wherein a first hit, such as insulin resistance (IR), resulted in fatty liver, and a subsequent second hit, such as oxidative stress, was desired to develop NASH (James & Day, 1998). It is now manifest, that the 2-hit hypothesis is not enough to characterize, the multiple pathways that may be interrelated and contribute to NASH (Buzzetti *et al.*, 2016).

Although the etiology and progression of NAFLD remain unclear, increasing research present that addition to insulin resistance and inflammation and circadian rhythmicity of hepatic metabolic genes are believed to play key roles in the pathogenesis of NAFLD (Ponziani *et al.*, 2019; Sharpton *et al.*, 2019). Gut microbiota also plays a critical role in the pathogenesis of NAFLD. Furthermore, the regulation of gut microbiota balances the circadian rhythm to maintain hepatic glucose and lipid metabolic homeostasis (Jiang *et al.*, 2020). Many studies have exposed that gut microbiota dysbiosis is related to NAFLD (Chi *et al.*, 2019; Wei *et al.*, 2020). In each stage of NASH from simple steatosis to NASH, fibrosis, and cirrhosis the composition of gut microbiota is different. Therefore, gut microbiota may be a useful predictor for NAFLD progression and severity (Aragonès *et al.*, 2019). Also, gut microbes can produce important metabolites such as short-chain fatty acids and indole and indole derivatives by fermenting carbohydrates and tryptophan which can impact the NAFLD disease process. Recent studies have identified that these metabolites have useful effects on preventing or alleviating obesity and NAFLD. Understanding

the mechanisms of how gut microbiota and metabolites are engaged in NAFLD pathophysiology can inspire us to find out potential strategies to prohibit or treat NAFLD/NASH (Jiang *et al.*, 2020).

The liver receives nutrients from the intestine such as gut hormones that participate in hepatic metabolism. For instance, glucagon-like peptide 1 (GLP-1) which is released by L cells of the small intestine stimulates pancreatic β islet cells to produce insulin. Also, GLP-1 works on the GLP-1 receptor, which exists on human hepatocytes, to decrease hepatic glucose production and ameliorate hepatic fat deposition and insulin resistance (Gupta *et al.*, 2010). Another gut hormone called insulin-like peptide 5 (INSL5), which is also an L cell-derived gut hormone and regulated by gut microbiota, is reported to decrease hepatic glucose production. In addition, glucose-dependent insulinotropic polypeptide (GIP), which is also a gut hormone released from K cells located in the duodenum and proximal jejunum, controls glucose homeostasis, and lipid metabolism. Fibroblast growth factors 15 and 19 (FGF15 and FGF 19), which are also from the gut, were notified, to ameliorate high-fat diet-induced hepatic fat accumulation and endoplasmic reticulum (ER) stress (Alvarez-Sola *et al.*, 2017). In particular, FGF 19 enhance hepatic glycogen and protein synthesis, reduced inflammation, and fibrosis in the liver injury through downregulating the expression of cholesterol 7α-hydroxylase (CYP7A1) and sterol-27-hydroxylase (CYP27A1) and thereby decrease bile acid synthesis (Zhou *et al.*, 2016).

Additional to gut hormones that control hepatic metabolism and inflammation, the gut microbiota is related to the development of NAFLD (Schnabl & Brenner, 2014). Patients with NAFLD show an increase in gut permeability and small intestinal bacterial overgrowth compared with those in healthy controls. Increasing the gut permeability caused by the change of intercellular tight junction likely contributes to the improvement and progression of NAFLD (Bibbò *et al.*, 2018). Inflammation is also observed in NASH. In NAFLD, a damaged intestinal barrier caused by nutrition stress rises the translocation of microbes and their products into the circulation, leading to liver inflammation and even fibrosis/cirrhosis (Chen *et al.*, 2019). Gut-derived antigens in the blood are considered prime causing factors of strong inflammatory responses in the liver. Although intestinal permeability is not the major cause of liver inflammation and fibrosis, due to increased intestinal permeability, the inflammatory responses to microbial antigen strongly influence the progression of the disease (Jiang *et al.*, 2020). The wall of gut bacteria contains LPS that activate the signaling pathways involved in liver inflammation and fibrogenesis through stimulating innate immune receptors, (TLRs). TLR4 is significantly increased in the hepatic and serum of NASH patients. Thus, high serum levels of TLR4 are considered as a biomarker for liver

fibrosis development (Cengiz *et al.*, 2015). Recently studies found the involvement of gut-derived endotoxin in the development of fructose-induced NAFLD (Spruss & Bergheim, 2009). LPS in gut bacteria can activate proinflammatory signals through TLR4, inducing IL-1β, and TNF-α production, as well as enhancing ROS production in hepatic infiltrating macrophages (Kim & Kim, 2017). Mechanistically, TLR4 enhances the progression from simple steatosis to NASH including a rise in ROS-dependent activation of X-box binding protein-1 (XBP-1) in KCs (Ye *et al.*, 2012). TLR4 also induces the TGFβ signaling pathway, stimulates the hepatic stellate cell, and increases extracellular matrix deposition, which all participate in the progression of the liver fibrosis (Dattaroy *et al.*, 2018). Furthermore, chronic liver inflammation and fibrosis can be caused by the interaction between LPS and TLR4 on the liver cells (Dapito *et al.*, 2012).

1.1.1 Role of the inflammatory responses in ALD and NAFLD

The primary cause of ALD and NAFLD is a dysregulated immune response to various factors such as bacteria, viruses, or macromolecules. The liver has several mechanisms to modulate immune function. It is considered as a major immunologic organ. Given its strong and specific blood supply route through the liver, it preserves a unique immune microenvironment. Stimulating the immune system of the liver for a long time can produce liver fibrosis which can progress to cirrhosis, and liver failure (Robinson *et al.*, 2016b). Cirrhosis is featured by a loss of architecture, the function of the liver, and the development of life-threatening complications. Hepatocytes, hepatic stellate cells, liver cholangiocytes, sinusoidal endothelial cells (LSECs), immune cells, and especially macrophages have appeared in the pathogenesis of liver fibrosis (Koyama & Brenner, 2017). Immunologically, KCs have a necessary role in phagocytosis to prevent the invasion of pathogenic organisms from the intestine (Robinson *et al.*, 2016b). In pathogenic situations, bone marrow-derived cells, like infiltrating macrophages, migrate to the liver and work in cooperation with the resident cells. However, the liver has a limited capacity to remove pathogenic organisms from the blood. This balance between immunity and tolerance is essential for liver function. The inflammatory processes in the liver are involved in both homeostasis and pathology (Robinson *et al.*, 2016b). Mechanisms in the liver act to resolve the inflammation issues to avoid the pathological consequences of excessive inflammation (Robinson *et al.*, 2016b). Excessive inflammation in the absence of infection leads to sterile liver injury, tissue damage, and remodeling; insufficient immunity allows for chronic infection and cancer (Koyama & Brenner, 2017). Hepatocytes and macrophages recognize the foreign body (bacterial endotoxin) by pattern recognition receptors (PRR), form microbial associated molecular patterns (MAMP) and damage-associated molecular patterns (DAMP) (Janeway, 1992). MAMPs, and DAMPs present in high

quantities in blood arriving from the portal vein, and both of them (MAMP and DAMPs) are phagocytosed and degraded by hepatocytes and KCs, without the production of inflammatory mediators that usually accompany PRR signaling. (Robinson *et al.*, 2016b). However, the excessive immune activation due to regular exposure to dietary and microbial products creates liver inflammation (Robinson *et al.*, 2016a). The macrophages that appear in the liver following injury are heterogeneous and may derive from various origins such as, liver-resident macrophages KCs and two patrolling populations of bone marrow monocyte-derived macrophages (MoMFs) as well as peritoneal macrophages for subcapsular regions of the liver (Guillot & Tacke, 2019). The various sources to these cells linked to contradictions in cell functionality as well as in responsiveness toward activating and recruiting signals, directly influencing the immune response (Guillot & Tacke, 2019). The understanding of the role of hepatic macrophages in liver diseases provides opportunities for the development of targeted therapeutics for chronic liver disease (van der Heide *et al.*, 2019b). Kupffer cells are self-renewing and usually non-migrating macrophages in the liver and are located in the liver sinusoids in contrast to macrophages originating from circulating monocytes (Guillot & Tacke, 2019). KCs are essential in the first reaction to injury, release cytokines and chemokines, and recruit monocytes via secretion of CCL2 and CCL5 chemokines (van der Heide *et al.*, 2019b). Recent studies have presented that the increase of hepatic macrophages from 3–5-fold in liver inflammation due to the infiltration of Ly-6C-high MoMFs–Promoting inflammation through stimulate MoMFs to release factors like TGF-β, IL-1β, platelet-derived growth factor (PDGF), and CCL2, which activate hematopoietic stem cells (HSCs) and progress the inflammation. Ly-6C-high MoMFs are pro-fibrogenic and proinflammatory. The Ly-6C-high MoMFs can be changed to Ly-6Clow macrophages that are pro-restorative, anti-fibrotic, and anti-inflammatory depended on microenvironmental signals (van der Heide *et al.*, 2019b). Significantly, the macrophages are responsible for the repair of liver fibrosis when the injury in the liver is removed (van der Heide *et al.*, 2019b). Comprehension of the mechanism of inflammation and fibrosis is critically to improve treatments for chronic liver diseases. (Koyama & Brenner, 2017). Hepatic steatosis is a predominant result of metabolic or toxic stress. This steatosis may develop to progressive hepatic inflammation and liver damage (Sayiner *et al.*, 2016). The liver is permanently exposed to exogenous protein obtain from the foods, chemicals, drugs, and microbiota in the gut that can happen even under physiologic conditions.

The majority of macrophages in the liver are KCs. KCs clear up any foreign material in the blood on the surface of sinusoidal cells (Abdullah & Knolle, 2017). KCs can distinguish between the

millions of red blood cells, platelets, and immune cells that flow by and choose pathogens that may have infringed barrier and entered the bloodstream. Such distinguish action is done in part through opsonins that tag the pathogen as foreign; these opsonins contain complement and more specifically C3b and inactivated C3b (iC3b) (Strey *et al.*, 2003). When the injury occurs in the liver, KCs turn to activated cells which can produce cytokines and signaling molecules. In addition, stimulating KCs present different markers of M1-like macrophages or M2-like macrophages that rely on the signals they receive from their environment (van der Heide *et al.*, 2019a). The equilibrium between proinflammatory M1 KCs, and anti-inflammatory M2 KCs is a regulator of liver inflammation (Tacke & Zimmermann, 2014). The major function of KCs is to sense and remove pathogens and dangerous molecules that pass through the portal circulation(Tacke & Zimmermann, 2014). KCs binds with pathogens through PRRs. PRRs include at least two families of sensing proteins: TLRs and NLRs, which reveal danger signals inclusive of DAMPs and alarmins. TLRs identify gut microbiota-derived bacterial products such as LPS and peptidoglycan. TLR has more than 13 members, but the most important is TLR4, TLR3 (Yamamoto & Takeda, 2010). KCs react to LPS via TLR4 to release different inflammatory cytokines including TNF-α, IL-1β, IL-6, IL-12, IL-18, and chemokines in granulomatous liver disease, ischemia/reperfusion liver injury, NASH, and alcoholic liver disease (Seki *et al.*, 2000; Tacke, 2017). Moreover, stimulation of many of these mediators worsens insulin resistance and metabolic syndrome (Tacke, 2017). Macrophages obtain from recruited bone marrow are an essential component of both acute and chronic liver inflammation and are implicated in the regression of liver disease (Tacke, 2017). Macrophages are classified into M1 macrophages that cause proinflammatory, M2 macrophages act as wound-healing and regulatory macrophages that work immunosuppressive phenotypes (Mosser & Edwards, 2008).

The pathogenesis of chronic liver inflammation caused by proinflammatory cytokines such as TNF-α, IL-6, and IL-1 which produced from M1 macrophages due to inducing of IFN-γ, LPS, and TNF-α, while M2 macrophages are induced by IL-4, IL-10, IL-13 and release IL-10, TGF-β, PDGF, and EGF, have anti-inflammatory effects and promote wound healing (Rosen & Golden-Mason, 2020). Also, M1 and M2 macrophages have separate metabolic actions (Xu *et al.*, 2015). M1 macrophages are related to rising glycolysis and the production of nitric oxide (NO) from arginine by inducible nitric oxide synthase (iNOS) (Munder *et al.*, 1998). However, M2 macrophages depend on fatty acid oxidation and metabolize arginine by arginase enzyme 1 (ARG1) (Koyama & Brenner, 2017; Munder *et al.*, 1998). Study has shown that massive heterogeneity in hepatic macrophages, with distinct functions and gene signatures, has been detected highlighting their

essential role in liver diseases (Xue *et al.*, 2014). The major hepatic macrophages are KCs that present during steady-state involved in homeostasis, hepatic metabolic or toxic damage that results in a huge infiltration of monocyte-derived macrophage (MOMFs) into the site of liver injury (Wang *et al.*, 2016).

Generally, KCs preserve homeostasis, but when the imbalance happens, this can lead to liver inflammation and fibrosis. The imbalance in KCs functioning can result in different liver diseases consequently liver inflammation. When the liver damage occurs, KCs react and communicate with hepatic stellate cells that are also known as the liver pericytes located in the space of Disse between parenchymal cells and LSECs of the hepatic lobule (Robinson *et al.*, 2016b). There are several functions in the liver such as storage vitamin A, hemodynamic functions, immuno-regulation, and extracellular matrix (ECM) remodeling, done by hepatic stellate cells. However, in liver injury, these cells transdifferentiate into activated proliferative, migratory, and contractile myofibroblasts, and secrete multiple pro-inflammatory and pro-fibrotic factors (Seki & Brenner, 2015). Moreover, these hepatic myofibroblasts elevate the differentiation of liver macrophages with pro-inflammatory and pro-fibrotic functions. Thus, the combination between KCs and hepatic stellate cells is the one of reasons for the development of liver fibrosis and hepatocellular carcinoma (van der Heide *et al.*, 2019b).

Liver homeostasis is necessary for tolerating and adjusting immune responses. Hepatic immune tolerance mainly relies on the reaction between KCs and Tregs to generate a local suppressive microenvironment, whilst the determination of progression of the liver disease and tissue regeneration following liver injury is related to the recruitment of MoMFs and crosstalk between hepatic stellate cells and KC. One of the most key significant to the improvement of the targeted therapies for liver diseases, particularly, targeting liver inflammation is to gain insights into phenotypic heterogeneity and functions of hepatic macrophages (van der Heide *et al.*, 2019b). The hepatic macrophages (KCs and MoMFs) are the main players in different kinds of liver disease (van der Heide *et al.*, 2019b).

Innate and adaptive immune responses can be activated by excessive alcohol and high-calorie diet consumptions. Alcohol and its metabolite, acetaldehyde enhance cytotoxic and (ROS) on hepatocytes which cause damage to the liver (Ju & Liangpunsakul, 2016). During ALD progression, the (KCs) M1 and M2 phenotypes increase significantly which led to an increase in intrahepatic inflammation such as high expression of inflammatory genes, M1 and M2 markers, cytokines, and chemokines (Gao & Bataller, 2011). M1 macrophages act as pro-inflammatory by

produce cytokines such as TNF-α while M2 macrophages work as anti-inflammatory producing chemokines like IL10. Recent research showed that M2 macrophages are increased at the beginning of alcoholic liver injury whereas, M1 macrophages present in severe alcoholic liver injury (Wan *et al.*, 2014). In ALD patients, LPS levels are high in the vascular circulation and in the liver (Osna *et al.*, 2017). High levels of LPS in KCs, HSCs, and LSECs lead to activate TLR4 signaling, which contributes to the regulation of angiogenesis and fibrogenesis, leading to fibrosis. Activation of TLR4 signaling in these cells KCs, HSCs, and LSECs) produce (ROS) as well as proinflammatory cytokines and chemokines that together with alcohol contribute to hepatocyte damage. Other factors contribute to hepatocyte damage include alcohol-induced activation of various immune cells (i.e., neutrophils, T cells, and other leukocytes) as well as alcohol's effects on the fat (i.e., adipose) tissue, which results in the production of damage-associated molecular pattern (DAMP) molecules (Osna *et al.*, 2017). Complement activation, TLR pathways, and LPS-mediated pathways, including inflammasome activation, could be potential therapeutic targets to develop new therapies for the treatment of ALD (van der Heide *et al.*, 2019a). Animal studies have been shown that long administration of alcohol is linked with signs of CD14/TLR4 activation of macrophages in the liver, which leads to an increase of CD14 as well as increased production of TNFα, MCP-1, and ROS (Enomoto *et al.*, 2001). Moreover, the exhaustion of liver macrophages during different mechanisms to prevent alcohol-induced liver inflammation can induce liver injury (Ju & Liangpunsakul, 2016; Koop *et al.*, 1997).

Recent studies have revealed that unhealthy long-term alcohol consumption may trigger KCs activation (Slevin *et al.*, 2020; Wu *et al.*, 2016). One of the mechanisms that contribute to increased sensitization of KCs to LPS in the alcoholic liver is ROS production (Thakur *et al.*, 2006). Production of ROS in KCs probably by induction of an enzyme (cytochrome P4502E1) and NADPH oxidase that involved in alcohol metabolism in the liver during prolonged alcohol exposure (Ju & Liangpunsakul, 2016). In 2006, Thakur shown that the pre-treatment of alcohol-fed rats by inhibitor agent for the NADPH oxidase normalized ROS production as well as reduced phosphorylation of the signaling molecule ERK1/2 and inhibited the production of the proinflammatory cytokine TNFα in KCs (Thakur *et al.*, 2006). Also, ROS-mediated Kupffer-cell sensitization in the alcoholic liver can be enhanced by the expression of multiple TLRs (Hritz *et al.*, 2008). In ALD, the TLR4 expression is essential for both the KCs and MoMFs. However, it is still unclear if the liver-resident Kupffer cell-specific TLR4 is the only TLR contributing to alcohol-mediated pathogenesis. Studies proposed that alcohol can induce ROS and increase KCS sensitization to endotoxin, which can prompt pro-inflammatory responses, and these are two

major players in the activation of KCs in the ALD (Haorah *et al.*, 2008; Ju & Liangpunsakul, 2016; Marchi *et al.*, 2016). Nowadays, the most important goal to treat ALD is to inhibit KCs activation (M1macrophages) that reduces the pro-inflammatory cytokines or induces M2 macrophages to release anti-inflammatory cytokine production (Mandal *et al.*, 2010).

KCs in the liver have many functions, not just protect against pathogens, it can also, help nourish and maintain the cells and ensure tissue homeostasis (Ju & Mandrekar, 2015). Regulation of inflammation by KCs is not only associated with the production of pro-inflammatory substances but also by producing anti-inflammatory substances, such as prostaglandin D2, which is sensed by HSC receptors. Producing prostaglandin D2 can programs HSCs to switch their production to anti-inflammatory factors, including transforming growth factor-β1 (TGF-β1), which promotes fibrogenesis. The KCs and HSCs have an important role in the development of alcohol-induced inflammatory changes and progression to fibrosis and cirrhosis (Osna *et al.*, 2017)

1.1.2. The nuclear regulatory factor κB (NFκB)

NFκB is a central regulator of cellular stress in most cells in the liver (Mandrekar & Szabo, 2009). The group of NFκB proteins like RelA/p65, RelB, c-Rel, and p50, live in the cytosol of resting cells as dimers in a complex with (IkB) molecules (Chen & Ghosh, 1999). When there are stress signals such as pathogen-derived, oxidative stress and other signals, these dimers are activated and translocated to the nucleus. Danger signals lead to the stimulation of the IKK kinase complex consisting of IKK/IKKβ/NEMO and phosphorylation of IKB. Phosphorylated IKB is then ubiquitinated and degraded by the proteasomal pathway (Mandrekar & Szabo, 2009). Dissociation of IkB reveals the nuclear translocation sites of NFκB permit nuclear translocation and DNA binding. NFκB forms p65/p50 heterodimers in macrophages and is linked to the promoter zone of different pro-inflammatory genes to result in gene transactivation (Mandrekar & Szabo, 2009). Fat deposition and inflammation in the liver can change gut permeability and barrier-induced infiltration of bacteria. Then bacteria products increase the NFκB–or c-Jun-N-terminal kinase (JNK), as well as of TNFα. Stimulation of NFκB in hepatocytes increased the release of cytokines and resulted in the recruitment and activation of KCs that mediate inflammation in the progression of NASH. Stimulation of NFκB prompts the expression of TNFα, Fas ligand (FasL), and TGFβ, which contributed to fibrosis in NASH (Cobbina & Akhlaghi, 2017). NFκB is one of the mechanisms that regulate pro-inflammatory cytokine production by activated hepatic macrophage. Chronic alcohol-mediated liver injury is related to stimulation of TLR4 by

circulating LPS on hepatic macrophages culminating in NFκB activation and pro-inflammatory cytokine production. Animal studies have shown that NFκB DNA binding in the liver increased in alcohol-fed murine models of ALD (Wheeler *et al.*, 2001). These studies revealed that chronic alcohol exposure primes innate immune cells in the liver to causing sustained NFκB activation and induction up LPS-stimulation resulting in increased expression of TNFα (Nanji *et al.*, 1999). Also, similar increased NFκB activation compared to controls in resident liver macrophages, monocytes from chronic alcoholic patients (Hill, 2000). Furthermore, Activation of NF-κB can be induced by many factors such as growth factors (like insulin), cytokines (TNF-α, TNF-β, IL-1, etc.) (Kaĭdashev, 2012).

1.1.3. Pathogenesis of ALD and NAFLD

Today, alcohol consumption is considered as one of the common health problems worldwide which caused around 3.3 million deaths each year. Excessive alcohol consumption is associated with many social and clinical issues such as economic problems, mental disorder, cardiovascular and liver diseases (Organization, 2019; Sudhinaraset *et al.*, 2016). Excessive alcohol drinking over decades causes damage to virtually every organ in the human body. The earliest and greatest degree of tissue injury is in the liver because the liver is considered the first site of ethanol metabolism (Lieber, 2000). The metabolism of alcohol (ethanol) takes place in the main parenchymal cells of the liver (hepatocytes), which form more than 70 percent of the liver (Boyer & Zakim, 1990). Hepatocytes produce the most essential ethanol oxidizing enzyme which is alcohol dehydrogenase (ADH). The oxidation process of ethanol by ADH is known as a major ethanol-oxidizing pathway which leads to converting the ethanol into acetaldehyde in the presence of nicotinamide adenine dinucleotide (NAD) which works as a cofactor, generating reduced NAD (NADH), and this process occurs in the cytosol (Osna *et al.*, 2017). Acetaldehyde is highly reactive and toxic. It can covalently bind to macromolecules such as proteins, lipids, and nucleic acids to form acetaldehyde adducts, which, in turn, can deactivate the structure and the function of these macromolecules (Mauch *et al.*, 1986). Hepatocytes reduce acetaldehyde toxicity is by rapidly oxidizing it to acetate using the enzyme aldehyde dehydrogenase 2 (ALDH2) inside mitochondria. The ALDH2 reaction is another oxidation-reduction step that creates NADH and acetate, the latter of which can diffuse into the circulation to be used in other metabolic pathways. Furthermore, creating more NADH reduces the normal intrahepatocyte NAD /NADH ratio, called the cellular redox potential. This alteration generates important metabolic shifts from oxidative metabolism toward reductive synthesis, supporting the formation of fatty acids, which contribute

to fatty liver development (Donohue Jr, 2007; Osna *et al.*, 2017). There is another major hepatic enzyme that catalyzes ethanol oxidation to acetaldehyde called cytochrome P450 2E1 (CYP2E1). The efficiency of CYP2E1 is lower than ADH but CYP2E1 has a higher capacity to binding ethanol. In addition, the hepatocellular release of CYP2E1 increases during chronic ethanol consumption. The CYP2E1 is an adaptive enzyme (Dilger *et al.*, 1997; Osna *et al.*, 2017). This enzyme directly reacts with ethanol to form a new structure that resists degradation by the ubiquitin-proteasome system and resulting in the accumulation of CYP2E molecules. Heavy drinkers suffer from several major bad condition impacts that cause by the increase of CYP2E1 that includes metabolic tolerance, they need to drink more alcohol to reach a level of intoxication that they previously achieved after drinking less alcohol. Also, fatly alcohol metabolism by the large amount of CYP2E1 makes liver cells at metabolic risk, that because a lot of CYP2E1 can also produce great amounts of various other reactive oxygen species (ROS), including hydroxyethyl radicals (i.e., free-radical forms of ethanol), superoxide anions (O2), and hydroxyl radicals (·OH) (Osna *et al.*, 2017). Excessive alcohol use creates a lot of free-radical forms which can cause oxidative stress. Exceeding the rate of ROS generation leads to an increase in the capacity of the liver to neutralize them with natural antioxidants such as glutathione and vitamins E, A, and C, or to remove them using antioxidant enzymes like Glutathione Peroxidase and Catalase (Fang *et al.*, 2002). Some studies have shown that chronic ethanol consumption can decrease the activities and amount of many antioxidant enzymes which worsens the hepatocytes' oxidant burden (Dong *et al.*, 2014). ROS are highly reactive molecules which can react with carbohydrates, nucleic acids, lipids, and proteins inside the cells and alter their functions that lead to cell structure damages. This reaction results in the generation of lipid peroxides which interact with proteins and with acetaldehyde to form huge adducts (e.g., malondialdehyde-acetaldehyde [MAA] adducts) that are capable of generating an immune response (Tuma *et al.*, 1996). Lastly, a high level of CYP2E1 increases the metabolic of other compounds such as analgesic and antipyretic medication acetaminophen that can cause toxicity from an overdose (Schiødt *et al.*, 2002). The chronic drinker can be susceptible to high risk for chronic liver disease or acute liver failure (Osna *et al.*, 2017).

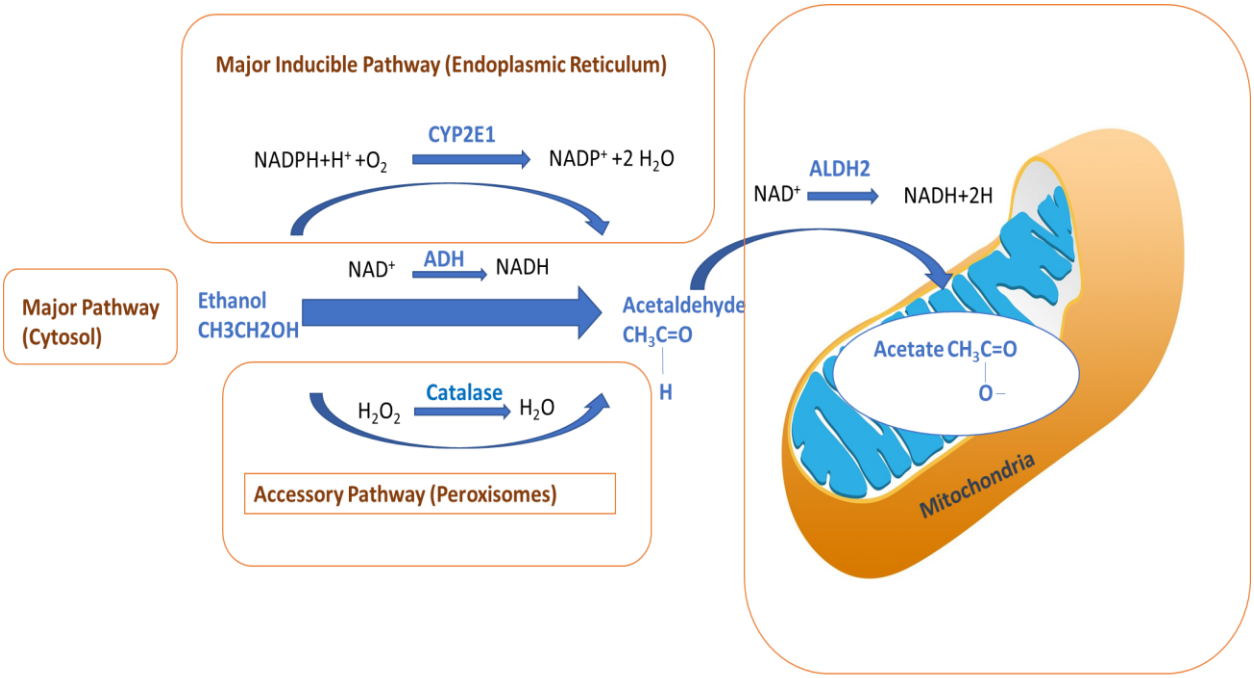


Figure 1.1. Major and minor ethanol-oxidizing pathways in the liver. The major ethanol-oxidizing pathway by ADH leads to converting the ethanol into acetaldehyde in the presence of nicotinamide adenine dinucleotide (NAD) which works as a cofactor, generating reduced NAD (NADH), and this process occurs in the cytosol. © Ebtisam Abosmaha.

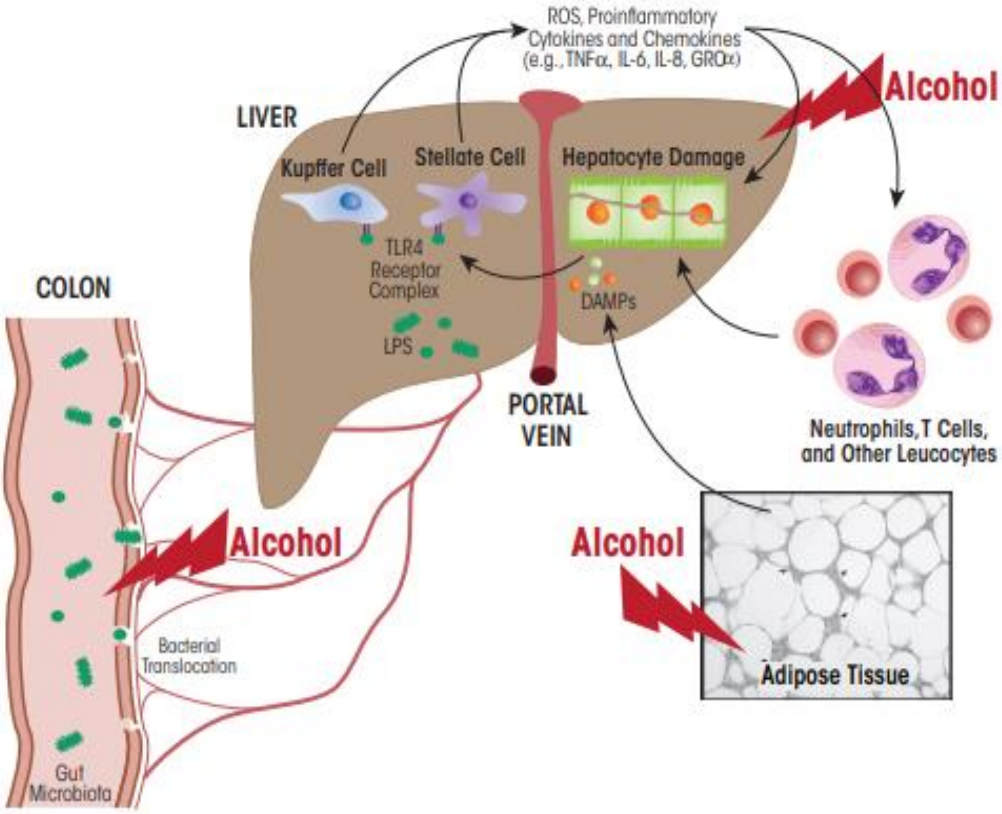


Figure 1.2. The role of inflammation response on ALD. Bacteria translocate from the gut lumen into the portal circulation to reach the liver. Then LPS which is one of the components of bacteria stimulates KCs and hepatic stellate cells by interacting with TLR4 (Osna et al., 2017).

1.2. Autophagy

Autophagy is a natural process that regulates the mechanism of the cell by removing or degrading unnecessary or damaged cell components (organelles, proteins, and metabolites) into the lysosome (Glick et al., 2010). Three kinds of autophagy have been identified: macroautophagy, chaperone-mediated autophagy (CMA) (Arias & Cuervo, 2011), and microautophagy (Gual *et al.*, 2017a). However, the most important type of autophagy is macroautophagy. It is the main catabolic pathway that form a double-membrane vesicle, the autophagosomes which is used to sequester cargo and subsequently transport the damaged components of the cell to the lysosome. The compounds and amino acids generated by degradation of macromolecules in the autophagy pathway are released into the cytoplasm for recycling or for energy production (Gual *et al.*, 2017a). The mechanisms of macroautophagy are complex and have different stages. The formation of the phagophore is controlled by the inhibition of the mammalian target of rapamycin complex 1 (mTORC1). mTORC1 is a major inhibitor of autophagy, which merges various signals like amino acids, glucose, and growth factors, and is negatively regulated by AMPK. Inhibition of mTORC1 activates the serine/threonine-protein kinase (unc-51-like kinase 1) ULK1, which forms a complex with at least three protein partners: FIP200 (focal adhesion kinase family interacting protein of 200 kDa), ATG (autophagy-related protein) 13 (ATG13), and ATG101 (Gual *et al.*, 2017a). This complex works as a node transform multiple signals into autophagosome formation. After the formation of the autophagosome, the ULK1 complex transfer to the initiation sites of autophagy and organizes the recruitment of a second kinase complex, the vacuolar protein sorting 34 (VPS34) complexes (Karanasios *et al.*, 2013). The VPS34 complex involves the class III phosphatidylinositol 3-kinase VPS34, as well as BECLIN-1, VPS15, and ATG14L (ATG14-like) (Zachari & Ganley, 2017). This complex is accountable for the manufacture of the phospholipid phosphatidylinositol 3-phosphate (PI3P) at the site of creating autophagosome, called the phagophore, which acts as a signaling molecule for the recruitment of PI3P-binding proteins such as WIPI2B WD repeat domain phosphoinositide-interacting protein 2 (WIPI2B) and doubles FYVE containing protein 1(DFCP1), this step called nucleation (Karanasios *et al.*, 2013; Zachari & Ganley, 2017). PI3P synthesized by the Vps34 complex, is an essential trigger for the elongation and closure of the autophagosome by two ubiquitin-like conjugation systems, Atg5-Atg12 and LC3 (Atg8)- phosphatidylethanolamine (PE) (Zachari & Ganley, 2017). Degradation is the last phase of autophagy that has a high quantity of factors to adjust the autophagosome-lysosome fusion and the lysosomal biogenesis, activation, reformation, and turnover. This phase is selectively recognized by autophagy adaptors, such as p62, a protein containing an LC3-interacting region, which allows selective degradation of the ubiquitinated cargo by autophagy.

Once the cargo has been engulfed, the autophagosome fuses with the lysosome to form the digestive autolysosome where cargo is degraded and recycled (Gatica *et al.*, 2018). Moreover, an essential recognizer of lysosomal biogenesis and autophagy is the transcription factor EB, TFEB (Settembre *et al.*, 2013). TFEB arrange the cellular responses to different stresses, including nutrient starvation, metabolic stress, and lysosomal stress, to maintain cellular homeostasis (Settembre *et al.*, 2013).

The second type of autophagy is CMA. It is the process in which isolates the polypeptides and soluble proteins which contain a KFERQ motif in their amino acid sequence (Arias, *et al.*,2011). These proteins are bound to a chaperone protein for translocation to lysosomes where binding to the lysosome-associated membrane protein type 2A (LAMP-2A) leads to protein internalization and degradation (Kaushik & Cuervo, 2018). The third type of autophagy is microautophagy. This type contains the direct isolation of cellular constituents within the lysosome through the invagination of the lysosomal membrane. Also, it considers as less studied compared with another type of autophagy (Parzych & Klionsky, 2014).

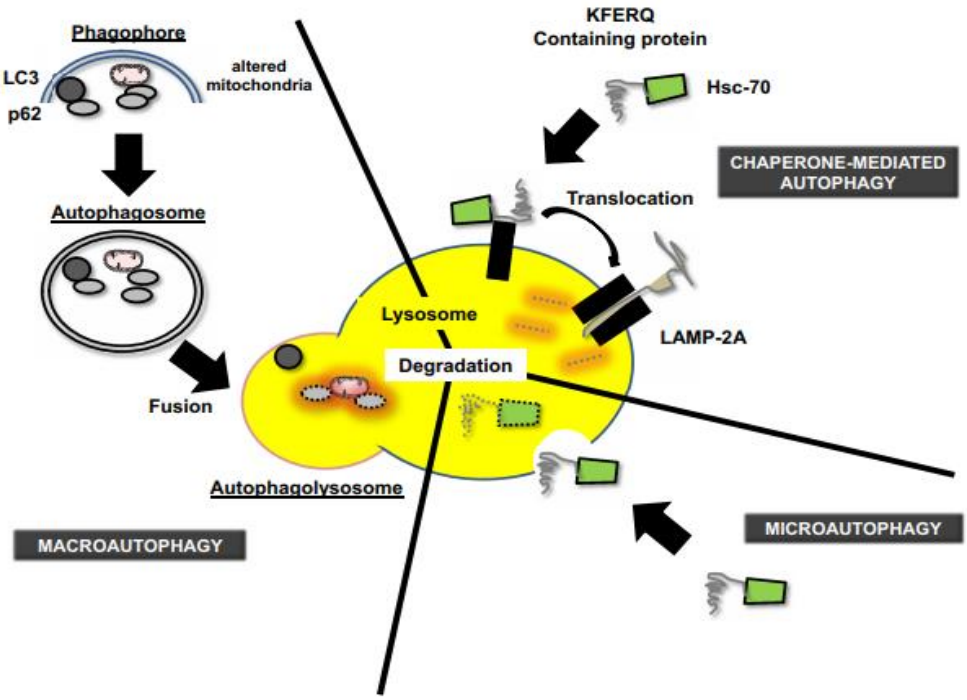


Figure 1.3. The autophagic pathways model. Macroautophagy, microautophagy and chaperone-mediated autophagy (Gual *et al.*, 2017b).

1.2.1 The Role of Autophagy in ALD and NAFLD

The essential regulators of the liver homeostasis in metabolic and detoxifying are macroautophagy and CMA. Both macroautophagy and CMA can eliminate aggregate-prone proteins, damaged mitochondria (mitophagy), and counteracting hepatocyte swelling (Madrigal-Matute & Cuervo, 2016). The physiological functions could be the regulation of metabolic pathways such as gluconeogenesis during fasting oxidation of fatty acids, and ketone body formation. Moreover, proteolysis by bulk autophagy produces amino acids used for gluconeogenesis, while selective autophagy of triglycerides stored in lipid droplets (lipophagy) produces fatty acids. Autophagy could regulate the level of VLDL particles through lipophagy, which liberates fatty acids degrades apolipoprotein B (Zamani *et al.*, 2017). Autophagy appears to play an important role not only in the normal condition of the liver, but also within the pathogenesis of liver infections such as the non-alcoholic and greasy alcoholic liver. Autophagy can regulate the lipid in the liver by engulfing and degrading lipid droplets. The study showed that increasing obesity can decrease autophagy levels in hepatocytes. For example, increasing fatty tissue on NAFLD leads to decrease autophagy (Zamani *et al.*, 2017). Moreover, another study demonstrates that lipid accumulation and endoplasmic reticulum stress increase in hepatocytes with the mice that have knockdown on Atg5 and Atg7 (autophagy genes) or mice who take the pharmacological inhibition of autophagy (3-methyladenine) (Lavallard & Gual, 2014). During liver injury conditions, macroautophagy protects hepatocyte by removing misfolded proteins, accumulated lipids (lipophagy), and damaged mitochondria (mitophagy) to decrease lipid peroxidation, ER, or oxidative stress, and provides nutrients to maintain cellular energy homeostasis.

1.2.2. Anti-inflammatory properties of macroautophagy in macrophages during ALD and NAFLD

Macroautophagy is a prime regulator of pro-inflammatory signaling (Deretic *et al.*, 2013). Autophagy organizes phagocytosis of pathogens and regulates monocyte differentiation into macrophages and the acquisition of phagocytic functions (Jacquel *et al.*, 2012). Moreover, inhibition of autophagy in macrophages has been shown to stimulate the proinflammatory profile, enhances IL-1 in two ways. The first way is the NLRP3 inflammasome pathway which activated by ROS (Chuang *et al.*, 2013; Liao *et al.*, 2012), and the second way is the inflammasome-independent pathway, which increases the production of IL-1 via a ROS/calpain dependent (Castillo *et al.*, 2012). Researchers showed that mice with lacking Atg5 in myeloid cells are highly susceptible to liver inflammation when exposed to carbon tetrachloride, and show higher hepatic

secretion of IL-1 and, increased recruitment of neutrophils and monocytes into the liver (Lodder *et al.*, 2015). Besides, in obese mice, impaired macrophage macroautophagy cause exacerbates hepatic immune response by promoting M1 macrophage polarization (Liu *et al.*, 2015). Interestingly, and although inhibition of macroautophagy in myeloid cells does not affect the ALD phenotype upon chronic exposure. It was reported that stimulating macroautophagy in macrophages limits hepatic inflammation and steatosis. One of the suitable potential targets to elevate macrophage macroautophagy is the cannabinoid receptor 2 (Denaës *et al.*, 2016).

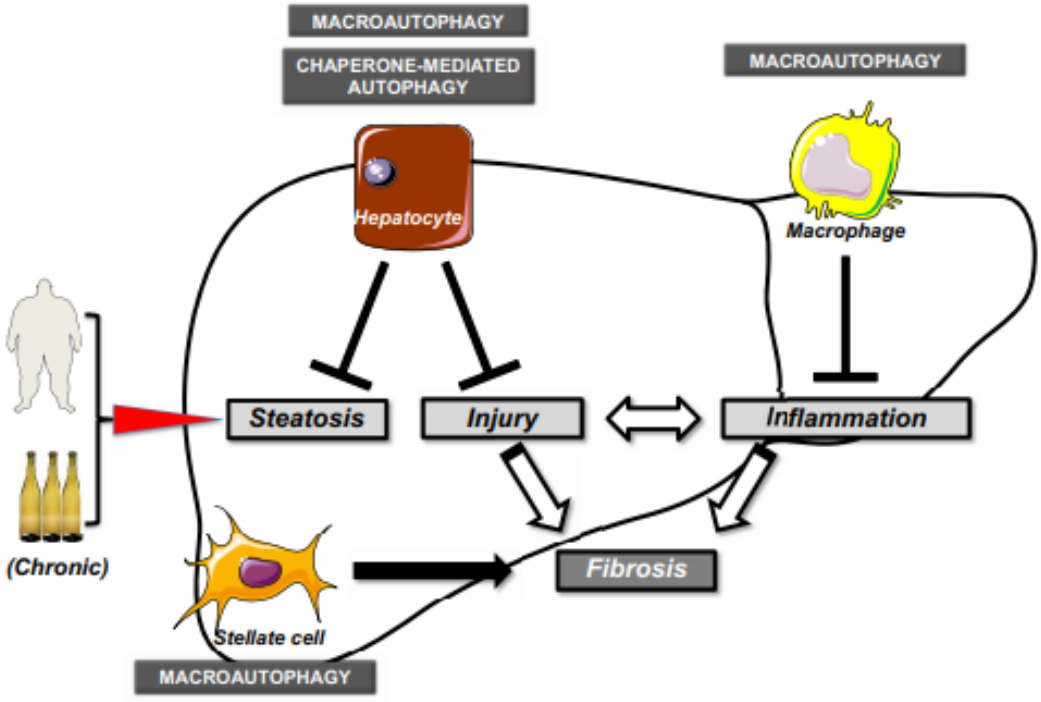


Figure 1.4. The effect of macroautophagy in ALD and NAFLD. CMA protects the hepatocytes against fat accumulation and prevent liver injury by removing altered mitochondria and decreasing cellular stresses (Gual *et al.*, 2017b).

1.3. Cannabis

Cannabis (*Cannabis sativa*) is a plant that belongs to the Cannabaceae family (*Magnoliopsida, Urticales*). The utilization of medical and psychoactive properties of cannabis dates back to 4000 B.C. Cannabis sativa has more than 500 ingredients, including 104 cannabinoids that have presently been identified. Tetrahydrocannabinol (THC) and cannabidiol (CBD) are cannabinoids that have been the most subject of scientific investigation into their pharmacological properties (Lafaye *et al.*, 2017). All the cannabis sativa plants have the same active ingredients but the differential of cannabis species characteristics depend on different concentrations and proportions, which do not only rely on the genomic background but also growing conditions and climate, meaning that they can be indicated as chemical varieties or chemovars, rather than strains. Each chemovar includes varying concentrations of cannabinoids, a class of mono- to tetracyclic C21 (or C22) meroterpenoids (Lewis *et al.*, 2018). Chemovars, also known as chemotypes, indicate the collapse of a plant species according to its chemical structure. Chemotypes classification is vital for growers and breeders. The determination of Certain chemical characteristics for example, whether a cannabis Indica plant has a greater CBD to THC ratio or vice versa (Lewis *et al.*, 2018). THC is the essential psychoactive cannabinoid in cannabis, and the adverse effects of acute or regular cannabis use are in direct relation to THC content in the product (Volkow *et al.*, 2014b). In 1964 the first isolation of THC in its pure form was by Gaoni and Mechoulam (Bruni *et al.*, 2018; Gaoni & Mechoulam, 1964). The extracts concentred of cannabis come as oils form are sticky and viscous appearance. Generally, preparation methods for Cannabis oils are comparatively simple and do not demand particular tools. In our study we use two formulations of full-spectrum cannabis; one which has (high CBD and low THC contents that are known as high CBD cannabis, and the other formal which has (low CBD and high THC contents) known as high THC cannabis. There is a growing body of proof suggesting that cannabinoids are useful for a range of clinical conditions, including pain, inflammation, epilepsy, sleep disorders, the symptoms of multiple sclerosis, anorexia, schizophrenia, and other conditions (Bruni *et al.*, 2018). Also, the progressive transformation of cannabinoids from herbal preparations into highly regulated prescription drugs is therefore progressing rapidly. The improvement of these drugs requires well-controlled clinical trials to be carried out to objectively establish therapeutic efficacy, dose ranges, and safety. One of the methods of cannabinoids administration is oral administration, but low oral bioavailability of cannabinoids has also led to feasible methods of administration, such as the transdermal route, intranasal administration, and transmucosal adsorption, which is being proposed (Bruni *et al.*, 2018). Over the past few years, the CBD oils

extracted from Cannabis sativa with low THC content have become very common use medically. Epidiolex is the first CBD-based drug that, was approved by the US Food and Drug Administration in June 2018, for the treatment of rare, severe epilepsy, further putting the spotlight on CBD and hemp oils. ("Hemp" is a term used to classify varieties of Cannabis that contain 0.3% or less THC content (by dry weight (Johnson, 2019). Presently, there is a preclinical and clinical study that support the use of CBD oils for many conditions suggesting its potential role as another option for treating challenging chronic pain or opioid addiction (van der Heide *et al.*, 2019b).

1.3.1. Cannabinoid receptors

The pharmacologic action of cannabinoids occurs by their interactions with cannabinoid receptors. Currently, there are two types of cannabinoid receptors that have been described. These include cannabinoid receptors 1 (CB1) and cannabinoid receptor 2 (CB2) which are two G-protein receptors identified within the endocannabinoid system that may play a part in liver diseases (Mallat *et al.*, 2011).

1.3.2. Impact of cannabinoids (CBD and THC) on inflammation and autophagy in ALD and NAFLD

The autophagy process is one of the protective mechanisms in the liver. Stimulation of autophagy in the liver can provide hepatocyte homeostasis and decreases hepatocyte oxidative stress and fat accumulation by clearing damaged mitochondria and lipid droplets from hepatocytes (Ding *et al.*, 2010; Ding *et al.*, 2011). Previous research showed that stimulates autophagy protection by CBD can affect ethanol toxicity and prevent alcohol-induced steatosis (De Ternay *et al.*, 2019; Yang *et al.*, 2014). CBD can activate autophagy by reduction of alcohol-mediated oxidative stress, prevention of JNK MAPK activation. CBD can stimulate autophagy in liver hepatocellular cells (HepG2 cells) that express CYP2E1, it showed an increase in flux from LC3-I to LC3-II which indicates increases in autophagy (Yang *et al.*, 2014).

The systemic immunoregulation properties of CBD involve complex molecular mechanisms which are still to be fully described. CBD can modulate many cellular signaling pathways impacting inflammation by way of the NF-κB pathway (Khaksar & Bigdeli, 2017; Rajesh *et al.*, 2010). Animal studies have provided that CBD could significantly decrease ethanol-induced liver damage via multiple mechanisms like inhibition of oxidative stress, adjustment of inflammation, death of stimulated HSC which responsible for fibrosis, inducement of autophagy, and inhibition of lipid

accumulation responsible for steatosis (Atalay *et al.*, 2019). In addition, THC can trigger different pharmacological actions from the CBD. It can suppress the immune response by inducing cell death or apoptosis in immune cell populations. THC significantly can induce apoptosis in murine macrophages, T cells, and B cells through activation of Bcl-2 and caspases (McKallip *et al.*, 2002; Zhu *et al.*, 1998). THC can induce a high level of apoptosis in naive lymphocytes when compared with mitogen-activated lymphocytes because activated cells suppressed the levels of CB2R on their cell surface (McKallip *et al.*, 2002). A lot of research showing that THC-induced apoptosis in antigen-presenting cells (McKallip, *et al.*, 2002). In bone marrow-derived dendritic cells (DCs), THC can induce apoptosis through ligation of both CB1 and CB2 and activation of caspase 2, 8, and 9 (McKallip, *et al.*, 2002). Another study showed that THC can affect immune response during inflammation by decrease IL-10 which acts as an anti-inflammatory cytokine and increase the proinflammatory cytokine IL-8 (Nagarkatti *et al.*, 2009) expression.

Pervious study demonstrated that, THC in the liver can inhibit the proliferation of hepatic myofibroblasts and stellate cells and promote their apoptosis, which may involve antifibrotic, hepatoprotective mechanisms (Dibba *et al.*, 2018). Cannabinoids (CBD and THC) not just have immunosuppression but also can induce autophagy. Many studies reported that cannabinoids such as (CBD and THC) can induce autophagy mechanisms in different types of cells. Cannabinoids stimulate autophagy, in an independent or receptor-dependent pattern. Activation of autophagy by cannabinoid treatment mostly through two various mechanisms: stimulation of AMPK or upregulation of ER stress response by increasing de novo synthesis of ceramide and upregulation of stress-regulated protein p8 and its downstream targets such as ATF4, CHOP, TRIB3, GRP78 and subsequent inhibition of the Akt/mTORC1 proteins, increasing levels in CHOP without an increase in GRP78 or TRIB3 can lead to mitochondrial-dependent apoptosis. Activation of autophagy by cannabinoids may lead to cell survival, cell death, or apoptosis (Costa *et al.*, 2016).

CHAPTER 2: HYPOTHESIS AND OBJECTIVES

ALD and NAFLD are major causes of morbidity and mortality globally (wong & huang, 2018b). There are no clinically approved treatments for progressive stages of chronic ALD and NAFLD with long-term efficacy in patients (shah et al., 2020). From epidemiologic studies, some individuals who used cannabis have some beneficial health effects on hepatic disease outcomes stemming from excessive alcohol and or high-calorie diet consumption (adejumo et al., 2018b; adejumo et al., 2017). In addition, the individuals who combine cannabis with alcohol have more beneficial health effects on the progression of ALD (adejumo et al., 2018b). In contrast, other individuals have observed no beneficial health effects of cannabis on ALD and NAFLD (adejumo et al., 2018b). Moreover, another study has shown that using cannabis could worsen ALD and NALD (Zhu & Peltekian, 2019). In this study, we aimed to elucidate the reason for these disparities of effect of cannabis on ALD and NAFLD outcomes and progression. Our hypothesis is full-spectrum cannabis formulations with varying contents of THC and CBD has differentially effects on the key cellular processes, such as inflammation and autophagy, that regulate ALD and NAFLD.

To verify this hypothesis in the current study our specific objectives are to determine in mouse macrophage cell line:

1. The effect of full-spectrum cannabis formulations (with varying CDB and THC contents) treatment on the production of TNF-α.
2. The effects of full-spectrum cannabis formulations (with varying CDB and THC contents) treatment sequential signaling events that drive inflammation through NF-kB.
3. How full-spectrum cannabis formulations (with varying CDB and THC contents) treatment modulate dysregulated autophagy in ALD and NAFLD.

The full-spectrum cannabis extracts contain the same active ingredients such as cannabinoids and terpenes. The difference between the two extracts is the amount of CBD and THC in each formula of the cannabis plant. Full-spectrum cannabis high CBD cannabis has high CBD and low THC contents while high THC full-spectrum cannabis has a high THC content and low CBD content.

CHAPTER 3. METHODOLOGY

3.1. Cell lines and alcohol treatment

Mouse macrophage cell line (RAW264.7 cells) was obtained from American Type Culture Collection (ATCC). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM)-low glucose (Sigma, Lot# RNBG7477) supplemented with 10% fetal bovine serum (FBS) and 100U/ml penicillin and streptomycin (Gibco, cat. #15140-163) at 37°C in 5% CO_2 incubator. Then, the cells were cultured in the absence of alcohol (control) or in the presence of either (50mM) ethanol 200proof (Sigma, lot# MFCD00003568) with or without LPS 100 ug/ml (Escherichia coli K-235, Sigma) for 72hr. In addition, the cells were treated with two full-spectrum cannabis formulations (with varying CDB and THC contents) for 8hr or 24hr as indicated. We used full-spectrum cannabis extracts which include the same active ingredients such as cannabinoids and terpenes. The difference between two extracts is the amount of CBD and THC in each formula of the cannabis plant. Full-spectrum cannabis high CBD cannabis has high CBD and low THC contents (cat. BT-CBD-0001) While the high THC full-spectrum cannabis has a high THC content and low CBD content (Cat. BT-THC-0001). The two different extracts from cannabis plant concentrations are CBD 20ug/ml and THC 0.1 ug/ml and THC 20ug/ml and CBD 0.1ug/ml. Both plant extracts have the same ingredients and the same amount of other active ingredients. Both formulations were obtained from the Société Québécoise du Cannabis (SQDC). Rapamycin 100ug/ml (Sigma Aldrich) was used as a positive control for autophagy.

3.2. Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants were collected and TNF-alpha levels was analyzed using the commercially available mouse ELISA kits (Biolegend, cat. 430904) according to the manufacturer's instructions. The 96 well plates are coated with 100 ul capture antibody 1X (mouse TNF-alpha for overnight in 4ºC). Absorbance was read at 450 nm within 30 minutes.

3.3. Fluorescent microscopy

RAW264.7 cells were treated with cannabis (high CBD and low THC) followed by LPS (100 ug/ml) stimulation for 45 minutes, then the cells were grown on glass coverslips for 6hr. The coverslips were then fixed with 4% formaldehyde for 10 minutes. Coverslips containing fixed cells were washed three times with ice-cold PBS and incubated in blocking buffer (PBS, 3% bovine serum

albumin (BSA), 10% FBS, 0.1% Saponin) for 30 minutes at room temperature (RT). For the detection of p65 nuclear translocation, fixed-permeabilized cells were incubated with anti-NF-kB p65 primary antibody [E379] (Abcam, cat. ab32536), in blocking buffer 1:200 for 1 hr at RT. Coverslips were then washed with ice-cold PBS and incubated with secondary Goat anti-Rabbit IgG, Alexa-Fluor 594 (red) conjugated antibody (Thermofisher, cat. # A-11035) for 1hr. After washing, coverslips were mounted on glass slides with Prolong Antifade with DAPI (Invitrogen). The images were acquired using confocal fluorescent microscopy (ZEISS,Jena, Germany) Olympus FV 1000 at 60X magnification.

3.4. Protein extraction and Western blot analysis

Cells were lysed in RIPA buffer (cat. #BP-115, Boston Bioproducts), supplemented with a protease inhibitor (cat.11852700 Roche). A Bradford protein assay was utilized to measure the total protein concentration. Equal amounts of protein 20 ug were displayed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The membranes were blocked for 1hr at RT with PBS containing 5% dry milk. Then, the membranes were incubated with primary antibodies (Table 3.1.) in PBS– 0.1% BSA. overnight at 4°C. Then, the membranes were washed with 0.1% Tween 20 in PBS and incubated for 1h at RT with secondary antibody (anti-rabbit or anti-mouse) in PBS–5% milk. Protein bands were revealed using the Clarity Western ECL reagent (Bio-Rad). Band intensity was quantified using a ChemiDoc MP imaging system (Bio-Rad).

Table 3.1: Primary antibodies used

Name	Catalog number/ Company	Dilution
Rabbit polyclonal anti-LC3B	L7543/ Sigma-Aldrich	1:1000
Monoclonal antibody anti-p62	H00008878/ Abnova	5:1000
Lysosome-associated membrane protein-1 LAMP1	53-1079-42/ Thermo-fisher	1:1000
Mouse monoclonal anti-actin	A5441/ Sigma-Aldrich	1:1000

3.5. Cell Proliferation Assay (MTS)

Cells were cultured in 96 well plates at density of $1x10^4$ cells per well and incubated for 2hr in 5% CO_2 incubator at 37°C. The cells were treated with various concentrations of cannabis

formulations and incubated for 24h. After 24h, 20 µL tetrazolium reagent was added and plate was incubated at 37°C in 5% humidified CO_2 for 2h. The absorbance was recorded at 490 nm.

3.6. Statistical analysis

All results were the outcome of at least 3 independents experiments. Data were presented as mean ± SD. Statistical analysis was carried out by using student t-test or one- and two-way analysis of variance for multiple comparisons ANOVA. A p-value of < 0.05 was considered statistically significant. Graph Pad Prism 8 software was used for analysis.

CHAPTER 4: RESULTS

4.1 Full-spectrum cannabis with varying THC and CBD contents differentially impact the TNF-α production following LPS stimulation in mouse macrophages cell line, RAW264.7

Increasing the leaky gut microbiome translocation play important role in the pathogenesis of ALD and NAFLD. These gut endotoxins stimulated hepatic innate immune cells KCs. We performed in-vitro stimulation of mouse macrophages (raw267.4 cells) with LPS to mimic this condition. We found that chronic alcohol treatment significantly increases the LPS induces TNF-α production compared with control (non-alcohol treated cells). By using different concentrations of full-spectrum cannabis (with high CBD and low THC contents) treatment, we found that 10ug/ml of full-spectrum cannabis had a slight reduction in the TNF-α production in both conditions non-alcohol treated cells following by LPS stimulation or chronic alcohol-treated cells following by LPS stimulation. However, 20 ug/ml of full-spectrum cannabis (with high CBD and low THC contents) treatment inhibited significantly the production of TNF-α in chronic alcohol-treated cells while with the non-alcohol-treated cells, there was non-significative decrease (Fig. 4.1). Also, there was a more significant reduction in the TNF-α production by using 30 and 40 ug/ml of full-spectrum cannabis (with high CBD and low THC contents) treatment in both conditions. As we saw from the graph the TNF-α production was decreased gradually with an increase in the cannabis dose in both conditions (Fig. 4.1.A). The toxicity assay was done to see the toxicity effect of all the cannabis doses that we used. To detect the toxicity, we used the MTS assay (Fig. 4.1.B). As we saw in (Fig. 4.1.B) all the treatment and cannabis doses that we used are not toxic.

On the other hand, the full-spectrum cannabis (with high THC and low CBD contents) treatment had a non-significative effect on TNFα production in both conditions non-alcohol treated cells and chronic alcohol-treated cells compared to baseline (control medium). Moreover, the full-spectrum cannabis (with high THC and low CBD contents) treatment in low concentrations (2.5 µg/ml and 5ug/ml) had a significant increase in the production of TNF-α in both conditions (non-alcohol treated cells and chronic alcohol-treated cells) following by LPS stimulation. In contrast, the full-spectrum cannabis (with high THC and low CBD contents) treatment with high concentration (7.5 µg,10ug) had no effect on LPS induced TNFα production on both conditions (non-alcohol treatment and chronic alcohol treatment) (Fig. 4.1.C). The various concentrations of full-spectrum cannabis (with high THC and low CBD contents), we had used are not toxic according to the MTS assay (Fig. 4.1.D).

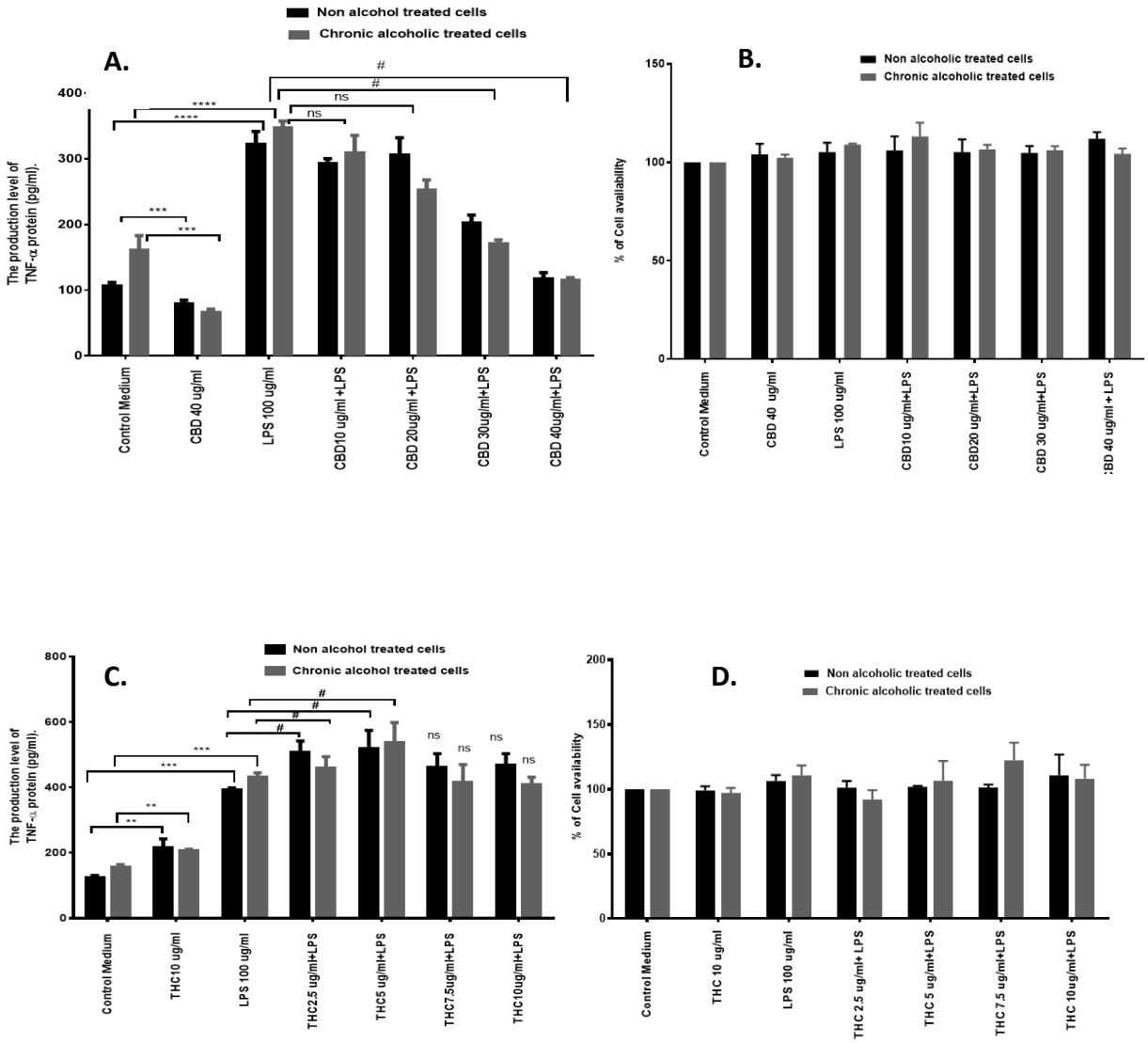


Figure 4.1.: Full-spectrum cannabis with varying THC and CBD contents differentially impact TNF-α production following LPS stimulation of macrophages (RAW264.7 cells). CBD= cannabis (with high CBD and low THC contents) treatment and THC= full-spectrum cannabis (with high THC and low CBD contents) treatment. **A.** The effect of the full-spectrum cannabis (with high CBD and low THC contents) on the production of TNF-α. **B.** Cell availability to the full-spectrum cannabis (with high CBD and low THC contents). **C**. The effect of the full-spectrum cannabis (with high THC and low CBD contents) on the production of TNF-α. **D**. Cell availability to the full-spectrum cannabis (with high THC and low CBD contents). n=3; ***p <0.001, ****p <0.0001 compered to control, #p <0.05 compered to LPS, ns= non-significant.

4.2. The full-spectrum cannabis with high CBD and low THC contents treatment can inhibit NF-kB signaling activation.

The production of pro-inflammatory cytokines by innate immune cells is modulated by numerous signaling mechanisms. To determine the direct effect of full-spectrum cannabis formulations in modulating inflammation signaling, we assessed NF-kB activation p65 unclear translocation by fluorescent microscopy. Our data suggest that full-spectrum cannabis with high CBD and low THC contents treatment can suppress alcohol-LPS induced TNF-α production by macrophages involving at least NF-kB signaling (Fig.4.2.). Normally p65 presents on the cytoplasm of cells but with LPS stimulation the p65 transfer to the nucleus. We assessed the non-treated macrophages (RAW264.7 cells), there is no transfer of p65 from the cytoplasm to the nucleus that means macrophages do not activated. However, during the LPS stimulation, we found that p65 was transferred to the nucleus which means macrophages are activated. Using full-spectrum cannabis with high CBD and low THC contents treatment following by LPS stimulation showed a decrease in p65 in the nucleus, showing that full-spectrum cannabis with high CBD and low THC contents significantly reduce LPS- induced NF-KB activation (Fig. 4.2.).

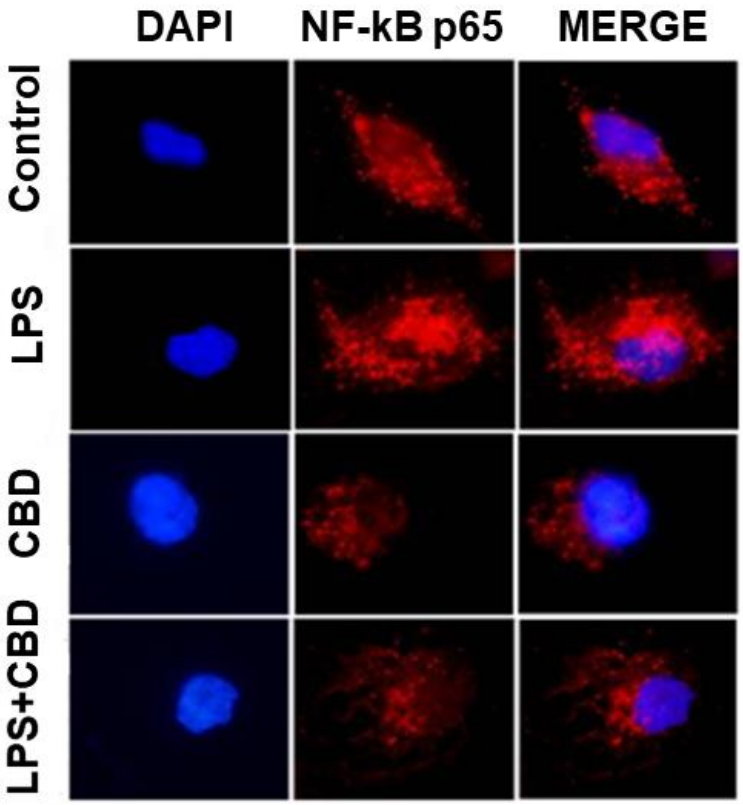


Figure 4.2.: CBD inhibits NF-kB p65 expression in the nucleus after LPS treatment. CBD= cannabis (with high CBD and low THC contents) treatment. **Immunofluorescence images showed a significant increase in the expression of NF-kB p65 in the nucleus after LPS treatment compared to control.** The cell nuclei were stained with DAPI (Blue). However, the expression of NF-kB p65 in the nucleus was markedly deceased after adding CBD to LPS compared to LPS alone.

4.3 The impact of full-spectrum cannabis with varying THC and CBD concentrations on the autophagy process in mouse macrophage cell line.

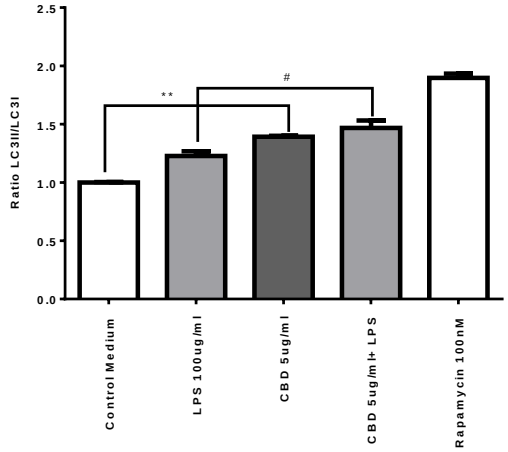
One of the characterizes of liver diseases is decreasing hepatic homeostasis which is the main cause of increased ROS oxidative stress, inflammation, perturbation of mitochondrial function, and cell death (Gual et al., 2017). We used western blot to detect auto-phagolysosome proteins (LC3, P62, LAMP-1). We evaluated the expression of two LC3 lipidation proteins (LC3II and LC3I). We used Rapamycin as a positive control for autophagy. We found that the rapamycin significantly increases LC3II/ LC3I ratio compared to the control (Fig. 4.3.A, B). We also found that LC3II/ LC3I ratio was significantly increased by both LPS and two full-spectrum cannabis (with varying THC and CBD concentration) treatment compared to control (Fig. 4.3.A, B). In addition, we observed that LC3II/ LC3I ratio was significantly increased after LPS stimulation followed by full-spectrum cannabis, high CBD and low THC, treatment compared to LPS alone (Fig. 4.3.A, B). However, LC3II/ LC3I ratio was markedly inhibited after LPS stimulation followed by full-spectrum cannabis (with high THC and low CBD contents) treatment compared to LPS alone (Fig. 4.3.A, B). Measuring LC3II/LC3I ratio only is not sufficient to determine the activation or impairment of autophagy flux, according to autophagy guidelines (Abdullah & Knolle, 2017). Therefore, we analyzed the p62 protein levels after LPS stimulation and two full-spectrum cannabis treatment (Fig. 4.3.C, D). We observed that p62 levels were significantly increased after rapamycin treatment, LPS stimulation, and two full-spectrum cannabis (varying CBD and THC concentration) treatment compared to control (Fig. 4.3.C, D). In addition, the p62 level was markedly increased after LPS stimulation followed by full-spectrum cannabis (with high CBD and low THC contents) treatment compared to LPS alone (Fig. 4.3.C). In contrast, we found that the p62 level was significantly reduced after LPS stimulation followed by full-spectrum cannabis (with high THC and low CBD contents) compared to LPS alone (Fig. 4.3D).

To detect the capacity of autophagosome degradation, we measured the lamp-1 protein levels after LPS stimulation and two full-spectrum cannabis (with varying CBD and THC contents) treatment compared with control. We identified that LAMP-1 protein levels were significantly decreased after rapamycin treatment and full-spectrum cannabis (with high CBD and low THC contents) treatment compared to control (Fig. 4.3.E). However, LAMP-1 protein level was not affected by LPS stimulation compared to control (Fig. 4.3.E). We also found that LAMP-1 protein expression was markedly increased after full-spectrum cannabis (with high THC and low CBD contents) compared to control (Fig. 4.3.F). In addition, we observed that LAMP-1 protein expression was potentially reduced after LPS stimulation followed by full-spectrum cannabis (with high CBD and low THC contents) treatment compared to LPS alone (Fig. 4.3.E). In contrast,

LAMP-1 protein level was insignificantly increased after LPS stimulation followed by full-spectrum cannabis (with high THC and low CBD contents) compared to LPS alone (Fig. 4.3. F).

Comparing between two full-spectrum cannabis treatment, we identified that treatment with the full-spectrum cannabis with high THC and low CBD contents induce more autophagy compared to full-spectrum cannabis with high THC and low CBD content treatment as indicated by LC3II/ LC3I ratio and p62 protein expression. There more autophagosome accumulation within the cell by full-spectrum cannabis with high CBD and low THC contents treatment compared to full-spectrum cannabis with high THC and low CBD contents treatment (Fig. 4.3.G, H). We also observed that treatment with full-spectrum cannabis with high THC and low CBD contents was increased more LAMP-1 expression that means more fusion and degradation of autophagosomes compared by cannabis with high CBD and low THC contents (Fig. 4.3.G, H).

A.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2.0
1.5
1.0
0.5
0.0
**
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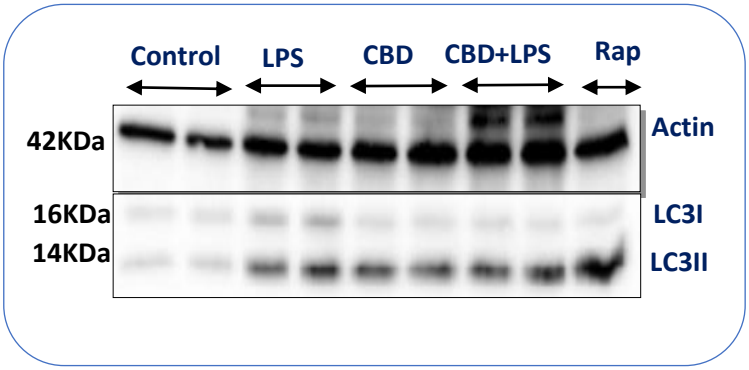
CBD 5ug/ml
CBD 5ug/ml+ LPS
Rapamycin 100nM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CBD
CBD+LPS
Rap
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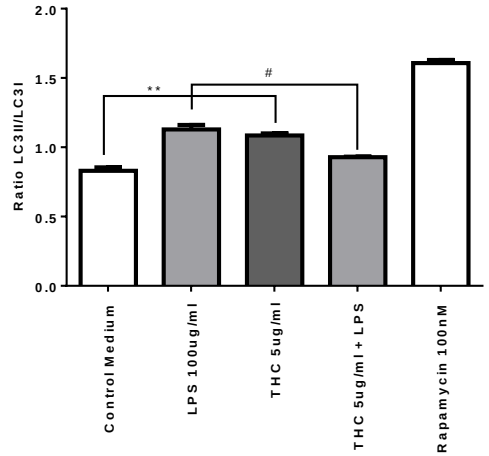
Actin
16KDa
LC3I
14KDa
LC3II

B.


Ratio LC3II/LC3I
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0.0
**
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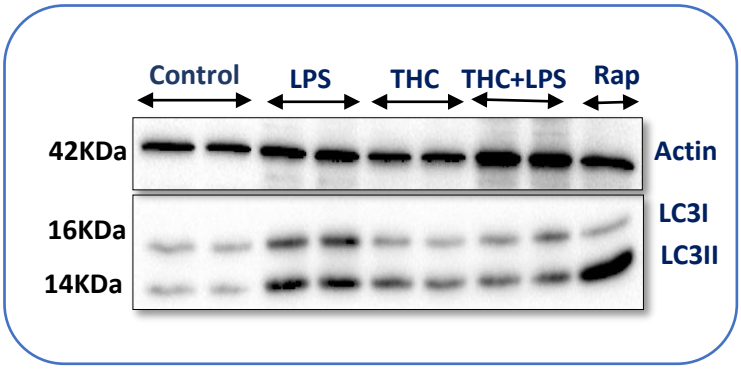
THC 5ug/ml
THC 5ug/ml + LPS
Rapamycin 100nM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THC
THC+LPS
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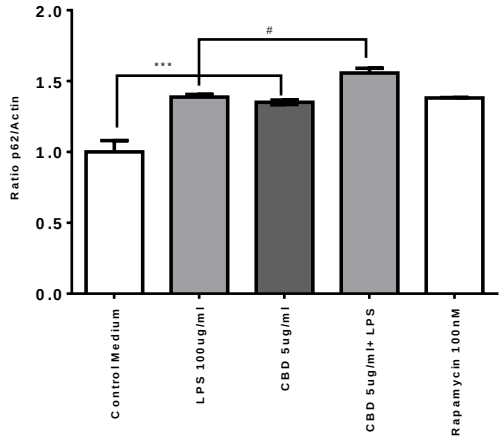
LC3II

C.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0.0
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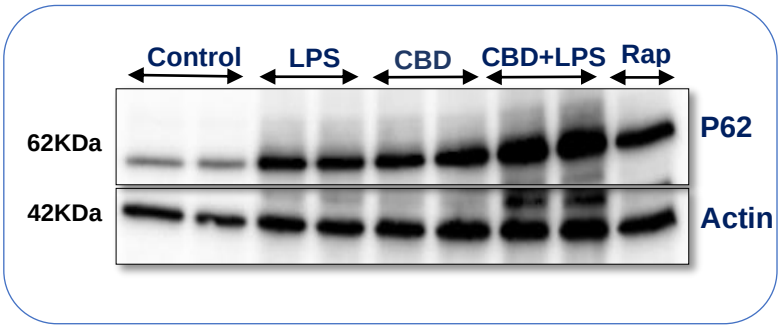
CBD 5ug/ml
CBD 5ug/ml+ LPS
Rapamycin 100nM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CBD+LPS
Rap
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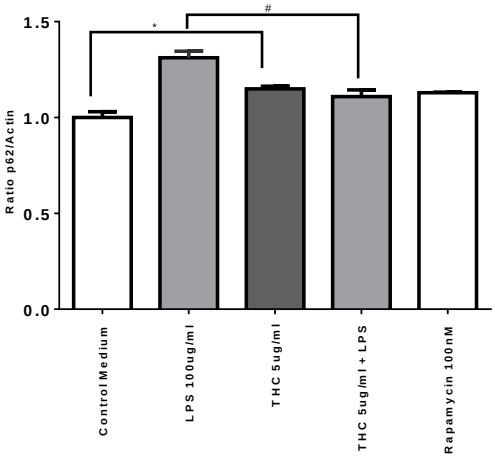
P62
42KDa
Actin

D.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*
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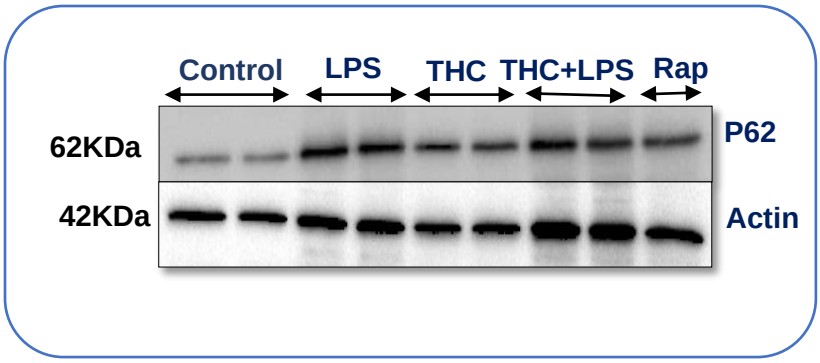
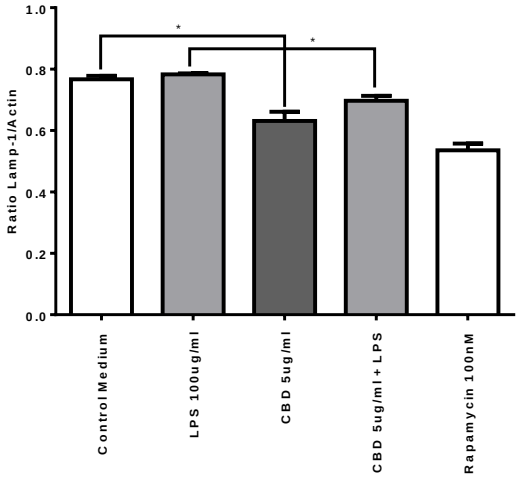
THC 5ug/ml
THC 5ug/ml + LPS
Rapamycin 100nM


Control
LPS
THC
THC+LPS
Rap
62KDa
42KDa
P62
Actin

E.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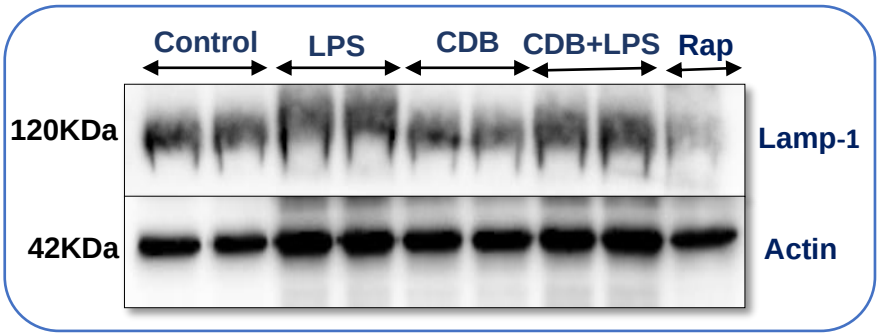
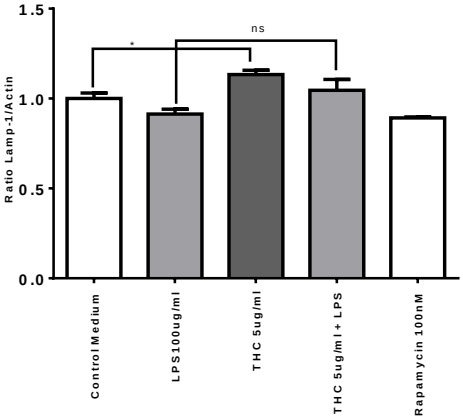
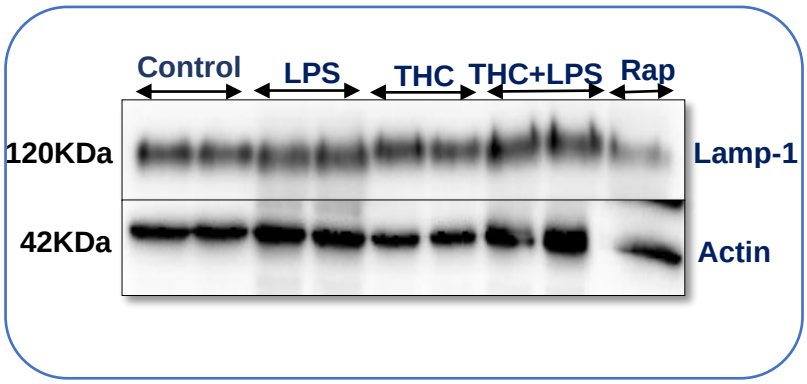
0.8
0.6
0.4
0.2
0.0
*
*
Control Medium
LPS 100ug/ml
CBD 5ug/ml
CBD 5ug/ml + LPS
Rapamycin 100nM


Control
LPS
CDB
CDB+LPS
Rap
120KDa
42KDa
Lamp-1
Actin

F.


Ratio Lamp-1/Actin
1.5
1.0
0.5
0.0
*
ns
Control Medium
LPS100ug/ml
THC 5ug/ml
THC 5ug/ml + LPS
Rapamycin 100nM


Control
LPS
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Actin

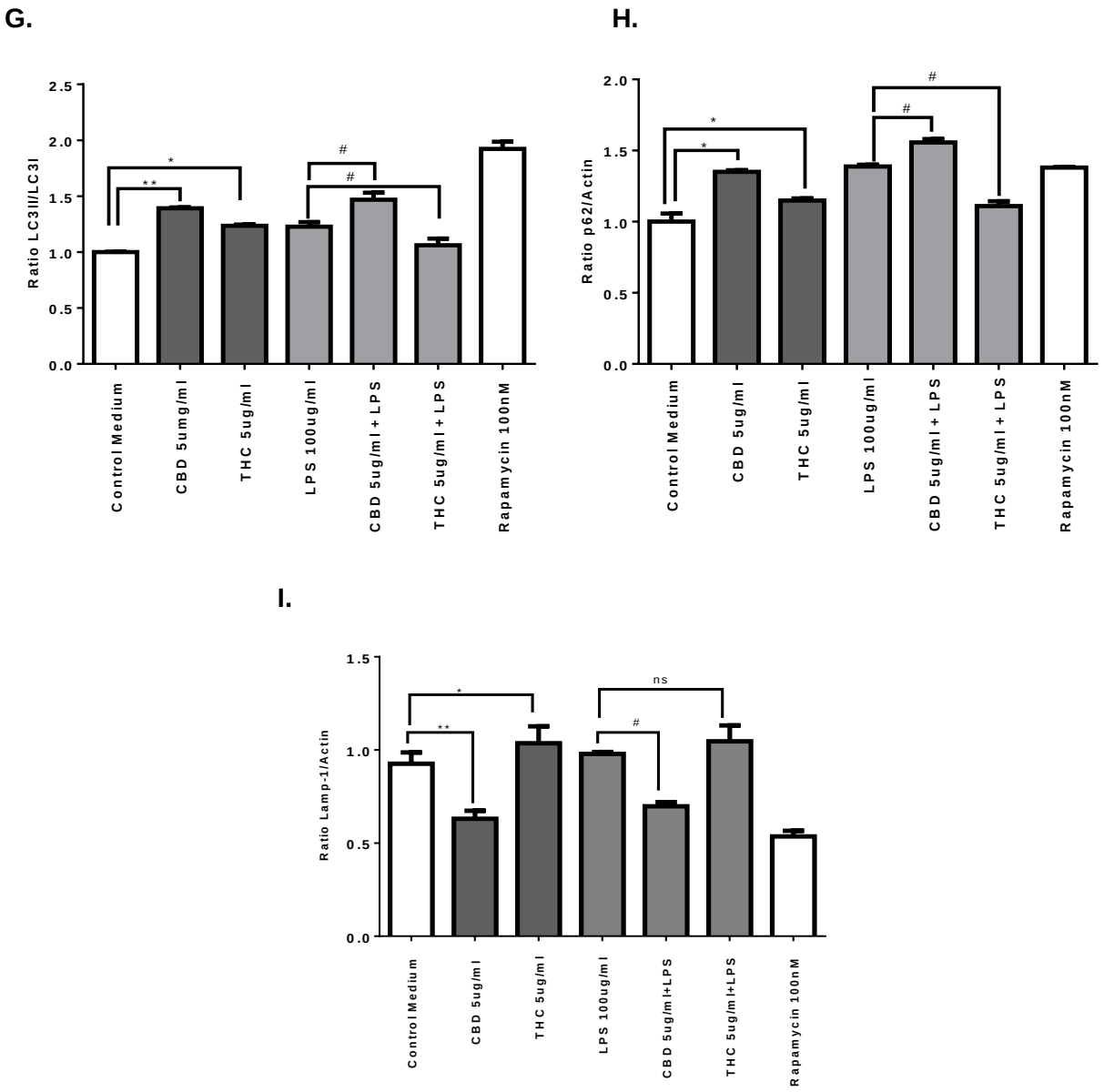

Figure 4.3.: The impact of full-spectrum cannabis with varying THC and CBD contents on the autophagy process in macrophages.CBD= cannabis (with high CBD and low THC contents) treatment and THC= full-spectrum cannabis (with high THC and low CBD contents) treatment. **A**. The ratio of LC3II/LC3I in spectrum cannabis high CBD and low THC contents treated cells. **B.** The ratio of LC3II/LC3I in full-spectrum cannabis (high THC and low CBD contents). **C.** The ratio of p62/ B actin in spectrum cannabis (high CBD and low THC contents) treated cells. **D**. The ratio of p62/ B actin in full-spectrum cannabis (high THC and low CBD contents). **E.** The ratio of Lamp-1/B actin in spectrum cannabis (high CBD and low THC contents) treated cells. **F.** The ratio of Lamp-1/B actin in full-spectrum cannabis (high THC and low CBD contents). **G.** Comparing the effect of two full-spectrum cannabis (with varying THC and CBD contents) on the LC3II/LC3I ratio. **H.** Comparing the effect of two full-spectrum cannabis (with varying THC and CBD contents) on the ratio of p62/ B actin. **I.** Comparing the effect of two full-spectrum cannabis (with varying THC and CBD contents) on the Lamp-1/B actin ratio. n=3; *p<0.05, **p<0.01, ***p<0.001 compared to control, #p<0.05 compared to LPS, ns= non-significant.

CHAPTER 5: DISCUSSION

ALD and NAFLD are considered as a common cause of liver diseases. Until today, there is no efficient treatment that used for ALD and NAFLD. A number of studies have reported that using cannabis has beneficial effect on ALD and NAFLD outcomes; however, other studies observed that cannabis has no beneficial effect or might worsen the disease in individuals that have ALD and NAFLD. In this study, we examined the effect of two full-spectrum cannabis formulations (with varying THC and CBD contents) on the progression of ALD and NAFLD by using in vitro model experiment. Our study indicated that using two full-spectrum medical/recreational cannabis formulations (with varying THC and CBD contents) can differentially impact inflammation response and autophagy process in the ALD and NAFLD. We found that full-spectrum cannabis (with high CBD and low THC) significantly suppressed inflammation by inhibiting TNF-α production and NF-kB activation, while full-spectrum cannabis (with high THC and low CBD) induced the autophagy. We also investigated that the full-spectrum cannabis (with high CBD and low THC contents) treatment was significantly reduced TNF-α production after LPS stimulation. However, full-spectrum cannabis (with high THC and low CBD contents) treatment has no significant effect on TNF-α production after stimulation with LPS. Based on the effect of cannabis (with high CBD and low THC contents) which suppressed TNF-α production that induced by LPS stimulation in macrophages, we determined NF-KB one of the pathways that increased the production of cytokines especially TNF-α, to check the NF-KB pathway we detected (p65 nuclear translocation) by using immune fluorescent. Under normal condition, p65 protein presents in the cytoplasm; however, after LPS stimulation the p65 translates to the nucleus (Giridharan & Srinivasan, 2018). Therefore, we wanted to investigate whether the cannabis (with high CBD and low THC contents) treatment inhibit translate of p65 from cytoplasm to nucleus. We observed that cannabis (with high CBD and low THC contents) treatment potentially inhibits p65 translation from cytoplasm to nucleus. Therefore, the differentiation of cannabis outcomes between individuals in ALD and NAFLD may be due to the difference in the cannabis contents.

Excessive alcohol and high-calorie diet consumptions in patients with ALD and NAFLD can activate the hepatic macrophage due to induce leaky gut microbiome translocation (Hills *et al.*, 2019). These gut endotoxins stimulate hepatic innate immune cells KCs to produce cytokines like TNF-α. These cytokines cause inflammation and lead to liver injure. We revealed that full-spectrum cannabis (with high CBD and low THC) treatment inhibited TNF-α cytokine in macrophages. Various studies have shown the effect of CBD on immune response including innate and adaptive responses, CBD inhibits many kinds of immune cells like T cells, neutrophils

and macrophages. It also suppresses pro-inflammatory cytokines secretion like TNF-α, IFN-γ, and IL-1α (Nichols & Kaplan, 2020; Rajan et al., 2016). Moreover, early study had been illustrated that CBD significantly inhibited the severity of liver inflammation and oxidative stress. Also, it attenuated the LPS-triggered NF-kB activation and TNF-α production in isolated KCs (Mukhopadhyay et al., 2011). On the other hand, we revealed that the full-spectrum cannabis (with high THC and low CBD contents) treatment did not inhibit TNF-α production by macrophages after LPS stimulation. Pervious study has shown that THC treatment reduced anti-inflammatory cytokine IL-10 and induced the proinflammatory cytokine IL-8 (Nagarkatti et al., 2009). In contrast, other researchers presented that THC reduced mRNA expression of IL-1α, IL-1β, IL-6, and TNF-α in isolated rat microglial cells after LPS stimulation (Puffenbarger et al., 2000).

The production of pro-inflammatory cytokines by activated hepatic macrophage is modulated by numerous of signaling mechanisms such as NF-κB pathway which is one of the mechanisms that regulate inflammation in the liver. Chronic alcohol-mediated liver injury through stimulation of TLR4 by circulating LPS on hepatic macrophages which results in culminating NF-κB activation and pro-inflammatory cytokine production (Wheeler et al., 2001). Some studies have shown that some active ingredients of cannabis like CBD can inhibit the modulation of many cellular signaling pathways which impacting inflammation including NF-κB pathway in activated macrophages and B cells (khaksar & bigdeli, 2017; rajesh et al., 2010). To determine the effect of full-spectrum cannabis formulations in modulating inflammation signaling, we assessed NF-κB activation by detecting p65 nuclear translocation using fluorescent microscopy. Our preliminary data suggest that full-spectrum cannabis with (high CBD and low THC contents) treatment can suppress alcohol-LPS induced TNF-α production by macrophages involving at least NF-κB signaling. Whereas, the full-spectrum cannabis with (high THC and low CBD contents) treatment does not decrease TNF-α production by macrophages after LPS. There are different methods that used to measure NF-κB activation such as electrophoretic mobility shift assay analysis of NF-κB DNA binding, transom DNA-binding ELISA, and F- kappa b activation assay kits using western blot to detect p65.

Cannabis can also affect the cellular homeostasis in the liver. The essential regulator of the liver homeostasis in metabolic and detoxifying is autophagy. In addition, autophagy is considered as a prim regulator of pro-inflammatory signaling (deretic et al., 2013). Previous study demonstrated that chronic alcohol exposure can reduce the autophagy process (gual et al., 2017). In contrast, other study reported that cannabinoids such as (CBD and THC) can induce autophagy mechanisms in different types of cells (costa et al., 2016). Previous research reports that CBD

can inhibit ethanol toxicity and prevent alcohol-induced steatosis by activation autophagy process (de ternay et al., 2019; yang et al., 2014). In our study, we found that cellular stress during ALD and NAFLD can induce a functional block in the autophagic flux that can prevent autophagosome degradation. Moreover, we revealed that two full-spectrum cannabis (with varying CBD and THC contents) treatments induce the autophagy process. We found that the expression of some basic molecules of the autophagy pathway was changed by using two spectrum formulations of cannabis. The protein levels of LC3II and p62 which are involved in phagophore and autophagosome formation were significantly increased by cannabis (with high CBD and low THC contents) treatment in both inactivated and activated macrophages. Whereas we observed insignificant increase in the protein levels of LC3II and p62 in inactivated macrophages after full-spectrum cannabis (with high THC and low CBD contents) treatment. However, the levels of these proteins were significantly decreased in activated macrophages after full-spectrum cannabis (with high THC and low CBD contents) treatment that means less of autophagosome accumulation and less of the function block.

A high level of LC3II and p62 that we showed in our experiments were associated with cannabis (with high CBD and low THC contents) treatment in both inactivated and activated macrophages. While LC3II and p62 expression were significant increased in inactivated macrophages but decreased in activated macrophages after full-spectrum cannabis (with high THC and low CBD contents) treatment. A Pervious study provides that CBD and THC induce autophagy in breast cancer by increasing autophagy marker LC3II and inhibiting beclin-1 (Costa et al., 2016). Also, THC induced autophagy in melanoma cells (Armstrong et al., 2015). Moreover, another evidence proposes that the CB2 receptor activation induces the autophagy pathway in macrophages leading to inhibit hepatic inflammation that caused by alcohol-induced steatosis (Denaës et al., 2016). in addition, another study has been shown that the CBD enhanced autophagy in both in vitro and in vivo experiments which played an important role to protect the liver from acute alcohol-induced steatosis (Yang et al., 2014). Strikingly, cannabinoids induce autophagy through the CB1 receptor in some cell-culture (Koay *et al.*, 2014; Salazar *et al.*, 2009), whereas in other cells they reduce autophagy (Hiebel et al., 2014). For example, THC reduces autophagy especially in the mouse striatum and this process contributes in the THC-induced impairment of motor coordination (Blázquez et al., 2020).

We identified that the autophagic degradation is impaired when we treated the cells with full spectrum of cannabis (with high CBD and low THC contents) by reducing (LAMP-1) in activated macrophages, while the full-spectrum cannabis (with high THC and low CBD contents) treatment

enhances autophagy degradation by increasing LAMP-1 level and inhabiting the accumulation of autophagosomes. Previous study has been shown that THC enhanced cytotoxic autophagy as an efficient strategy to drive melanoma cell death (Armstrong et al., 2015). Also, CBD induced autophagy in human glioblastoma multiforme (GBM) (Ivanov et al., 2020). We identified that level of LAMP-1 increased by the full spectrum cannabis (with high THC and low CBD contents) treatment on both inactivated and activated macrophages which means more degradation of autophagosomes. On the contrary, full-spectrum cannabis (with high CBD and low THC contents) decreased the level of LAMP-1 in both inactivated and activated macrophages which means more accumulation of autophagosome and more functional block.

Comparing two full-spectrum formulations of cannabis, we found that full-spectrum cannabis (with high THC and low CBD) treatment induced more autophagy than full-spectrum cannabis (with high CBD and low THC) treatment as indicated to p62 and LAMP-1 expression. Full-spectrum cannabis (with high CBD and low THC) treatment increases autophagosome accumulation within the cell compared to full-spectrum cannabis (with high THC and low CBD) treatment which seems to be more function block with full-spectrum cannabis (with high CBD and low THC) treatment as indicated to L3II/LC3I ratio. On the other hand, full-spectrum cannabis (with high THC and low CBD) treatment had more fusion and more degradation of autophagosome as indicted to LAMP-1and L3II/LC3I ratio.

Using the RAW264.7 cells, we revealed that two full-spectrum formulations of cannabis with varying contents of (THC and CBD) impacted the inflammation stage and autophagy process in ALD and NAFLD differently. Pervious study demonstrated that CBD might be very promising therapy for alcoholic liver diseases that related to inflammation, oxidative stress, and steatosis (Wang et al., 2017).

Further research is needed to investigate the effect of full-spectrum cannabis on the progression of liver diseases by using animal models. In addition, more studies are needed to investigate the cannabis receptors such as CB1, CB2 receptors, that might be associated with the development of liver diseases. These future studies should take into account cannabis pharmacology related to (THC), (CBD), routes of administration (oral, vaporization, smoking...etc.), and dosing recommendations. Adverse effects of cannabis medicine should be considered. For instance, THC has potential neurologic adverse effect. The total daily dose-equivalent of THC must be limited to 30 mg/day or less (MacCallum & Russo, 2018). In contrast, CBD is less potent; therefore, it may require higher doses for its adjunctive therapy on pain, inflammation, and

alleviation of THC-linked anxiety, and tachycardia. Preferably combine THC with CBD to avoid psychoactive sequelae and development of tolerance (MacCallum & Russo, 2018).

CHAPTER 6: CONCLUSION

In conclusion, our study revealed that full-spectrum cannabis formulations with varying contents of THC and CBD can differentially impact key cellular processes that regulate metabolic liver diseases. High CBD: low THC cannabis suppressed inflammation while high THC: low CBD enhanced more complete autophagy. Taken together, this might account for observed clinical differences in patient outcomes amongst individuals with ALD and NAFLD who additionally use cannabis.

CHAPTER 7: PERSPECTIVE OR FUTURE WORK

7.1. Counting the exosomes released for the RAW264.7cells that followed treated by Cannabis formulations using NanoSight.

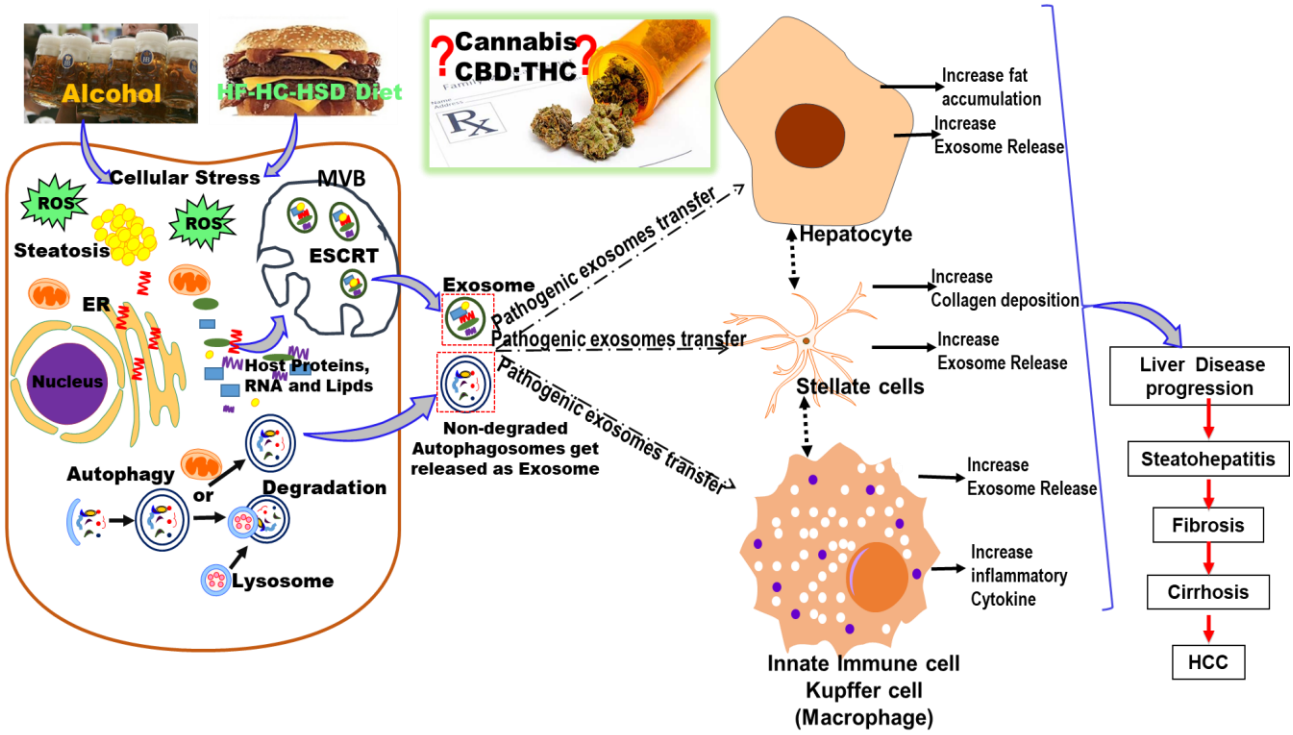

Figure7.1.1: The impact of cannabis on the release of autophagosomes in ALD and NAFLD. Once there is cellular stress either excessive alcohol or diet. There is an autophagy functional block that might lead to the release of autophagosomes. Non-degraded autophagosome get as exosomes. These exosomes content a pathogenic particle molecular That transfer to liver cells such as hepatocytes, stellate and immune cells like KCs. That leads to liver disease progression (steatohepatitis, fibrosis, cirrhosis, and Hepatocellular carcinoma (HCC)). ©Terence Bukong.

7.2. Detect how cannabis (with an equal amount of CBD and THC) impact inflammation and dysregulation of autophagy on ALD and NAFLD using in-vitro cell systems.

We will do the same experiments that we performed in this study, however, we will use full-spectrum cannabis (with an equal amount of CBD and THC). We will investigate the effect of full-spectrum cannabis (with an equal amount of CBD and THC) on the cytokines production, and how it impacts signaling pathways that drive inflammation through NF-kB. In addition, we will

detect how full-spectrum cannabis formulation (with an equal amount of CBD and THC) modulates dysregulated autophagy in ALD and NAFLD. The experiments will be performed by using Enzyme-linked immunosorbent assay (ELISA), Fluorescent microscopy and Western blot analysis.

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