An *in vitro* investigation on *Dillenia indica* L. for its beneficial effects against pathological lipogenesis and adipogenesis

By

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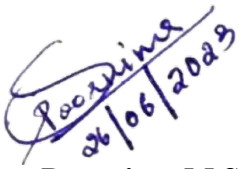

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Poornima M S

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ABBREVIATIONS

ABBTS	:	2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)
ACC	:	Acetyl CoA carboxylase
AceCS1	:	Cytoplasmic acetyl CoA synthetase
ACLY	:	ATP-citrate lyase
ACOX-1	:	Peroxisomal acyl-coenzyme A oxidase-1
Adipo R1	:	Adiponectin receptor 1
ADP	:	Adenosine diphosphate
ALT	:	Alanine transaminase
AMBRA1	:	Activating molecule in Beclin 1-regulated autophagy
AMP	:	Adenosine monophosphate
AMPK	:	AMP-activated protein kinase
ASK1	:	Apoptosis signal regulating kinase 1
AST	:	Aspartate transaminase
AT	:	Adipose tissue
Atg	:	Autophagy related genes
ATG16L1	:	Autophagy related-16 like 1
ATG7	:	Autophagy related-7
ATGL	:	Adipose triglyceride lipase
ATP	:	Adenosine triphosphate
BSA	:	Bovine serum albumin
CD36	:	Cluster of differentiation 36
CEBP-α	:	CCAAT/enhancer binding protein alpha
CPT-1	:	Carnitine Palmitoyl transferase 1
CVC	:	Cenicriviroc
CVDs	:	Cardiovascular disease
DAMPs	:	Damage associated molecular patterns
DCFH-DA	:	2,7-dichlorodihydrilfluorescein diacetate
DI-EA	:	Dillenia indica ethyl acetate extract
DI-HET	:	Dillenia indica hydroethanolic extract (70%)
DI-HX	:	Dillenia indica hexane extract
DMEM	:	Dulbecco's modified eagle medium

DMSO	:	Dimethyl sulphoxide
DNL	:	De novo lipogenesis
DPPH	:	2,2-diphenyl-1-picryl-hydrazyl-hydrate
EDTA	:	Ethylenediamine tetra acetic acid
ER	:	Endoplasmic reticulum
FABP4	:	Fatty acid binding protein-4
FAS	:	Fatty acid synthase
FBS	:	Fetal bovine serum
FDA	:	Food and Drug Administration
FFAs	:	Free fatty acids
FGF19	:	Fibroblast growth factor 19
FGF21	:	Fibroblast growth factor 21
FXR	:	Farnesoid X receptor
GLP-1	:	Glucagon like peptide 1
HCC	:	Hepato cellular carcinoma
HDL	:	High density lipoprotein
HMGCR	:	Hydroxymethylglutaryl-coenzyme A reductase inhibitor
HPLC	:	High performance liquid chromatography
IBMX	:	3-isobutyl-1-methylxanthine
IL-10	:	Interleukin-10
IL-12	:	Interleukin-12
IL-1β	:	Interleukin-1β
IL-6	:	Interleukin-6
IR	:	Insulin Resistance
JNK	:	Phosphorylated JNK
LC3	:	Light chain 3
LCMS	:	Liquid chromatography-mass spectrometry
LKB-1	:	Liver Kinase B-1
LXR-α	:	Liver X receptor alpha
MAPK	:	Mitogen activated protein kinase
MEME	:	Minimum essential medium eagle
MetS	:	Metabolic syndrome
MTT	:	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide]

NAFL	:	Non-alcoholic fatty liver
NAFLD	:	Non-alcoholic fatty liver disease
NASH	:	Non-alcoholic steatohepatitis
NF-κB	:	Nuclear factor kappa B
NLRP-3	:	NLR family pyrin domain containing 3
norUDCA	:	Norursodeoxycholic acid
OA	:	Oleic acid
OCA	:	Obeticholic acid
ORO	:	Oil Red O stain
p-ACC	:	Phosphorylated ACC
p-ACLY	:	Phosphorylated ACLY
p-AMPK	:	Phosphorylated AMPK
PBS	:	Phosphate buffered saline
PGC-1α	:	Peroxisome proliferator-activated receptor-gamma coactivator-1α
p-JNK	:	Jun-N-terminal kinase
p-LKB-1	:	Phosphorylated LKB-1
PPAR-α	:	Peroxisome proliferator-activated receptor-alpha
PPAR-β/δ	:	Peroxisome proliferator-activated receptor-beta/delta
PPAR-γ	:	Peroxisome proliferator-activated receptor-gamma
PVDF	:	Polyvinylidene di fluoride
RIPA	:	Radio immunoprecipitation assay
RNS	:	Reactive nitrogen species
ROS	:	Reactive oxygen species
SCD-1	:	Stearoyl CoA desaturase 1
SDS	:	Sodium dodecyl sulphate
SGLT2	:	Sodium-glucose co-transporter 1
SIRT-1	:	Sirtuin 1
SOD	:	Superoxide dismutase
SREBP-1c	:	Sterol regulatory-element-binding protein-1c
T2D	:	Type 2 diabetes mellitus
TCA cycle	:	Tricarboxylic acid cycle
TG	:	Triacylglycerides
TLR4	:	Toll like receptor 4

TNF-α	:	Tumour necrosis factor alpha
Tris HCL	:	Tris hydroxymethyl aminomethane hydrochloride
TRP	:	Total reducing power
WAT	:	White adipose tissue
WHO	:	World Health Organization

Chapter 1

Introduction

1.1 Pathological lipogenesis

Lipids are vital metabolites that serve as fuel for energy, act as a structural component of the cell membrane, and also aids in cell signaling (Jeon et al., 2023). Lipids are produced through *de novo* lipogenesis (DNL) and obtained through dietary sources. Pathological lipogenesis occurred due to the imbalance in lipid metabolism. Excessive and inadequate lipogenesis aids in aberrant lipid homeostasis. Scientific studies report that pathological lipogenesis leads to the progression of metabolic diseases and associated comorbidities (Ameer et al., 2014). DNL metabolic pathway results in the production of triacylglycerides (TG) utilizing glucose and acetyl CoA as the substrate. Hence, the lipogenic pathway controls the transfer of carbons from glucose to fatty acids through a coordinated set of enzymatic processes. Major organs involved in the DNL pathway are the liver and adipose tissue. Hence, the liver and adipose tissue plays an important role in the regulation of the overall energy balance in the body (Wallace & Metallo, 2020).

More carbohydrate intake is associated with elevated levels of lipid accumulation. Because carbohydrates are converted to TG through DNL. In particular, intake of fructose has a strong impact on DNL (Ameer et al., 2014). This excess TG build-up in the liver and adipose tissue contributes to the release of free fatty acids in the circulation. That causes lipotoxicity in the body and further leads to insulin resistance (IR). IR is another major mechanism that contributes to pathological lipogenesis because the higher levels of insulin in circulation stimulate lipogenesis (Wolf et al., 2014). IR results in elevated levels of glucose in the blood. Hence, IR plays a crucial role in the progression of Type 2 diabetes mellitus (T2DM) (Wolf et al., 2014). Transcription factors also play a crucial role in pathological lipogenesis. Aberrant regulation of transcription factors like sterol regulatory binding protein-1C (SREBP-1C) and CAAT/enhancer binding protein-α (C/EBP-α) contribute to the activation of lipogenic protein and enhanced DNL (Strable & Ntambi, 2010).

Abnormal lipogenesis leads to an increased accumulation of lipids in adipose tissue. Which further contributes to the development of obesity. Excess lipid accumulation in adipose tissue results in adipose tissue hypertrophy (increase in cell size) and hyperplasia (increase in cell number) which further enhance the lipid storage capacity of the cell. Hence lipid storage in adipose tissue leads to IR and creates a pro-inflammatory environment (Exley et al., 2014).

The liver is the major organ involved in the production of glucose and lipids. Upregulated lipogenesis, impaired fatty acid oxidation along with the release of free fatty acid from adipose tissue promotes excess fatty acid uptake in the liver. Besides, during IR lipid accumulation will increase in the liver. That further contributes to the development of nonalcoholic fatty liver disease (NAFLD) and contributes to oxidative stress, inflammation, and fibrosis. Therefore, IR and the development of NAFLD are interrelated. Hence pathological lipogenesis affects whole-body energy homeostasis due to chronic low-grade inflammation, oxidative stress, IR, and increased level of FFA. All these trigger fat deposition in the heart and lead to atherosclerosis and cardiac complications. Therefore, dysregulation of DNL leads to the consequences like obesity, T2D, cardiovascular diseases, and NAFLD (Sanders & Griffin, 2016). Pathological lipogenesis and its associated diseases can be considered an attractive area of scientific research. Hence analyzing the molecular mechanism and identification of possible drug targets are essential for the management of disease due to dysregulated lipid metabolism.

1.2 Adipogenesis

Adipose tissue functions as the fat-storing tissue as well as the endocrine organ. Adipose tissue especially white adipose tissue (WAT) regulates lipid mobilization and storage in the body. By releasing numerous adipokines it acts as an endocrine organ to regulate systemic metabolism. Additionally, it acts as insulation and protects the body (Scheja & Heeren, 2019).

The maturation process of preadipocyte to mature adipocyte is called adipogenesis. Adipogenesis is a well-regulated process in which PPAR-γ functions as the major regulatory protein (Song et al., 2018). An important peculiarity of adipose

tissue is that it can adapt to the needs of the body through adipose tissue plasticity. That is, it shows cellular hypertrophy and hyperplasia (Luo & Liu, 2016).

Sedentary lifestyle and positive energy balance lead to lipid accumulation in adipose tissue through DNL. Hence excess lipid buildup contributes to IR in adipose tissue as well as the release of FFAs and proinflammatory cytokines. The release of proinflammatory cytokines attracts the infiltration of macrophages to the adipose tissue. It will further enhance inflammation in adipose tissue. Studies have reported that obese mice and humans have shown increased infiltration of macrophages to adipose tissue and associated elevated levels of inflammation (Sakers et al., 2022). Hence dysfunctional adipogenesis is associated with the development of metabolic syndrome, obesity, cardiovascular disease, and NAFLD (Hassan et al., 2012). Owing to the importance of adipogenesis in physiology of the living system, the importance of the same has been explained in this thesis in relation to NAFLD.

1.3 Role of lipogenesis in the genesis of NAFLD

The occurrence of NAFLD has increased globally with an increase in diabetes and metabolic syndrome. Fat accumulation in the liver without other recognized liver diseases or without heavy alcohol use is known as NAFLD (Bellentani & Marino, 2009). It is a spectrum of liver abnormalities that ranges from nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH) (Friedman et al., 2018). NAFL is a non-progressive simple steatosis, while NASH is a progressive form that leads to cirrhosis and hepatocellular carcinoma (HCC). The most significant risk factor for NAFLD and NASH in an individual is the warning of the occurrence of metabolic syndrome (MetS). In fact, because of the common risk factors such as diabetes mellitus, hypertension, and obesity, patients with NAFLD are twice as likely to die from cardiovascular disease as from liver disease (Lindenmeyer & McCullough, 2018). NAFLD significantly contributes to liver-related morbidity and death worldwide (Whalley et al., 2007).

Now NAFLD is considered as the hepatic manifestation of MetS. That means ectopic fat buildup in the liver, such as that seen in NAFLD, is typically accompanied by an increase in the release of hepatokines, inflammation, hepatocyte damage, and hepatocyte death (Arrese et al., 2016). Aberrant lipid and glucose metabolism might

lead to a buildup of hepatic fat due to the dysregulation of hepatic transcription factors and nuclear receptors. NAFLD affects extrahepatic organs, regulatory pathways, and many physiological systems (Armstrong et al., 2014). Recent research shows a connection between NAFLD and other chronic conditions like polycystic ovary syndrome, osteoporosis, sleep apnea, psoriasis, and colorectal cancer (Musso et al., 2013). Hence NAFLD is a complex disease condition involving several cell signaling pathways and contributes to the progression of other comorbidities.

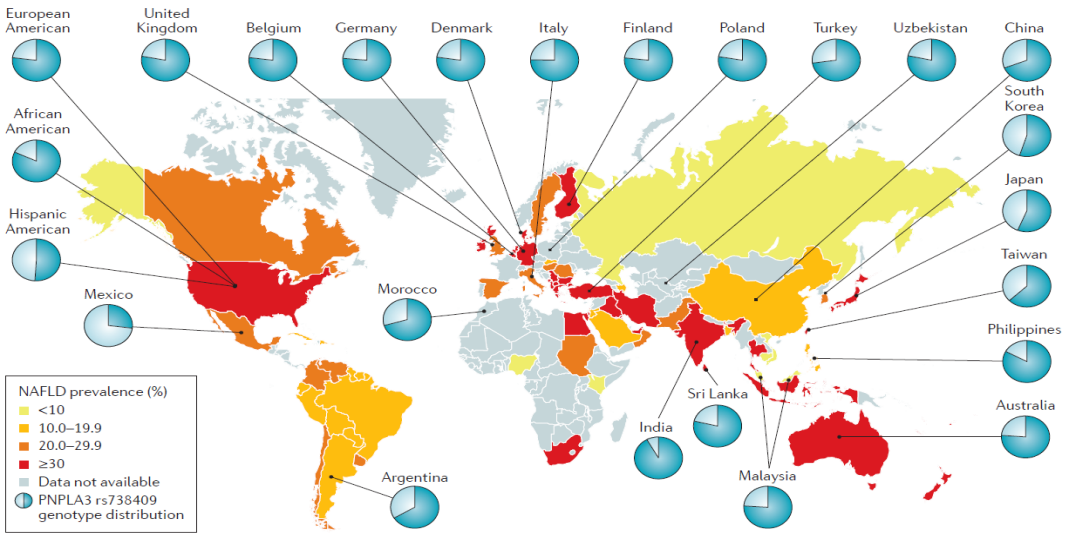
Treatment and medications for NAFLD management are still under investigation. This treatment ambiguity is partly a result of our limited knowledge of the underlying mechanisms that promote the onset and progression of NAFLD. Despite consistent advancements in understanding the aetiology of NAFLD, discovering therapeutic targets, and expanding drug development, there are still many unanswered questions, and there is no FDA-approved treatment for NAFLD for absolute recovery. Since surplus lipogenesis is critical in the genesis of NAFLD, this thesis mainly deals with various molecular pathways associated lipid metabolism in HepG2 cells and modulation with medicinal plant extract during surplus lipogenesis.

1.4 Prevalence of NAFLD

Over the past century, dramatic lifestyle changes have profoundly impacted the world's population, as seen by the emerging chronic liver disease like NAFLD. Although there are regional variations in the epidemiology and demographics of NAFLD, generally parallel to the incidence of obesity, a significant number of patients are lean. NAFLD prevalence is currently estimated to be 24% worldwide. All continents have high rates of NAFLD; however, the highest rates have been seen in South America (31%) and the Middle East (32%), followed by Asia (27%), the USA (24%), and Europe (23%), and less frequently in Africa (14%) (Lonardo et al., 2016) (Figure.1.1). Notably, liver transplants are anticipated to be performed the most frequently by 2030 due to NAFLD (Byrne & Targher, 2015). Because being overweight during childhood and adolescence increases the likelihood of developing NAFLD later in life, liver-related morbidity or mortality thresholds are attained earlier. Due to a lack of accessible, non-invasive diagnostic procedures, it is difficult to determine the prevalence of NAFLD in adults and children in the general population. A liver biopsy is the "gold standard" for assessing the severity of NAFLD. However, using it on

healthy populations is neither practical nor ethical. Nevertheless, the prevalence rate of NAFLD in children in the general population is probably between 5% and 10% (Yu & Schwimmer, 2021). Race and ethnicity also affect the prevalence of NAFLD in children. Compared to white children (8.6%), children of Hispanic ethnicity (11.8%) and Asian race (10.2%) have a greater frequency of NAFLD (Smith & Perito, 2018).

India is the seventh-largest and most populated nation in the world. NAFLD has become a significant public health issue in India and other areas of the world due to the easy availability of calorie-dense foods, sedentary lifestyles, and the current epidemics of diabetes mellitus (DM) and obesity. It could substantially influence the country's healthcare resources due to increased liver transplants associated with hepatocellular carcinoma (HCC) in India. Recent research studies have shown that in India, the prevalence of NAFLD varies between 9% and 53% among the general population (Duseja et al., 2015, 2019). All these scientific data reveal the considerable burden of hepatic and extrahepatic illness by NAFLD, which has become a significant public health concern in India. Hence, teaching children and teenagers about healthy lives in organizations like schools and institutions may be the most urgent concern. Additionally, initiatives are needed to alter the public's and physician's perceptions of this ongoing silent pandemic.



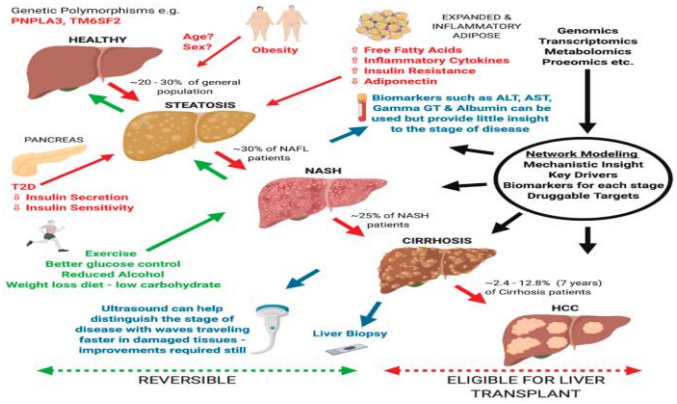
Younossi et al., Nature Reviews Gastroenterology & Hepatology, 2018.

Figure.1.1. Worldwide prevalence of NAFLD

1.5 Pathogenesis of NAFLD

The fundamental process behind the onset and progression of NAFLD is complex and multifaceted. Several theories were proposed to explain NAFLD development. The “first hit” is hyperinsulinemia and insulin resistance, preceding obesity, which causes hepatic steatosis and increases the absorption of non-esterified fatty acids in the liver and esterification to produce triacylglycerol. The “second hit” contributes to the development of nonalcoholic steatohepatitis through oxidative stress. Inflammatory cascades and fibrogenesis are triggered by the “second hit” (Marzuillo et al., 2015). However, it soon became clear that this perspective does not adequately reflect the complexity of human NAFLD, which is characterized by multiple simultaneous variables that interact to drive the development and progression of the disease in genetically predisposed individuals. As a result, the obsolete two-hit explanation for the progression of NAFLD has been replaced with a “multiple-hit” hypothesis. The “multiple-hit” hypothesis states that genetic factors, along with changes in dietary habits and environment, lead to insulin resistance, obesity with adipocyte proliferation, and changes in gut microbiota can all contribute to the development of NASH (Buzzetti et al., 2016).

The first stage in the pathophysiology of NAFLD is TG buildup, which is brought on by an imbalance between TG production and breakdown. Factors like nutrition, hormones, microbiota, IR, FFA, genes, and inflammation play a major role in the pathogenesis of NAFLD. Dietary lipids, fatty acids, carbohydrates, and lipids produced by DNL have all been regarded as crucial components of the synthesis of TG in human livers (Chiu et al., 2018). For TG synthesis, the liver takes free fatty acids (FFAs) from plasma. Large amounts of fat decompose during IR in obese patients with metabolic syndrome, increasing the plasma concentrations of FFAs from adipose tissue (Delarue & Magnan, 2007). This causes more intrahepatic lipids to accumulate (steatosis) and more triglycerides to be secreted as very low-density lipoprotein. Through various processes, lipotoxicity further compromises insulin signaling, results in oxidative damage, and stimulates inflammation and fibrosis (Gao & Tsukamoto, 2016). The development of cirrhosis and HCC in NAFLD patients and the transition from NAFL to NASH are thought to be caused by these subsequent consequences (Carr et al., 2016) (Figure. 1.2).



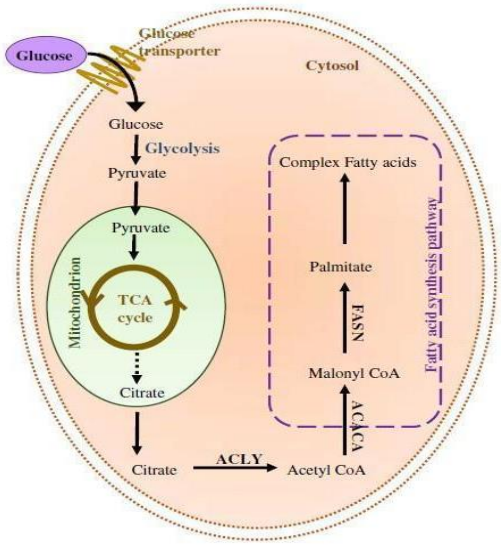
Blencowe et al., Genes, 2019.

Figure.1.2. An overview of nonalcoholic fatty liver disease (NAFLD) progression from a healthy liver to hepatocellular cancer (HCC).

1.6 *De novo* lipogenesis and NAFLD

De novo lipogenesis (DNL) is a tightly regulated metabolic process in which excess carbohydrate is converted to fatty acids (FAs). Based on the metabolic state of the body, FAs are either used for the production of TG and stored or rapidly metabolized for energy production via β-oxidation of FAs. In humans, DNL is primarily active in the liver and adipose tissue. By glycolysis, glucose is converted to pyruvate; then, pyruvate is converted to acetyl CoA, which enters into the tricarboxylic acid (TCA) cycle. During the TCA cycle, citrate is formed in mitochondria that leave the mitochondria and enter the cell cytoplasm. The enzyme ATP-citrate lyase (ACLY) converts cytoplasmic citrate to acetyl CoA. Thereafter, acetyl-CoA is converted to malonyl-CoA by the enzymatic action of acetyl-CoA carboxylase (ACC). The primary biosynthetic enzyme, fatty acid synthase (FAS), utilizes malonyl-CoA as a substrate to create 16-carbon saturated palmitate. FAS, which has been classified as a liver housekeeping protein, is thought to be a marker for lipogenesis. Palmitate is subsequently transformed into complex fatty acids through a variety of processes. The primary end product of DNL is palmitate. However, it can also produce stearate and shorter fatty acids (Ameer et al., 2014; Figure. 1.3). In some clinical or physiological situations, the DNL is dysregulated. Hence normal lipid balance in the body may be disturbed by the DNL abnormalities in the main lipogenic tissues like the liver and adipose tissue. Studies have reported that increased hepatic expression of numerous

genes involved in DNL has been linked in persons with NAFLD (Kohjima et al., 2008; Mitsuyoshi et al., 2009).



Ameer et al., Metabolism, 2014.

Figure.1.3. *De novo* lipogenesis

1.7 Drug discovery and development scenario of NAFLD treatment

NAFLD drug development is an active research area in which finding medications and targets to treat this multifaceted disease condition. However, finding medications for NAFLD is getting more difficult because of the complexity of the disease. Drug development for NAFLD mainly focuses on addressing certain specific aspects like prevention of fat build-up, inflammation, fibrosis, and correcting metabolic dysfunction. Hence the major drug targets for NAFLD are enzymes and receptors involved in lipid metabolism.

Targeting hepatic inflammation and fibrosis is another crucial factor in NAFLD treatment. The progression of NAFLD is associated with inflammation and fibrosis. IR also has a huge impact on NAFLD pathogenesis. Hence drug-target reducing IR is also helpful in alleviating the disease progression. Hence the ongoing research in preclinical and clinical studies is focused on target-based studies. Therefore, there is hope for effective drug development in the coming future.

Various drug classes are currently being explored for the treatment of NAFLD. These include drugs that act as agonists of nuclear receptors, such as FXR agonists (including FGF19), PPAR agonists, and thyroid hormone receptor-β agonists. In addition to that, there are inhibitors of chemokine receptors, SGLT2 inhibitors, SCD-1 inhibitors, FAS inhibitors, and analogues of enterohepatic hormones (GLP-1 and FGF21), which are also under investigation as potential treatment options for NASH (Rau & Geier, 2021). The following are some of the major drugs that were being investigated for NAFLD

Obeticholic acid (OCA) is a synthetic bile acid derivative that functions as a farnesoid X receptor (FXR) agonist. FXR is a nuclear receptor that regulates bile acid and lipid metabolism in the liver (Venetsanaki et al., 2019). Clinical studies have shown that OCA is effective in NAFLD patients. It exerts its effect in lowering inflammation, lipid accumulation, and liver fibrosis (Mudaliar et al., 2013; Polyzos et al., 2020). Hence OCA is considered a viable treatment approach. However, more studies are required to test its safety and efficacy.

In addition to OCA some other FXR agonist like tropifexor and cilofexor have shown antisteatotic properties. But they showed adverse effects like pruritus and changes in serum cholesterol levels (Lucas et al., 2020; Patel et al., 2020). To reduce the adverse effects there are several research attempts are going on to evaluate its potential therapeutic benefits and dosing.

Cenicriviroc (CVC) is a dual antagonist of chemokine receptors CCR2 and CCR5. These receptors aid in the migration of inflammatory cells to the liver (Marra & Tacke, 2014). Inhibiting these receptors during NAFLD progression will be helpful for the reduction of inflammation and associated fibrosis in NAFLD patients. Studies are demonstrated the promising therapeutic effect of CVC. However further research is needed to know the long-term safety and effectiveness (Anstee et al., 2020).

Elafibranor is a PPAR-α agonist. PPAR-α is a nuclear receptor. Activation of PPAR- α in the liver leads to lipolysis, reduced inflammation, and fibrosis. Clinical studies have reported its therapeutic benefits. Although more studies are required to confirm its long-term safety and efficacy (Westerouen Van Meeteren et al., 2020).

Selonsertib is another drug candidate that selectively inhibits apoptosis signal-regulating kinase 1 (ASK1). Inhibition of ASK-1 helps in the reduction of inflammation and oxidative stress. Studies with selonsertib treatment demonstrated the therapeutic effects in NAFLD patients (Rinella & Noureddin, 2020). Nevertheless, more research studies are needed to confirm its effectiveness and safety profile.

Aramchol is a stearoyl CoA desaturase-1 (SCD-1) inhibitor. SCD-1 is a key enzyme in the production of monounsaturated fatty acids is SCD-1. So, inhibiting SCD-1 helps in reducing lipogenesis and inflammation associated with over-lipid accumulation in cells (Ratziu et al., 2018). TVB-2640 is a fatty acid synthase (FAS) inhibitor. FAS is a major enzyme that is involved in the formation of fatty acids. Excess fatty acid formation leads to excess TG synthesis. Clinical studies have shown that the administration of TVB-2640 for 12 weeks had shown a significant reduction in lipid accumulation in the liver and also reduces inflammation. However, more studies are required to know the long-term safety and effectiveness (Loomba et al., 2021).

A few other therapeutic drug candidates for NAFLD are Ileal Bile Acid Transporter (IBAT) inhibitors, Nor-Ursodeoxycholic acid (norUDCA), pegylated FGF21, GLP-1 analogues (liraglutide, semaglutide), thyroid hormone receptor-β agonists (resmetirom and VK2809). These candidates target various pathways involved in NAFLD and show promising effects in improving liver health (Rau & Geier, 2021).

Hence the development of effective treatment for NAFLD requires targeting multiple cell signaling pathways and different cellular targets. The possible drug candidates give hope for improved NAFLD management. Therefore, more scientific research is needed in this field to understand NAFLD pathophysiology, new target identification, and possible drug development based on long-term safety and efficacy profile.

1.8 Targets for NAFLD treatment

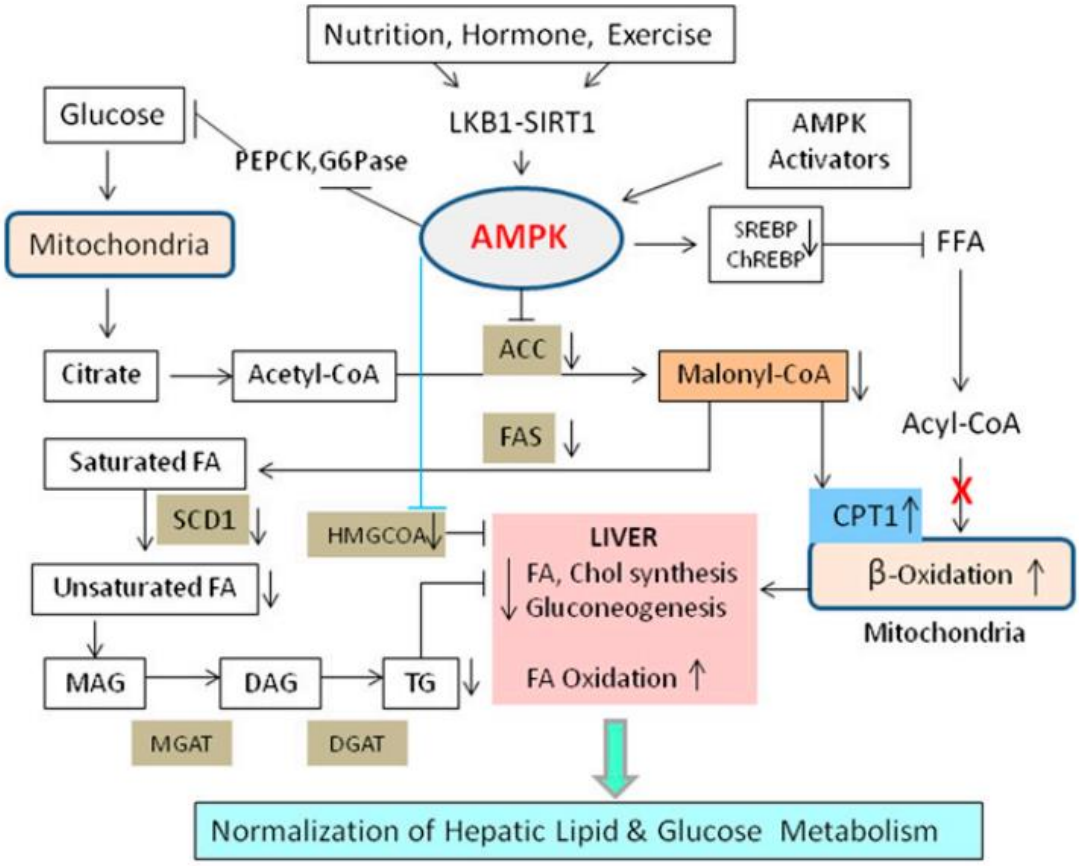
1.8.1 AMPK signaling pathway

AMPK is a heterotrimeric serine/threonine protein kinase with α, β, and γ subunits. AMP-activated protein kinase (AMPK) functions as an energy sensor of the cell. Because AMPK responds to cellular AMP/ATP and ADP/ATP ratio changes, both ratios rose during an energy shortage. All eukaryotic cells contain AMPK. Activation of AMPK occurs by the phosphorylation of Thr172 at the α-subunit of AMPK by upstream kinase. AMPK can also be activated by the allosteric binding of AMP to the γ-subunit. AMPK can detect minor changes in AMP concentration in the cell (Hardie et al., 2016). To maintain energy balance, AMPK is crucial in cellular metabolism (Carling et al., 2012).

When AMPK is active, it promotes catabolic pathways that produce ATP and inhibits anabolic pathways that deplete ATP to restore energy homeostasis. Hence AMPK activation inhibits cellular anabolic pathways like lipogenesis. As mentioned earlier, upstream kinase can regulate AMPK. For example, Liver kinase B1 (LKB-1) is a primary upstream kinase of AMPK, a necessary element in cell metabolism that is required for maintaining energy homeostasis. LKB-1 regulates lipid metabolism in its uptake, *de novo* synthesis, and fatty acid β-oxidation. SIRT-1 is another enzyme regulating LKB-1(Kwon et al., 2018). The role of SREBP-1C in the regulation of lipogenesis in the liver has been well-established by several lines of evidence (Hao et al., 2016). It is known that activated AMPK also inhibits SREBP-1C. Activated AMPK, in turn, will downregulate the expression of acetyl CoA carboxylase (ACC) and FAS. Hence fatty acid synthesis is reduced, thereby lipid accumulation also reduced (Jayachandran et al., 2020). Hence AMPK plays a major role in lipid metabolism.

This particular impact on lipid metabolism perfectly describes AMPK's pivotal role in maintaining homeostasis in cellular metabolism. Hence, AMPK was identified as a possible target protein for NAFLD treatment. Metformin is a common medication for treating T2D, which works in part by activating AMPK in the liver and skeletal muscle. Most experts believe that increasing AMPK activation is a practical treatment approach (von Loeffelholz et al., 2021). Scientific studies have shown that

AMPK activation and associated lipid catabolism were impaired in NAFLD patients (Kohjima et al., 2008; Figure. 1.4).



Srivastava et al., Journal of lipid research, 2012.

Figure1.4. The AMPK signaling system regulates lipid and glucose metabolism in the liver.

1.8.2 SIRT-1 and NAFLD

Sirtuins are silent information regulator 2 (Sir2) family members and class III histone/protein deacetylases. Mammals have seven distinct sirtuins (SIRT1–7), with SIRT-1 being the most widely studied. SIRT-1 controls protein activation by deacetylating a range of proteins that are crucial in the pathophysiology of metabolic disorders (Colak et al., 2011). SIRT-1 has recently been demonstrated to play important roles in the regulation of lipid and glucose homeostasis, control of insulin secretion and sensitivity, anti-inflammatory effects, control of oxidative stress, and improvements in

endothelial function brought on by increased mitochondrial biogenesis and β-oxidation capacity (Hou et al., 2008). Now NAFLD is considered as the hepatic manifestation of metabolic syndrome. The liver, which serves as the body's primary metabolic organ, responds to dietary and hormonal cues to control many important lipid metabolism processes, including β-oxidation of fatty acid, lipogenesis, and lipoprotein absorption and secretion (Van den Berghe, 1991). Hepatic steatosis is caused when nutrient intake exceeds β-oxidation in hepatocytes, dysregulation in lipoprotein production, and excretion abnormalities. The liver's transcriptional network gradually fuels an inflammatory process that eventually results in NASH, liver cirrhosis, hepatocellular cancer, and liver disease-related death (McCullough, 2004).

SIRT-1 has been demonstrated to be crucial to the dynamics of NAFLD pathogenesis in a number of recent investigations. The primary mechanism underlying the pathophysiology of NAFLD is insulin resistance (Lewis & Mohanty, 2010). SIRT-1 activation improves glucose-dependent insulin secretion, which normally declines with aging, and boosts ATP generation in β-cells. Research studies also demonstrated that SIRT-1 activation helps in the secretion of insulin from β-cells of the pancreas (Bordone et al., 2005).

It has been demonstrated that SIRT-1 activation in the liver results in a regulatory effect via the LKB/AMPK pathway (Hou et al., 2008). A number of tissues express the enzyme AMPK, which is essential for maintaining cellular energy balance (Winder & Hardie, 1999). The liver's acetyl-CoA carboxylase (ACC) is deactivated by it, which causes fatty acid oxidation. Increased fatty acid transport and oxidation result from ACC inactivation. A study demonstrated that hepatocyte-specific loss of SIRT-1 (via hepatocyte-specific deletion of SIRT-1) results in PPAR-α signal failure and a reduction in fatty acid β-oxidation (Purushotham et al., 2009).

The inflammatory process, which is regarded as the "second hit" following steatosis and leads to the development of NASH, is one of the critical steps in the pathophysiology of NAFLD. Recent research has shown that SIRT-1 activation has major anti-inflammatory effects. A growing body of research suggests that NF-κB activation has a role in the pathophysiology of NASH and the emergence of HCC

(Pikarsky et al., 2004). The RelA/p65 subunit is deacylated by SIRT-1 activation, which inhibits NF-κB signaling (Schug et al., 2010).

Many medicinal plants contain polyphenol resveratrol (trans-3,5,4'-trihydroxystilbene). It has been demonstrated that resveratrol is a powerful activator of SIRT-1. Resveratrol significantly increases SIRT-1 activity and encourages the cellular production of the SIRT1 protein in mammalian cells (Baur & Sinclair, 2006). Clinical studies also reported the beneficial effect of resveratrol in NAFLD patients (Berman et al., 2017). Consequently, SIRT-1 activation has been regarded as a possible therapeutic target to prevent the development and progression of NAFLD.

1.8.3 Acetyl CoA carboxylase (ACC) and NAFLD

The hepatic buildup of extra fatty acids in the form of triglycerides is a hallmark of NAFLD. There is always a balance between DNL in the liver and β-oxidation within the mitochondria. Hence, the steady-state level of triglycerides in the liver is typically low. Metabolic distress conditions like insulin resistance cause dyslipidemia and increased fatty acid uptake in the liver from blood plasma. IR and increased FA uptake will cause elevated DNL in the liver. DNL plays a significant role in the metabolism of fatty acids. The enzyme acetyl-CoA carboxylase (ACC), which is involved in DNL, converts acetyl-coenzyme A (acetyl-CoA) to malonyl-CoA. Malonyl-CoA generated by ACC is primarily employed as a substrate for the production of fatty acids. Malonyl-CoA, a by-product of ACC, inhibits carnitine palmitoyltransferase I (CPT-1), a transporter for FA absorption in mitochondria, which means that ACC also controls fatty acid oxidation. As a result, ACC has become a target in NAFLD treatment (Tong, 2005). The primary kinase that regulates the activity of the ACC is AMPK.

When cellular energy is low, AMPK is activated, which phosphorylates ACC and causes it to become inactive. By doing so, DNL is decreased, while fatty acid oxidation is increased (Imai & Cohen, 2018). Hence ACC has received interest as a possible therapeutic target in NAFLD due to the methods through which it regulates DNL and fatty acid oxidation. Therefore, recent clinical studies have demonstrated ACC inhibitors' effect on NASH patients (Calle et al., 2021). The use of ACC inhibitors in the treatment of hepatocellular cancer is a related potential use. In tumour progression, elevated FA synthesis occurs to deal with the increased metabolic requirements of cell

growth. It has been acknowledged that ACC may be used as a method in cancer chemotherapy to inhibit lipogenesis (Imai & Cohen, 2018).

1.8.4 Fatty acid synthase (FAS) and NAFLD

A crucial enzyme in the DNL pathway, fatty acid synthase (FAS), converts the malonyl-CoA produced by ACC into saturated long-chain fatty acids (Ferguson & Finck, 2021). NAFLD upregulates FAS expression and activity in the liver, and preclinical research in obese mice shows that inhibiting FAS improves insulin sensitivity while reducing triglyceride levels in the liver (Wu et al., 2011). In a study after ten days of treatment, a phase I trial found that the FAS inhibitor decreased DNL, hepatic lipid buildup, and blood alanine transaminase (ALT) levels in comparison to baseline values (Syed-Abdul et al., 2020). Hence elevated levels of FAS play a significant role in the pathophysiology of NAFLD.

1.8.5 SREBP-1C and NAFLD

There are three distinct isoforms of the family of transcriptional regulators known as sterol regulatory element-binding proteins (SREBPs) in humans. These are SREBP-1a, SREBP-1C, and SREBP-2 (Horton et al., 2002). SREBPs, membrane-bound transcription factors that control the expression of genes involved in lipid synthesis, positively control the expression of genes encoding lipogenic enzymes such as ACC and FAS (Kohjima et al., 2008). SREBP-1C mRNA and its functional nuclear protein form are elevated in ob/ob mice livers, supporting the notion that SREBP-1C overexpression can cause hepatic steatosis (Browning & Horton, 2004). As anticipated, enhanced lipogenesis caused by SREBP-1C overexpression in transgenic mice livers causes steatosis (Ferre & Foufelle, 2010). SREBP-1C thus plays a significant role in the aetiology of fatty liver. SREBP-1C is suppressed when AMPK is active, preventing the synthesis of fatty acids in the liver. Hence SREBP-1C is regulated by AMPK activation (Yap et al., 2011).

1.8.6 Role of SREBP-2 and HMGCR in NAFLD

Hepatic lipotoxicity is a practical conceptual framework for comprehending the aetiology of NASH. Cholesterol, free fatty acids, and their derivatives, as well as diacylglycerol and ceramides, are compounds that contribute to lipotoxicity. In a

minority of patients with NAFLD, lipotoxicity increases hepatic inflammation and fibrosis, leading to NASH and even progression to cirrhosis and HCC. Even though the underlying molecular mechanisms causing the development of inflammation and fibrosis that characterize progressive NASH remain unknown, recent research now suggests that hepatic free cholesterol is a significant lipotoxic substance crucial in the development of experimental and human NASH. Lipotoxic substances act on hepatocytes, liver Kupffer cells, stellate cells, and subcellular organelles like mitochondria and ER in the liver. That can further lead to insulin resistance, inflammation, apoptosis, cell death, and fibrosis. There is significant evidence of NAFLD-related disruption of hepatic cholesterol homeostasis, which results in elevated levels of hepatic cholesterol. Hence NAFLD has been linked to substantial dysregulation of cholesterol homeostasis, which results in high hepatic cholesterol levels by increasing both cholesterol production and uptake and decreasing cholesterol elimination.

The primary method of eliminating too much bodily cholesterol is the hepatic excretion of cholesterol in bile, either as bile acids or cholesterol. There are multiple mechanisms to maintain normal cholesterol levels to prevent hepatic cholesterol accumulation in the liver. Therefore, the primary routes in which hepatocytes take in cholesterol are 1) endogenous cholesterol synthesis (by the activity of HMGCR), 2) LDL and chylomicron remnants are taken up through LDL receptor-dependent endocytosis and further processing in the endosomal/lysosomal region, and 3) Scavenger receptor class B type I (SR-BI), directly absorbs HDL cholesterol.

The primary mechanisms that hepatocytes use to remove cholesterol are conversion to 1) bile acids (CYP7A1 and CYP27A1 are the rate-limiting enzymes) and excretion of bile acids by BSEP 2) excretion of cholesterol into bile by ABCG5/G8 3) insertion into VLDL and released into the bloodstream, and 4) cholesterol is effluxes onto circulating apolipoprotein AI and HDL.

Hence there must be a balance between cholesterol accumulation and cholesterol elimination from hepatocytes. The primary regulator of cholesterol homeostasis is known to be SREBP-2. SREBP-2 functions as an ER-anchored precursor and primarily modifies cholesterol production by controlling the expression of many biosynthetic

genes (Horton et al., 2002). The rate-limiting enzyme for cholesterol synthesis is the 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGCR), a glycoprotein localized in the ER. The SREBP-2 activation pathway controls the transcription of the HMGCR gene, which is one of its target genes.

Regarding liver conditions, investigations revealed that patients with NASH had higher levels of hepatic SREBP2 expression (Min et al., 2012). Hence inhibition of SREBP-2 activation and, thereby, the reduced expression of HMCGR or the direct inhibition of HMGCR is considered a novel target in NAFLD treatment. Statin is one such drug that inhibits HMGCR, hence suggested therapy to inhibit excess cholesterol accumulation during NAFLD (Dong et al., 2020). Activation of AMPK directly inhibits SREBP-2. Thus, inhibition of SREBP-2 downregulates HMGCR protein levels (Zhang et al., 2018).

1.8.7 Peroxisome proliferator-activated receptors (PPARs) and NAFLD

Nuclear receptors known as peroxisome proliferator-activated receptors (PPARs) are ligand-activated receptors and control inflammation as well as lipid and glucose metabolism. Three mammalian isotypes of the nuclear receptor superfamily of PPARs are present, that includes PPAR-α, PPAR-β/δ, and PPAR-γ. FA and prostaglandins are natural PPAR ligands (Tailleux et al., 2012). In humans, fibrates are synthetic PPAR-α ligands frequently used to treat hypertriglyceridemia. Glitazones and thiazolidinediones, which are artificial PPAR-γ ligands, have powerful insulin-sensitizing and antidiabetic benefits, but they also have adverse side effects, including weight gain, edema, and bone fractures. Synthetic PPAR-β/δ ligands are in the course of development with promising results in treating dyslipidemia. The liver is one of the tissues with high levels of PPAR-α expression. PPAR-α controls FA transport and β-oxidation to reduce fat accumulation in the liver (Staels et al., 2008). In addition, PPAR-α has an anti-inflammatory property by inhibiting the NF-κB gene (Zeng et al., 2014). Therefore, finding PPAR-α agonists with fewer side effects is a challenging research area, and it is a crucial target protein in NAFLD treatment.

1.8.8 Inflammation and NAFLD Progression

Significant features of NAFLD progression are inflammation, hepatocyte injury, and cell death. Innate immune activation is critical in initiating and worsening hepatic inflammation during NAFLD (Arrese et al., 2016). Excess lipid overload in hepatocytes causes lipotoxicity, which further enhances the progression of inflammation and associated complications in liver tissue. Lipid buildup in hepatocytes promotes the release of proinflammatory cytokines. Hence it attracts immune cells to the liver and elicits innate immune activation. Continuous inflammation for an extended period leads to hepatic injury and fibrosis formation. Studies have reported that hepatocyte death occurs during NASH progression.

More specifically, the significance of surface-expressed pattern recognition receptors (PRRs), crucial in identifying cell damage and pathogen invasion, as significant players in NAFLD/NASH is being revealed (Garcia-Martinez et al., 2015). Damage-associated molecular patterns, or DAMPs, are intracellular molecules or dying hepatocytes that might release during lipotoxicity. These molecules can operate on numerous immune cells in the hepatocytes to start a physiological wound-healing response to repair tissue damage. However, continuing these signals may cause an exaggerated reaction that could end in an extensive inflammatory response with tissue inflammation and severe scarring, which would then cause progressive fibrosis and, eventually, cirrhosis.

DAMP activation leads to the release of inflammatory cytokines like TNF-α and interleukin-6 (Feldstein, 2010). DAMP also binds to PRRs like TLR-4 receptors and aids in the progression of inflammation (Kesar & Odin, 2014). Significant emphasis has recently been paid to the function of inflammasome activation in NAFLD/NASH. Activation of the NLRP3 inflammasome is linked to hepatocyte pyroptosis, an inflammasome-dependent cell death process. The absence of this receptor reduces inflammation and fibrosis in experimental NASH, highlighting the importance of this pathway (Cannito et al., 2017).

JNK, a mitogen-activated protein kinases (MAPK) family member, mediates cellular reactions to various intra- and extracellular stressors. The first indication that JNK signaling has a mechanistic role in NAFLD was that JNK mediates two principal risk factors for NAFLD development – obesity and insulin resistance. Research studies have

shown that JNK mediates lipid buildup and liver damage in NAFLD experimental models. Through the stimulation of hepatic stellate cells, JNK signaling may also aid in the formation of hepatic fibrosis in response to chronic injury (Czaja, 2010). Immune activation is crucial in causing and enhancing hepatic inflammation in NAFLD/NASH. In conclusion, inflammation causes the progression of fibrosis, cirrhosis, and cancer during NAFLD. Scientific studies have reported that reducing inflammation alleviates the severity of NAFLD progression (Mosca et al., 2021).

1.8.9 Autophagy and NAFLD

A basic cellular process called autophagy uses lysosome-mediated degradation to eliminate molecules and subcellular components such as nucleic acids, proteins, lipids, and organelles to support the cell's homeostasis, differentiation, development, and survival (Aman et al., 2021). The mechanism of autophagy starts with a semi-circular membrane bilayer that encloses metabolic waste that needs to be degraded. After that, a closed autophagosome is formed by the slow collapse of the bilayer membrane structure. The autophagy substrates eventually get broken down when the autophagosome joins with a lysosome to produce an autolysosome. Breakdown products are then released into the cytoplasm. This breakdown product can further be used for anabolic pathways.

Autophagy-related genes (Atg) are primarily responsible for the formation of autophagosomes (Wesselborg & Stork, 2015). Autophagy, a crucial mechanism in addition to lipase for modulating endogenous fat metabolism, is directly linked to the aetiology of NAFLD. NAFLD is caused by excess lipid deposition in hepatocytes. One of the major causes of NAFLD is defective autophagy (Li et al., 2017).

As mentioned earlier, autophagy is regulated by autophagy-related genes (Atg) genes. Autophagy operates at a basic level and upregulates in response to various stimuli and stressful situations. Stress factors that trigger autophagy include amino acid shortage, serum starvation, growth factor deprivation, hypoxia, and exposure to chemicals and harmful substances. There are typically three phases to autophagosome formation. (1) Nucleation: The signaling processes that cause the initial phagophore membrane formation. (2) Expansion: The processes of membrane modeling and trafficking that contribute to the phagophore expansion and its cytoplasmic cargo

sequestration. (3) Closure: Phagophore closure to form an autophagosome involves membrane scission and fusion processes (Søreng et al., 2018).

During normal physiological conditions, suppression of autophagy occurs because the mTORC1-dependent phosphorylation of ULK1 and Atg13 results in the inactivation of the ULK1/2 complex. However, the ULK1/2 complex is dephosphorylated in stressful conditions, and mTORC1 is inhibited. In order to start autophagy, activated ULK1/2 phosphorylates ATG13 and FIP200 (Jung et al., 2010). Beclin1, AMBRA1, and other proteins govern the development of the first phagophore and membrane nucleation. Beclin1 is under the control of the activation of the ULK1/2 complex. Along with other proteins, ATG16L-1, ATG-7, and LC3 help the expansion, phagophore closure, and autophagosome formation. Finally, to attain lytic capability, autophagosomes merge with lysosomes (Kocaturk & Gozuacik, 2018).

Studies have reported that during the initial stage of NAFLD, autophagy helps to reduce fat accumulation in hepatocytes. Besides, studies have also shown that after 18 weeks of a high-fat diet, lipid buildup and an increase in liver fat content are seen in Atg knockout mice. Autophagy plays a different role in the advanced stages of NAFLD. Autophagy activation in hepatic stellate cells (HSCs) is associated with liver fibrosis and release of energy. Lipid droplets seen in abundance in HSCs lead to a surplus of extracellular matrix, collagen, and actomyosin, which lead to liver fibrosis (Blaner et al., 2009). Hence increased autophagy is induced in the early stages of NAFLD, which helps the liver to break down lipids and slows the progression of the illness. However, in the advanced stage of NAFLD condition, down-regulation of autophagy inhibits the progression of the disease. Autophagy presents a complex problem for the therapy of liver fibrosis since it plays a dual function in the condition. Nevertheless, the study of autophagy in NAFLD is still in its early stages and needs more investigation.

1.9 Current therapeutic strategy for NAFLD

Along with a rise in the frequency of obesity and MetS, NAFLD is now one of the most prevalent causes of chronic liver disease. Currently, there are no FDA-approved medications for NAFLD. As a result, the main objectives of treatment have been to reduce insulin resistance and weight loss through lifestyle changes, endoscopic or surgical intervention, and medication (Mundi et al., 2020).

(1) *Lifestyle modification*: Research studies have shown that weight loss significantly improved the NAFLD condition and histological features. One such study with 293 patients reported that NASH resolved in 58% of patients who lost 5% of their body weight, and also NASH resolved in 90% of patients who lost 10% of their body weight (Vilar-Gomez et al., 2015). Hence weight loss management is a major lifestyle modification in NAFLD patients. Moreover, a nutritious diet with caloric restriction is used in many trials, and some diets may have extra benefits, particularly those that increase insulin sensitivity. An example of one such diet is the Mediterranean diet which has 20% energy from protein, 40% energy from carbs, and 40% MUFA and omega-3 fatty acids provide the energy. Studies also showed that compared to the control diet (50% of calories come from carbohydrates, 30% from fat, and 20% from protein), the Mediterranean diet led to a higher reduction in hepatic steatosis (Ryan et al., 2013).

(2) *Medications*: As mentioned earlier, no approved NAFLD medication exists. However, we can manage the disease to a certain extent by giving some medications related to MetS, IR, and vitamins and supplements. Hence physicians recommend treatment that emphasizes weight loss, metabolic disorders, dyslipidemia, and diabetes to lessen insulin resistance and enhance the functioning of the liver. Medications like metformin, thiazolidinediones, glucagon-like peptide-1 (GLP-1) analogues, sodium-glucose cotransporter-2 (SGLT2) inhibitors, HMGCR inhibitors (statins), orlistat, vitamins, and supplements are used in the management of NAFLD. (3) *Endoscopy and Bariatric surgery*: For those who have struggled to lose weight and keep it off with lifestyle changes or medication, endoscopic and bariatric surgery are therapeutic procedures that have become viable options (Mundi et al., 2020).

Early detection and treatment of obesity and its concurrent conditions, such as NAFLD, are essential for weight loss and improved insulin sensitivity. Modest-intensity exercise is also incorporated, along with a change in lifestyle, calorie deficit diet, medications, and supplements are helpful to improve hepatic steatosis.

1.10 Natural compounds and NAFLD

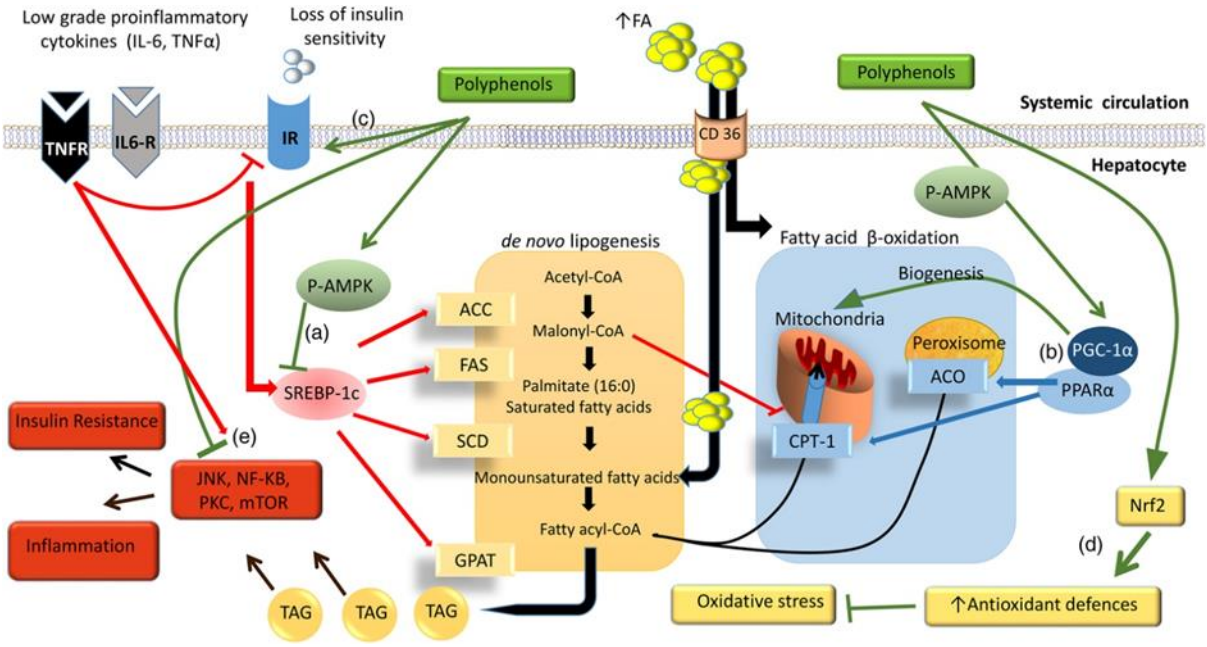
Studies of natural substances have emerged as an alluring strategy for overcoming lifestyle diseases. It has been demonstrated that several natural substances positively affect the cellular processes involved in the onset and development of various diseases. These data indicate a viable approach for NALFD's multifaceted and complex pathophysiology. Implementing these results into the clinical setting has been unsuccessful in spite of the reported findings on experimental models because of differences in the effective dosage, bioavailability, treatment duration, variations in the purity of the drug, and a lack of standardization.

Phytochemical components in medicinal plants are primarily responsible for the various pharmacological effects against diseases. According to their function in fundamental metabolic processes, the phytochemical constituents of plants can be divided into primary and secondary metabolites. Primary metabolite participates in the plant's growth, development, and reproduction. Secondary metabolites help ecological functions like defense mechanisms in plants. In traditional medicine, secondary plant metabolites are crucial in treating various illnesses. However, in modern medicine, they contribute to identifying and producing lead compounds to treat several diseases. According to their chemical structures, secondary plant metabolites are divided into numerous categories. They are phenolics, terpenoids, and nitrogen-containing compounds. The most significant group of secondary metabolites found in plants are likely phenols. They all include one or more phenolic groups (Chinou, 2008). Plant polyphenols show medicinal properties like anti-inflammatory, antioxidant, antihepatotoxic, anticancer, antifungal, antibacterial, and antiviral.

Several *in vitro* and *in vivo* studies have reported that plant extract treatment is an attractive approach to alleviate several metabolic diseases. Hence, using natural remedies as an alternate method for treating NAFLD has recently attracted increasing interest among physicians. NAFLD is marked by a high level of fat deposits (>5.5 %) in the liver, followed by fibrosis and inflammation. And insulin resistance plays a crucial role in the development and progression of NAFLD (Tarantino et al., 2021). Natural substances have been investigated in preclinical and clinical settings, demonstrating their positive benefits on conditions like NAFLD. Studies have reported that polyphenols have hepatoprotective benefits by promoting fatty acid oxidation and

controlling insulin resistance, oxidative stress, and inflammation, which are the key pathogenetic variables connected to the transition from simple fat storage to NASH (Rodriguez-Ramiro et al., 2016) (Figure.1.4). For example, silymarin/silybin from Silybum marianum, or milk thistle shows the hepatoprotective effect and also effective against NAFLD in clinical studies (Loguercio et al., 2012). A natural polyphenol called resveratrol found in many plants and spices and is effective against NAFLD. Clinical studies done in 50 NAFLD patients show a significant reduction in release of proinflammatory cytokines like NF-κB and reduced hepatic steatosis compared to the control group (Faghihzadeh et al., 2014). An isoquinoline alkaloid obtained from natural plants, berberine shows hepatoprotective function through the activation of the AMPK pathway (Tillhon et al., 2012) (Figure. 1.5). Clinical studies also showed the medicinal property of berberine by reducing weight gain and improving total serum cholesterol, TG, apolipoprotein B, alanine transaminase (ALT) and aspartate transaminase (AST) (Yan et al., 2015). Asian nations have employed curcumin in the turmeric plant for hundreds of years as a food spice. Due to its therapeutic characteristics, curcumin has recently gained much attention as a natural polyphenol. Clinical studies done on NAFLD patients show its promising therapeutic benefit against NAFLD. After eight weeks, curcumin treatment significantly decreased total serum cholesterol, non-HDL-C, LDL-C, HDL-C, TG, ALT, AST, and uric acid (Panahi et al., 2016). Several other plant-based compounds have also been reported for their beneficial effect against NAFLD.

Steatosis, oxidative stress, inflammation, and fibrosis are the major pathological features of the NAFLD condition. Additionally, the quest for a treatment for NAFLD has been complicated by the multiplex effects of the disease and its related comorbidities. Hence finding plant-based lead compounds should be a reliable and effective strategy for NAFLD drug development.



Rodriguez et al., Proceedings of the nutrition society, 2016.

Figure.1.5. The impact of polyphenols on nonalcoholic fatty liver disease (NAFLD) and possible cell signaling pathways.

1.11Metabolic syndrome, NAFLD, and obesity

Now NAFLD is considered the hepatic manifestation of metabolic syndrome (Buzzetti et al., 2016). The term "MetS" refers to a group of metabolic conditions, including abdominal obesity, hypertension, dyslipidemia, and hyperglycemia (Eckel et al., 2005). NAFLD prevalence rises concurrently with increases in obesity, MetS, and T2D. Obesity and overweight are the primary causes of NAFLD and metabolic disorders. Metabolism-related disorders like MetS, diabetes, and NAFLD are caused by abnormalities in fat distribution, adipose tissue (AT) function, and insulin resistance (IR) (Shulman, 2014). Adipose tissue stores fat and releases it when the body needs more energy.

Moreover, it functions as an endocrine organ, which releases adipokines. AT can regulate whole-body homeostasis through its complex cell signaling pathway. The two types of adipose tissue that make up; white adipose tissue are subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT). SAT expandability makes it the ideal

location for storing fat. However, fat deposition in VAT is associated with metabolic syndrome (Godoy-Matos et al., 2020).

Since IR promotes lipolysis, raises the flow of FFAs to the liver, and stimulates hepatic DNL, IR is a key element in the development of NASH. Dysfunctional AT produces adipokines and inflammatory cytokines while lowering the anti-inflammatory proteins. Increased hepatic lipid accumulation (through faster FFA input and DNL) results in liver lipid overload. It is well known that adipose tissue significantly affects the buildup of lipids in the liver, primarily via releasing FFA, adipokines, and cytokines. On the other hand, the cause of IR is the accumulation of intracellular lipids.

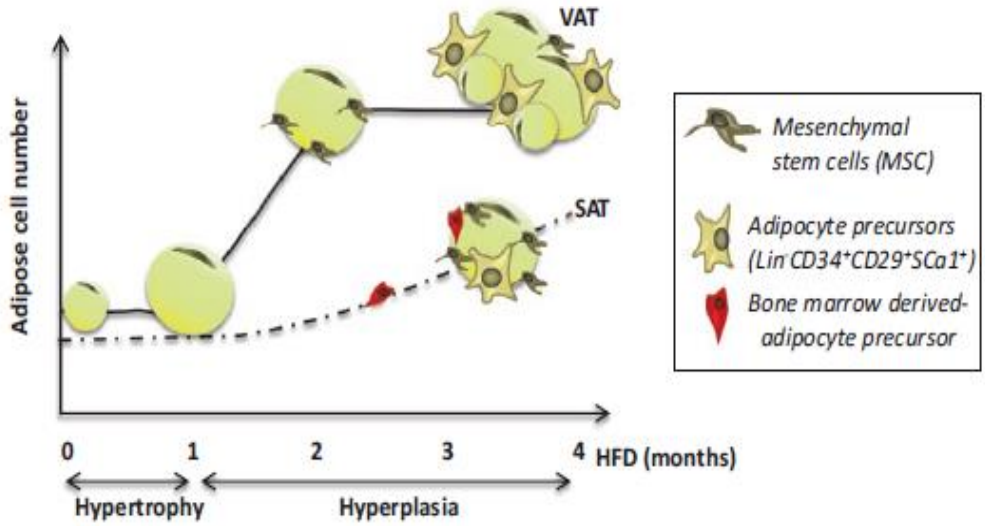
Additionally, in experiments, adiponectin has been demonstrated to influence the regulation of hepatic inflammation (Dietrich & Hellerbrand, 2014). Clinical investigations also provided evidence of the central role of adiponectin signaling in the pathophysiology of NAFLD and hepatic IR. Leptin, among adipokines, has also been proposed to have a role in the aetiology of NAFLD (Parola & Marra, 2011). Hence the crosstalk between adipose tissue and liver plays a major role in the aetiology of NAFLD progression. Therefore, these cytokines can alter hepatic metabolism by acting on specific receptors that may serve as the target of particular pharmacological interventions for NAFLD treatment.

1.12 White adipose tissue and physiological importance

Adipose tissue can be defined as the specific tissue with lipid regulating capacity. Adipocyte store fat and function as a reservoir of energy. White, beige, and brown adipocytes are the three primary types of adipocytes found in placental mammals. While beige and brown adipocytes are specialized thermogenic cells capable of expending energy in the form of heat, white adipocytes (WAT) are specialized for lipid storage and release (Sakers et al., 2022). The most prevalent type of adipose tissue, found in nearly every part of the body, is WAT. Subcutaneous or visceral are the two anatomic categories used to classify the primary WAT depots. In humans, the peritoneal cavity houses the omental and mesenteric visceral fat depots. The abdominal and gluteofemoral deposits of subcutaneous fat found beneath the skin often account for 80% or more of the total amount of fat in people (Karastergiou & Fried, 2017).

The WAT is a crucial endocrine organ that secretes various hormones and other substances known as adipokines. In addition to boosting insulin sensitivity (adiponectin), insulin resistance (resistin), and inflammation (TNF-a, IL-6, IL-1b, IL-8, and IL-18), adipokines also play essential roles in controlling the entire body metabolism (Funcke & Scherer, 2019). The WAT metabolism quickly changes to satisfy the organism's varying energy requirements during fasting, feeding, cold, and exercise periods. WAT metabolism alternates between two competing metabolic programs, pushing food intake and driving nutrient release.

Various effector proteins and transcriptional regulators work in concert to cont rol the metabolic plasticity of white adipocytes in response to hormonal and neurologi cal cues. After eating and during positive energy balance, WAT absorbs nutrients from the circulation and synthesizes lipids via DNL. Adipose tissue undergoes adipocyte hypertrophy and adipocyte hyperplasia (Figure.1.6). Adipocyte hypertrophy is associated with dysfunctional WAT metabolism that leads to tissue inflammation, fibrosis, and hypoxia (Burhans et al., 2018). Inflammatory cytokines, primarily but not exclusively tumour necrosis factor-α (TNF-α), are released into circulation by necrotic, hypertrophic, and hypoxic adipocytes. Fatty acids are transported to the liver rather than being retained in adipocytes. Adipose-liver interaction pathways influence the emergence and development of liver disease. Adipokines mediate and worsen the liver injury, while fatty acids cause hepatic steatosis and inflammation. Hence adipose tissue substantially contributes to the pathophysiology of obesity, insulin resistance, and NAFLD (Parker, 2018).



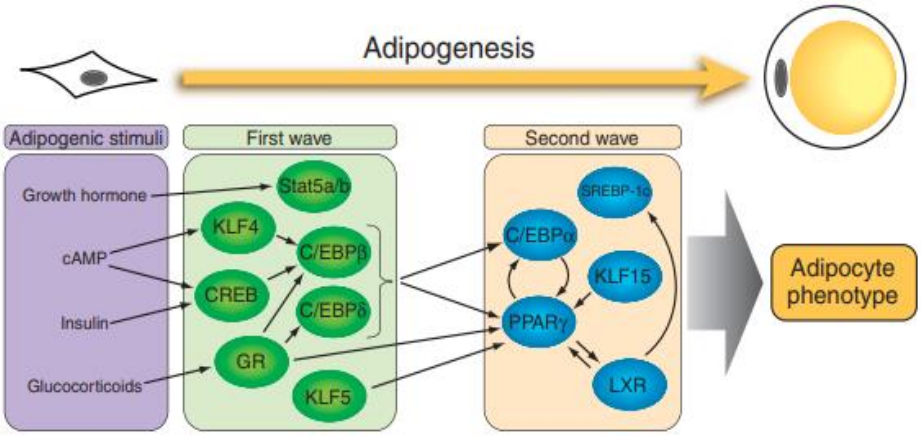
Engin, AB and Engin, A., Springer, 2017.

Figure.1.6. Adipose tissue hypertrophy and hyperplasia

1.13 Adipose tissue differentiation and its transcriptional regulation.

It is critical to understand adipocyte formation to comprehend how they contribute to systemic metabolic regulation. Adipogenesis is the process by which fibroblast-like preadipocytes are converted to mature fat-storing cells. The lineage commitment of mesenchymal stem cells (MSCs) into preadipocytes is the first stage of adipogenesis. A fibroblast-like preadipocyte clonal cell line was used to examine *in vitro* transcriptional regulation of adipogenesis (Richard et al., 2020). When preadipocytes reach confluence, their growth is halted. After differentiation is induced, they re-enter the cell cycle and proceed through multiple rounds of proliferation (a process known as mitotic clonal expansion). Preadipocytes terminally differentiate into mature adipocytes at the end of this brief proliferative period as they start synthesizing lipids and taking on mature fat cell characteristics. Adipogenesis is regulated by several transcription factors (Richard et al., 2020). Hence the prevailing consensus is that a transcriptional cascade regulates adipogenesis. Notably, C/EBP-β/δ/α and PPAR-γ tightly control the differentiation of stem cells into mature adipocytes. PPAR-γ is a nuclear transcription factor that plays a major role in adipocyte differentiation (Kawai & Rosen, 2013). C/EBP-β opens the chromatin to start the differentiation of adipocytes and stimulate PPAR-γ and C/EBP-α expression. In early adipogenesis, C/EBP-β and

C/EBP-δ are expressed in culture. SREBP-1C expression was activated after C/EBP-α and PPAR-γ expression during adipogenesis. According to current research, SREBP-1C is involved in the final stages of adipocyte maturation (Figure.1.7) Activation of SREBP-1C promotes adipocyte genes that are involved in lipogenesis (Tang & Lane, 2012).



Siersbæk, R. and Mandrup, S., Cold Spring Harbor symposia on quantitative biology, 2011

Figure1.7. Transcriptional regulation of adipogenesis

1.14 3T3-L1 as cell line model to study adipocyte differentiation

A popular *in vitro* model of white adipocytes is differentiated 3T3-L1 adipocytes. Because of their capacity to accumulate lipids, 3T3-L1 cells were separated and multiplied from the Swiss 3T3-L1 cells of mouse embryos (Green & Kehinde, 1975). To assist in cell differentiation to mature adipocytes, after growth arrest, this preadipocyte cell line is treated with a variety of pro-differentiation inducers, such as insulin, synthetic glucocorticoids like dexamethasone, and the phosphodiesterase inhibitor 1-methyl-3-isobutyl xanthine (IBMX) (Russell & Ho, 1976). These inducers facilitate PPAR-γ expression because they stimulate the signaling cascade. A key regulator of adipocyte differentiation is PPAR-γ. Compelled expression of PPAR-γ alone is sufficient to start the adipocyte differentiation cascade. That further activates other proteins and transcription factors relevant to adipogenesis and associated lipogenesis (Zebisch et al., 2012).

1.15 *Dillenia indica* L.

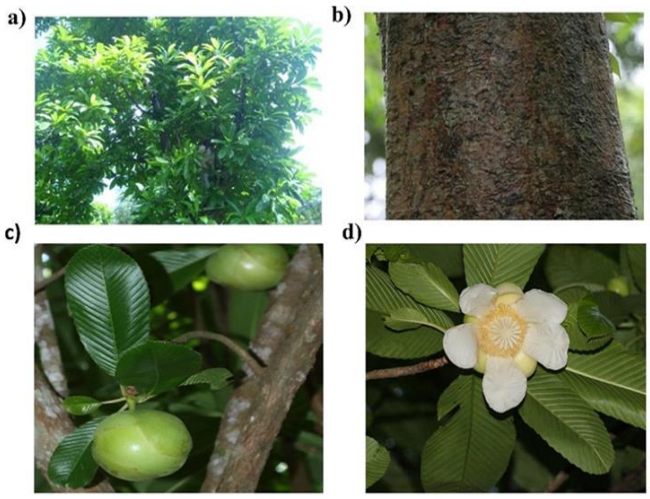
Through the decades, vast knowledge and practices regarding phytomedicine have been transmitted through the generations. For many years, the primary resources for treating a variety of human ailments were medicinal plants. Many of these plants are rare, endemic, and restricted to forest areas. The tribal and folk tribes of India's many forested regions have used a variety of plant species. Still, their medicinal and pharmacological value is unknown because these plants are rare. There are numerous plant species that Indian tribal cultures have used, but little is known about their medicinal and pharmacological properties. Among these medical plants, *Dillenia indica* L. stands out for its intriguing therapeutic properties despite their relative lack of popularity.

The common name for *Dillenia indica* L. is elephant apple belonging to the family Dilleniaceae. This evergreen deciduous and medium size tree is widely distributed throughout the tropical seasonal regions of several Asian nations and in India from the Himalayas to south India. The tree is 15 m tall with five white petals in flower, five yellow stamens, oval green leaves that range in size from 15 to 36 cm, and 5–12 cm of huge, tough, greenish yellow fruits that are edible (Chakre, 2010) (Figure.1.8).

Various rural cultures employ different parts of *Dillenia indica* L. in their traditional medicine systems to treat conditions like diabetes, indigestion, asthma, jaundice, and rheumatic pain. Traditional healers of the Khamptis, rural residents of Assam's Dhemaji district, and ethnic groups in Northeast India's Dibru-Saikhowa Biosphere Reserve all frequently use this plant for a variety of therapeutic purposes. In Ayurveda, it is recognized as a "vat" suppressor and "pitta" enhancing drug (Rai & Sajwan, 2020). The Mizoram tribal community uses fruits extensively as a traditional medicine to heal mouth ulcers, diarrhoea, and jaundice. Additionally, it has been discovered that leaf, fruit, and bark extracts have therapeutic qualities; as a result, they are used orally in the tribal regions of north-eastern India to treat cancer, diabetes, and stomach issues. The extracts from the leaves and bark have antioxidant properties and are also used as laxatives and astringents (Singh & Saha, 2019). Additionally, it is a typical culinary component in Assam and is used to make curries, pickles, and jams (Chakre, 2010).

Flavonoids and triterpenoids (of the lupene type) are the important chemical ingredient classes isolated from *Dillenia indica* L. The variety of phytochemistry in *Dillenia indica* L. is additionally enhanced by additional isolated chemicals, such as phytosteroids, diterpenes, ionones, phenolics, anthraquinones, alcohols, and ketones. To date, around 34 compounds are isolated from *Dillenia indica* L. These include Kaempferol, Myricetin, Quercetin, Dillenetin, Rhamnetin, Isorhamnetin, Kaempferide, Kaempferide 3-*O*-diglucoside, 3,5-Dihydroxy-4, 3- dimethoxy flavone-7-*O*-*β*-D--glucopyranoside, 5,7-Dihydroxy-4-methoxyflavone-3-*O*-*β*-D-glucopyranoside, (+)-Dihydroxykaempferol, (+)-3-Methoxydihydroquercetin, (+)-Dihydroisorhamnetin, Dihydrokaempferide, Dihydrokaempferide, 7-diglucoside, 4,5,7,3′,4'-Pentahydroxyflavan-3-*O*-*β*-D- - glucopyranoside, Leucocyanidin, Naringenin, Naringenin 7-diglucoside, 3,5,7-Trihydroxy-2-(4-hydroxy- benzyl)-chroman-4- one, Lupeol, Betulin, Betulinaldehyde, Betulinic acid, 3β -Hydroxylupane-13β,28- lactone, β-Sitosterol, Stigmasterol, Stigmasteryl palmitate, Cycloartenone, Gallic acid, 1,8-Dihydroxy-2-methylanthraquinone-3-*O*-β-D-glucopyranoside, n-Hentriacontanol, n-Heptacosan-7-one, and n-Honatriacontan-18-one (Singh & Saha, 2019). Therefore, isolating these compounds could spur research and a magnificent quest to discover new chemical components in this plant, which will be helpful for figuring out the plant's medical properties.

Kingdom	: Plantae
Division	: Magnoliophyta
Subdivision	: Angiospermae
Class	: Magnoliopsida
Subclass	: Dilleniidae
Order	: Dilleniales
Family	: Dilleniaceae
Genus	: *Dillenia*
Species	: *indica* Linnaeus



Gandhi, D. and Mehta, P., Journal of applied pharmaceutical Science, 2013.

Royal botanical gardens Kew, Plant of the World online

Figure.1.8. a) *Dillenia indica* L. tree b) *Dillenia indica* L. tree bark c) *Dillenia indica* L. fruit with leaves d) *Dillenia indica* L. flower with leaves

1.16 Aims and objectives

The NAFLD results from a complicated interplay of lipid overload, IR, hormonal, inflammatory, dietary, and hereditary variables. NAFLD is the collective term for these intricate metabolic, hepatic, and extrahepatic effects. Adipose tissue plays a significant role in the progression of NAFLD. NAFLD can lead to atherogenic dyslipidemia, altered glucose metabolism, and increased CVD risk. Pharmaceutical management for NAFLD is still under investigation. To date, no specific medication for NAFLD is available. This treatment ambiguity is partly a result of our limited knowledge of the underlying mechanisms that promote the onset and progression of NAFLD. Because of the fewer side effects and more medicinal properties, plant-based drugs are becoming more and more popular. Surplus lipogenesis is a major factor in the genesis of NAFLD. In addition, altered adipogenesis is a risk factor for NAFLD due to its significant impact on liver function. Based on these, this thesis deals with studies on various biochemical pathways associated with NAFLD and effect of plant extract of *Dillenia indica* L. Therefore, the present research employed two experimental models to study *Dillenia indica* L. extract's effect against pathological lipogenesis and

adipogenesis. First, *in vitro*-model is oleic acid-induced NAFLD in HepG2 cell lines to find the therapeutic property of *Dillenia indica* L. against abnormal lipogenesis. Second, *in vitro*-model is to find the effect of plant extract against adipogenesis in 3T3-L1 cell lines. The objectives of the present study are the following

- Extraction and partial characterization of *Dillenia indica* L. leaf extract.
- Validation of the beneficial effect of extract against NAFLD *in vitro* models emphasizing pathological lipogenesis and adipogenesis.
- Detailed investigation on the effect extract on various proteins involved in lipid metabolism signaling pathway.
- Role of inflammation and autophagy signaling during lipid accumulation in cells and the effect of the extract.

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Chapter 2

Extraction and partial characterization of *Dillenia indica* L. plant extract for *in-vitro* analysis

2.1 Introduction

NAFLD is a severe health problem affecting people worldwide. It is commonly linked to obesity, unhealthy eating habits, and insufficient exercise. The liver is a crucial organ in energy metabolism, vital in processing, partitioning, and metabolizing macronutrients and transporting and storing metabolites. The liver also plays a significant role in detoxification. Abnormal lipid accumulation in the liver is the primary cause of NAFLD (Pei et al., 2020). Several plant species have been reported to have hepatoprotective functions through lipid-lowering activity. Phytochemicals in plants also support cellular defenses against inflammation and promote antioxidant capacity.

D. indica comes under the family Dilleniaceae and is commonly known as elephant apple. It is an evergreen tree native to warm temperate regions like India, Indo-Malaysian, and tropical Australia. It is an evergreen tree that may grow up to 15 meters tall. The flower has five white petals and yellow stamen, the leaves are oval, broad, and green, and the fruit is edible, hard, and greenish-yellow in colour (Saiful Yazan & Armania, 2014). Evidence indicated that *D. indica* has a variety of therapeutic properties, including those that are anticancer (Kumar et al., 2010), antibacterial, antioxidant (Abdille et al., 2005), analgesic, anti-inflammatory, and antidiabetic, as well as its associated problems, including hyperlipidaemia, diabetic nephropathy, and neuropathy (Kumar et al., 2011). Studies have reported that *D. indica* has been used in traditional medicines in different countries. In ethnomedicinal practice, *D. indica* fruit has been used to treat jaundice, fever, and fatigue, used as a laxative, and prevent dandruff and hair loss (jelly-like part of the fruit) (Islam et al., 2014). Leaves are used to treat ailments like fever, cough, constipation, intestinal disease, jaundice, cancer, malaria, and malaria-like symptoms (Kabidul Azam et al., 2016; Singh & Saha, 2019). The flowers of *D. indica* are used to treat dysentery. The stem bark was used for treating

cough, cold, fever, blood cancer, and urinary disease (Sarmah et al., 2008; Singh & Saha, 2019). The root of *D. indica* has been used to cure food poisoning and biliousness (Quattrocchi, 2012). Hence in traditional medicine, most of the plant part of *D. indica* has been exploited for therapeutic purposes.

Numerous scientific studies have reported the presence of medicinally significant phytoconstituent like flavonoids and triterpenoids in *D. indica*. In addition, other bioactive substances such as phytosteroids, diterpenes, ionones, phenolics, anthraquinones, alcohols, and ketones were also reported. There are reports on phytochemical constituents from *D. indica* (Singh & Saha, 2019). Although *D. indica* has a wide spectrum of medicinal properties, very few studies have been done on its chemical makeup and pharmacological effects. Also, more scientific studies are needed regarding the quantification of bioactive constituents in *D. indica*. Studies indicate that *D. indica* has strong therapeutic potential and should be further investigated for its chemical composition and pharmacological properties. Several gaps in our current understanding of *D. indica* necessitate further biological research on its plant extracts. Therefore, more research is crucial to examine the effectiveness of its plant extracts against different ailments and look for the molecular pathway underlying its activity. Further research is necessary to assess the adverse effects, safety profile, and various biological functions of *D. indica* to facilitate their clinical use for human health.

In traditional medicine, different parts of *D. indica* are used to cure jaundice (Saiful Yazan & Armania, 2014). There are some scientific studies to show the hepatoprotective properties of *D. indica* in *in-vitro* and *in-vivo* (Reddy et al., 2010). However, the details of the molecular mechanisms by which it exerts its pharmacological activity have yet to be scientifically studied. The present chapter deals with the partial characterization of *D. indica* leaf extract for its composition and the evaluation of its beneficial effects against NAFLD in the *in vitro* system.

2.2 Materials and methods

2.2.1 Chemicals

Minimum essential medium eagle (MEME) was from HiMedia (Mumbai, India). Hanks balanced saline solution (HBSS), phosphate buffer saline (PBS), fetal bovine serum (FBS), penicillin-streptomycin antibiotics, and trypsin-ethylenediamine tetraacetate (EDTA), were bought from Gibco, USA. Dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), oleic acid, Oil Red O stain and 2, 7- dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich Co. USA. The triglyceride assay kit was from Cayman, USA. All other chemicals and solvents (Hexane, Ethyl acetate, Ethanol, and Isopropanol) were of analytical grade.

2.2.2 Preparation of *D. indica* leaf extracts

D. indica was collected from Malampuzha, Palakkad district of Kerala (Latitude: 10◦ 49’ 29.87″ N; Longitude 76◦ 41 0.89” E) during the month of January 2020 and identified with the help of a taxonomist and a voucher specimen of the same was kept in the herbarium at CSIR-NIIST (AC-3/2020) for future reference. The extracts of the leaf of *D. indica* were prepared following the method of Prathapan et al. (2013). The plant leaves were dried, ground to powder, and extracted (240 g) with n-hexane (1 L) to remove low polarity contaminants and filtered through the Whatman No.1 filter paper. Then the supernatant was concentrated using rotavapor at reduced pressure, to get *D. indica* n-hexane extract (DI-HX). After this, the powder was extracted further with ethyl acetate at ambient temperature (27 ± 1 ◦C) under stirring for 6 h, and the extraction procedure was repeated until the solvent became colourless. To get the DI-EA extract, the supernatant was filtered using Whatman No. 1 filter paper, concentrated under reduced pressure in a rotavapor, and then lyophilized. Again, the powder of DI-EA extract was extracted using hydroethanolic solvent (70% ethanol) and processed as above to get the DI-HET extract. The lyophilized leaf extracts (DI-HX, DI-EA, and DI-HET) were stored at 4 ◦C until use. Each extract (DI-HX, DI-EA, and DI-HET) was evaluated for its lipolytic activity by Oil Red O staining against oleic acid (OA) induced lipogenesis in HepG2 cells.

2.2.3 Polyphenol profiling of *D. indica* using HPLC

20 µL of the DI-HX, DI-EA, and HET extract solutions and the standard compounds (1 mg/mL) were loaded into the HPLC system separately after being filtered via a 0.45 µm PTFE filter. The analysis was done using a Shimadzu Prominence UFLC system (LC-20AD system controller, Phenomenex Gemini C18 column (250 x 4.6 mm, 5 µm), CTO-20A column oven, Rheodyne injector (USA) with a 20 lL loop volume, and SPD-M20A diode array detector). The qualitative analysis of polyphenols was done with HPLC analysis according to the slightly modified (Rodríguez-Delgado et al., 2001) method. The eluted fractions were observed at 280 nm while the column was kept at room temperature. Sample peaks were located by comparing the retention periods of sample peaks to those of standard peaks. Data collection and analysis were conducted using LC LabSolutions software (Arun et al., 2017).

2.2.4 Polyphenol profiling of *D. indica* using liquid chromatography and mass spectrometry (LCMS/MS)

Based on literature support (Akter et al., 2022; Fu et al., 2015; M. M. Islam et al., 2013; Kaur et al., 2016; Meeprathom et al., 2018; Singh & Saha, 2019), the compounds mentioned in Table (2.1, 2.2, & 2.3) were identified and quantified from leaves of *D. indica* extracts (DI-HX, DI-EA, and DI-HET) using LCMS/MS (Nexera with LCMS-8045, Shimadzu, Japan) (Abraham et al., 2020).

2.2.5 Cell culture and treatments

Hepatocellular carcinoma cell line (HepG2) was purchased from National Centre for Cell Sciences, Pune, India. Cells were cultured and maintained in modified eagle medium (MEME) supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin solution. The cells were cultured in a humidified atmosphere with 5% CO2 and 37 ◦C. Cultures were used at 80% confluency. For subculturing, the cells were washed with pre-warmed PBS and detached by treating with 0.25% trypsin. The experimental group consists of.

(a) Control group: HepG2 cells without any treatment are designated as C.

(b) OA group: HepG2 cells were incubated with 100 μM of OA for 24 h and indicated as OA.

(c) DI-HX-5, DI-EA-5, or DI-HET-5 group: HepG2 cells were pretreated with 5 μg/mL of extract for 2 h, followed by 100 μM of OA for 24 h.

(Hexane extract of *D. indica* leaves is designated as DI-HX, Ethyl acetate extract of *D. indica* leaves is designated as DI-EA and 70% hydroethanolic extract of *D. indica* leaves is designated as DI-HET)

(d) DI-HX-10, DI-EA-10, or DI-HET-10: HepG2 cells were pretreated with 10 μg/mL of extract for 2 h, followed by 100 μM of OA for 24 h.

(e) PC group: Cells were pretreated with fenofibrate (20 μM) for 2 h followed by OA (100 μM) for 24 h designated as PC.

Fenofibrate is a safe and effective medication approved by the FDA for the treatment of hypertriglyceridemia, primary cholesterolemia, and dyslipidemia (Sidhu & Tripp, 2021). Since there are no effective drugs for NAFLD, fenofibrate is commonly used as a positive control in most of the studies (Swapna et al., 2020).

2.2.6 Preparation of BSA- OA complex

To prepare BSA-OA complex, first, OA was dissolved in 0.1 N of NaOH at room temperature to get OA stock solution (10 mM). Then, fatty acid-free BSA (10% in MEME) was added to the stock solution to provide a final concentration of 1 mM. The final concentration of the solution was 100 μM after it had been diluted with 1% culture medium (Cousin et al., 2001).

2.2.7 Cell viability assay

For cell viability, HepG2 cells were seeded in 96-well plates at a density of 5×10^3 per well, and MTT assay was performed after treating the cells with different concentrations of DI-HX, DI-EA, and DI-HET for 24 h (5, 10, 20, 50, 100, 200 and 400 μg/mL). Cytotoxicity of fenofibrate was also studied with different concentrations (5, 10, 20, 30,40, 50,100, and 200 μM) (Mosmann, 1983).

2.2.8 Oil Red-O-staining

After the respective treatment, the cells were washed twice with PBS and fixed in 4% paraformaldehyde for 1 h. After fixation, the cells were washed with PBS and permeabilized with 0.1% Triton X 100, followed by Oil Red O (ORO) staining for 20 min at room temperature. The cells were then visualized under a light microscope. Absorbance was spectrophotometrically measured at 490 nm (Swapna et al., 2020).

2.2.9 Quantification of triglyceride content

With the use of a triglyceride colorimetric test kit (Cayman, USA), the concentration of triglyceride was determined. The assay was based on the enzymatic lipase action on triglycerides and the absorbance was measured at 530–550 nm (Shyni et al., 2021).

2.3 Results

2.3.1 HPLC polyphenol profiling

All three extracts were subjected to HPLC analysis. The polyphenol content in the hexane (DI-HX), ethyl acetate (DI-EA), and hydroethanolic (DI-HET) extracts of *D. indica* leaves was estimated using high-performance liquid chromatography (HPLC). The following thirteen standards of polyphenol were employed for HPLC analysis (1 mg/mL concentration) (Table. 2.1)

SL No	Name of polyphenol	Retention time (min)
1	Gallic acid	7.724
2	Catechol	12.773
3	Chlorogenic acid	23.807
4	Caffeic acid	25.349
5	Syringic acid	26.298
6	p-Coumaric acid	27.567
7	Ferulic acid	28.629
8	Ellagic acid	31.155
9	Myricetin	31.814
10	Cinnamic acid	33.329
11	Quercetin	34.531
12	Kaempferol	37.586
13	Apigenin	38.322

Table 2.1 shows the details of polyphenol standards with retention for HPLC analysis.

Nine polyphenols were identified in the DI-HX extract; These are syringic acid, p-coumaric acid, ferulic acid, cinnamic acid, caffeic acid, apigenin, myricetin, kaempferol, and gallic acid. Eleven polyphenols were identified in the DI-EA extract, which consists of syringic acid, ferulic acid, kaempferol, p-coumaric acid, ellagic acid, caffeic acid, gallic acid, cinnamic acid, quercetin, myricetin, and apigenin. DI-HET consists of ten polyphenols with syringic acid, kaempferol, ferulic acid, cinnamic acid, gallic acid, p-coumaric acid, myricetin, quercetin, apigenin, and caffeic acid. The chromatogram for DI-HX, DI-EA, and DI-HET are shown in Figure.2.1. HPLC profile indicates the rich source of polyphenolic compounds in all three extracts.

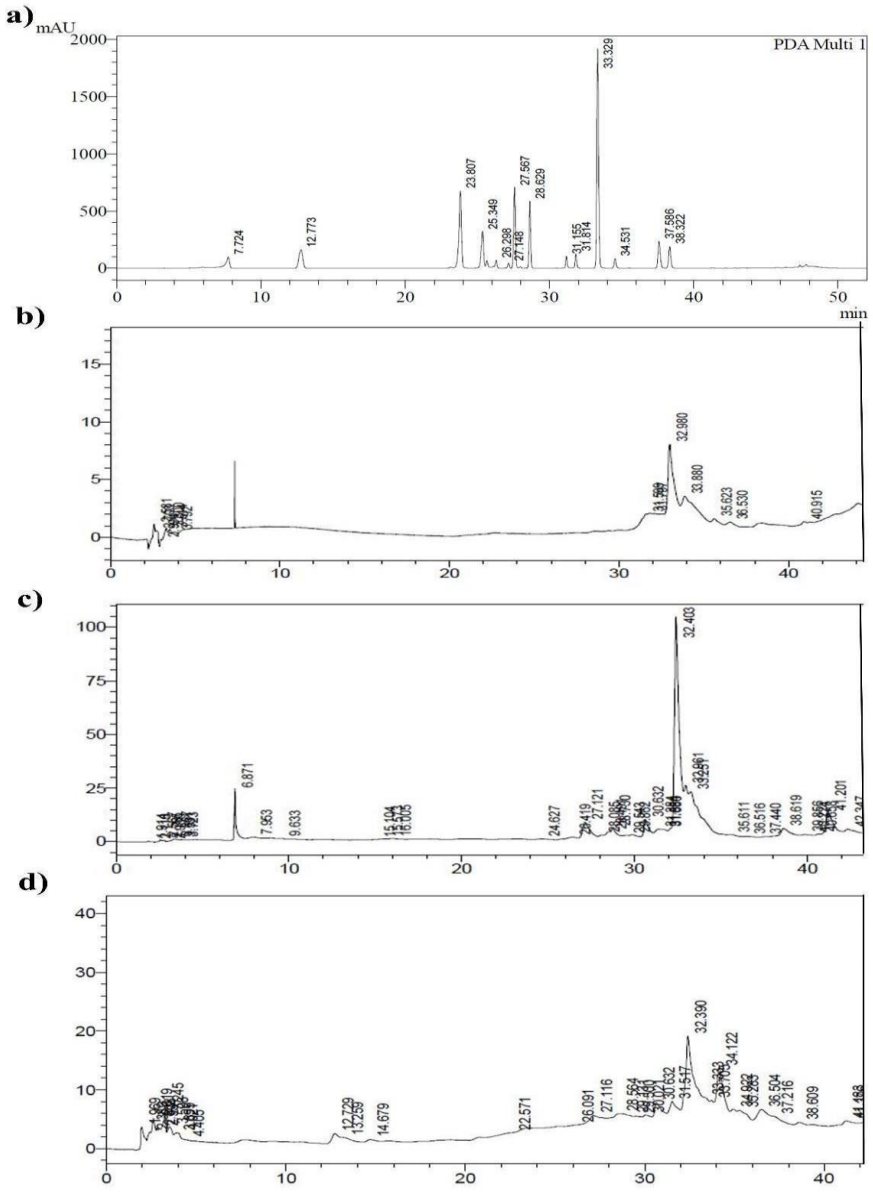

Figure.2.1. HPLC chromatogram of DI-HX, DI-EA, and DI-HET extracts a) HPLC Chromatogram of standard compounds with retention time in minutes-(1) gallic acid, (2) catechol, (3) chlorogenic acid, (4) caffeic acid, (5) syringic acid, (6) p-coumaric acid, (7) ferulic acid, (8) ellagic acid, (9) myricetin, (10) cinnamic acid, (11) quercetin, (12) kaempferol and (13) apigenin. The retention times were 7.724, 12.773, 23.807, 25.349, 26.298, 27.567, 28.629, 31.155, 31.814, 33.329, 34.531, 37.586, and 38.322, respectively. **b) HPLC Chromatogram of DI-HX extract:** 1) syringic acid, 2) p-coumaric acid, 3) ferulic acid, 4) cinnamic acid, 5) caffeic acid, 6) apigenin, 7) myricetin 8) kaempferol, and 9) gallic acid. **c) HPLC Chromatogram of DI-EA**

extract 1) syringic acid, 2) ferulic acid, 3) kaempferol, 4) p-coumaric acid, 5) ellagic acid, 6) caffeic acid, 7) gallic acid, 8) cinnamic acid, 9) quercetin, 10) myricetin, and 11) apigenin. **d) HPLC Chromatogram of DI-HETextract:**1) syringic acid, 2) kaempferol, 3) ferulic acid, 4) cinnamic acid, 5) gallic acid, 6) p-coumaric acid, 7) myricetin, 8) quercetin, 9) apigenin and 10) caffeic acid.

2.3.2 LCMS/MS polyphenol profiling

The LCMS/MS data revealed the abundance of polyphenols in all three extracts (for details, see chromatograms figures. 2.2. a, b, c & d, which are depicted in tables 2.2, 2.3 &2.4.

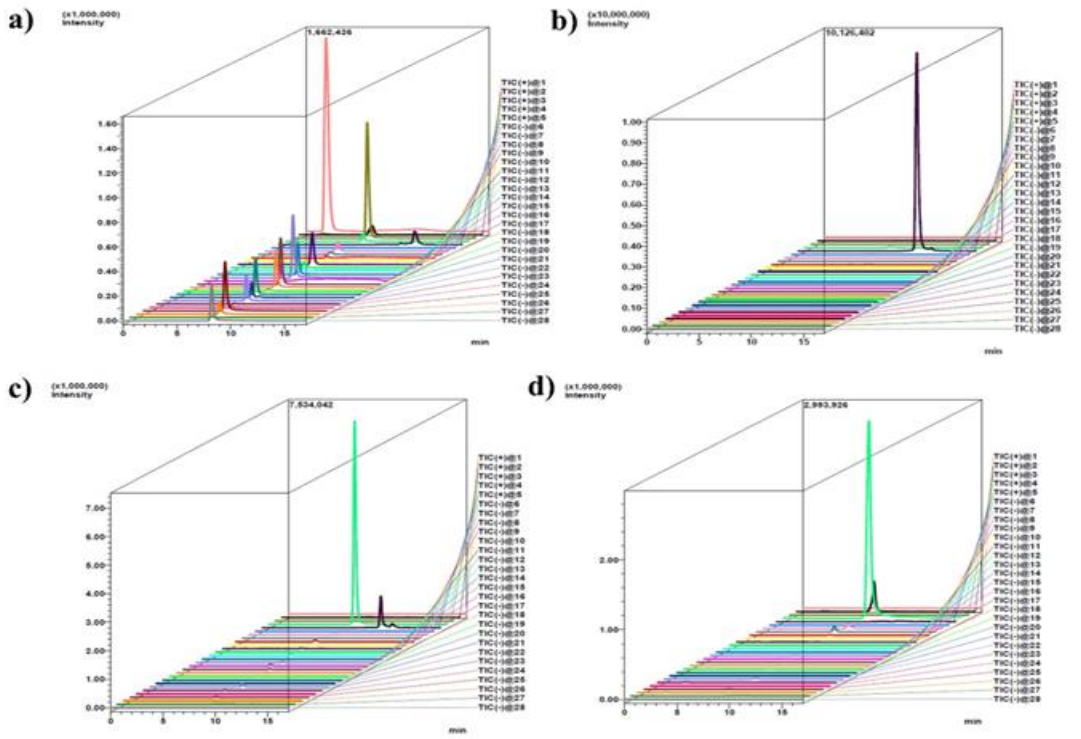

Figure.2.2. Phytochemical analysis of DI-HET by LCMS/MS method a) LCMS/MS chromatogram of standard compounds with retention time in minutes 1) Catechol (1.87 min), 2), Catechin(6.75 min), 3) Quinine(6.88 min), 4) Naringenin(7.28 min), 5) Tocopherol (12.87 min) 6) Gallic acid (1.91 min) 7) Chlorogenic acid (6.81 min) 8) Epicatechin (6.77 min), 9) Syringic acid (7.20 min) 10) Vanillic acid (6.79 min) 11) Caffeic acid (6.91 min) 12) Epigallocatechin(2.01 min) 13) Ferulic acid (7.36 min) 14) Myricetin (7.65 min), 15) Quercetin (7.92 min), 16) p-

Coumaric acid (7.34 min), 17) Luteolin (7.92 min), 18) Apigenin (8.18 min), 19) Kaempferol (7.83 min) 20) Rutin (7.34 min), 21) Diadzein (7.91 min), 22) Hesperetin (7.86 min) 23) Shikimic acid (1.76 min) 24) Ellagic acid (7.56 min), 25) Morin (7.74 min), 26) Genstein(7.81 min), 27) Cinnamic acid (7.93 min), 28) Chrysin (8.39 min) b) LCMS/MS Chromatogram of DI-HX showing corresponding peaks of polyphenols with retention time. c) LCMS/MS chromatogram of DI-EA showing corresponding peaks of polyphenols with retention time. d) LCMS/MS chromatogram of DI-HET showing corresponding peaks of polyphenols with retention time.

SL No	Name of polyphenol in DI-HX extract, peak position in chromatogram & retention time	Concentration(µg/g)
1	Vanillic acid-10 (6.79 min)	118.417 µg/g
2	Naringenin-4 (7.8 min)	111.691 µg/g
3	Tocopherol-5 (12.87 min)	69.455 µg/g
4	Syringic acid- 9 (7.20 min)	32.389 µg/g
5	p-Coumaric acid-16 (7.34 min)	27.498 µg/g
6	Ferulic acid-13 (7.36 min)	18.2 µg/g
7	Catechin- 2 (6.75 min)	12.373 µg/g
8	Cinnamic acid-27 (7.93 min)	8.103 µg/g
9	Epi catechin- 8 (6.77 min)	7.00 µg/g
10	Caffeic acid- 11 (6.91 min)	1.728 µg/g
11	Apigenin-18 (8.18 min)	1.443 µg/g
12	Myricetin-14 (7.65 min)	1.396µg/g
13	Kaempferol-19 (7.83 min)	1.371 µg/g
14	Chrysin- 28 (8.39 min)	1.101 µg/g
15	Genistein- 26 (7.81 min)	1.034 µg/g
16	Hesperetin-22 (7.86 min)	0.827 µg/g
17	Epigallocatechin-12 (2.01 min)	0.791 µg/g
18	Gallic acid- 6 (1.91 min)	0.768 µg/g
19	Luteolin-17 (7.92 min)	0.129 µg/g
20	Quinine- 3 (6.88 min)	0.054 µg/g

Table 2.2 shows the details of polyphenols with retention time and their corresponding concentration (µg/g) identified in the DI-HX extract.

SL No	Name of polyphenol in DI-EA extract, peak position in chromatogram & retention time	Concentration(µg/g)
1	Naringenin-4 (7.8 min)	10792.06 µg/g
2	Syringic acid- 9 (7.20 min)	626.079 µg/g
3	Vanillic acid-10 (6.79 min)	602.714 µg/g
4	Ferulic acid-13 (7.36 min)	259.943 µg/g
5	Tocopherol-5 (12.87 min)	140.849 µg/g
6	Kaempferol-19 (7.83 min)	86.568 µg/g
7	p-Coumaric acid-16 (7.34 min)	68.383 µg/g
8	Catechin- 2 (6.75 min)	23.209 µg/g
9	Epi catechin- 8 (6.77 min)	15.651 µg/g
10	Ellagic acid (7.56 min),	15.059 µg/g
11	Caffeic acid- 11 (6.91 min)	14.646 µg/g
12	Luteolin-17 (7.92 min)	13.968 µg/g
13	Gallic acid- 6 (1.91 min)	13.958 µg/g
14	Cinnamic acid-27 (7.93 min)	10.61 µg/g
15	Quercetin-15 (7.92 min)	5.057 µg/g
16	Morin (7.74 min)	4.821 µg/g
17	Myricetin-14 (7.65 min)	3.444 µg/g
18	Hesperetin-22 (7.86 min)	2.468 µg/g
19	Daidzein (7.91 min)	1.726 µg/g
20	Apigenin-18 (8.18 min)	1.441 µg/g
21	Chrysin- 28 (8.39 min)	0.991 µg/g
22	Genistein- 26 (7.81 min)	0.954 µg/g

Table 2.3 shows the details of polyphenols with retention time and their corresponding concentration (µg/g) identified in the DI-EA extract.

SL No	Name of polyphenol in DI-HET extract, peak position in chromatogram & retention time	Concentration(µg/g)
1	Naringenin-4 (7.8 min)	6768.581 µg/g
2	Catechin- 2 (6.75 min)	687.073 µg/g
3	Epi catechin- 8 (6.77 min)	539.969 µg/g
4	Shikimic acid-23 (1.76 min)	126.535 µg/g
5	Syringic acid- 9 (7.20 min)	75.687 µg/g
6	Vanillic acid-10 (6.79 min)	67.82 µg/g
7	Kaempferol-19 (7.83 min)	60.159 µg/g
8	Tocopherol-5 (12.87 min)	7.011 µg/g
9	Ferulic acid-13 (7.36 min)	3.382 µg/g
10	Cinnamic acid-27 (7.93 min)	3.094 µg/g
11	Gallic acid- 6 (1.91 min)	2.681 µg/g
12	Luteolin-17 (7.92 min)	1.751 µg/g
13	p-Coumaric acid-16 (7.34 min)	1.646 µg/g
14	Myricetin-14 (7.65 min)	1.161 µg/g
15	Quercetin-15 (7.92 min)	1.119 µg/g
16	Hesperetin-22 (7.86 min)	1.017 µg/g
17	Apigenin-18 (8.18 min)	1.002 µg/g
18	Caffeic acid- 11 (6.91 min)	0.964 µg/g
19	Chrysin- 28 (8.39 min)	0.884 µg/g
20	Epigallocatechin-12 (2.01 min)	0.703 µg/g
21	Genistein- 26 (7.81 min)	0.579 µg/g
22	Quinine- 3 (6.88 min)	0.413 µg/g

Table 2.4 shows the details of polyphenols with retention time and their corresponding concentration (µg/g) identified in DI-HET extract.

2.3.3 Effect of extract on the viability of HepG2 cells

DI-HX was not toxic up to 50 µg/mL in HepG2 cells. DI-EA, and DI-HET were not toxic up to 100 µg/mL in HepG2 cells (Figures.2.4a, b & c). Based on viability data, 5 and 10 µg/mL of DI-HX, DI-EA, and DI-HET extracts were selected for further studies.

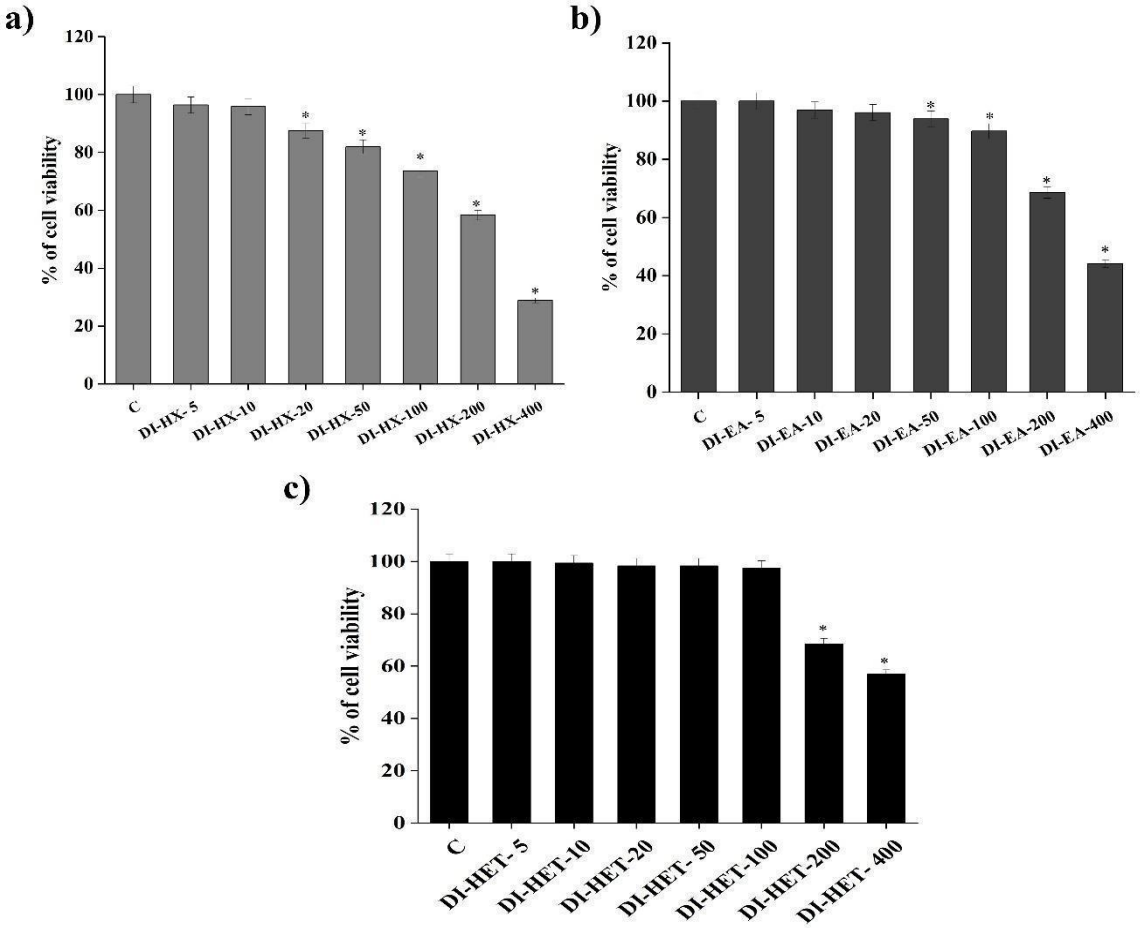

Figure.2.3 Bar diagram showing cell viability: a) DI-HX- 5, 10, 20, 50, 100, 200, and 400 (µg/ml), b) DI-EA- 5, 10, 20, 50, 100, 200, and 400 (µg/ml) c) DI-HET- 5,10,20,50,100,200 and 400(µg/ml). Values are expressed as mean ± SEM where n = 6. * indicates significantly different from the control group.

2.3.4 Effect of fenofibrate on viability of HepG2 cells

Cytotoxicity of fenofibrate was also studied with different concentrations (5, 10, 20, 30,40, 50,100, and 200 μM). Fenofibrate was found to be nontoxic up to 100 μM.

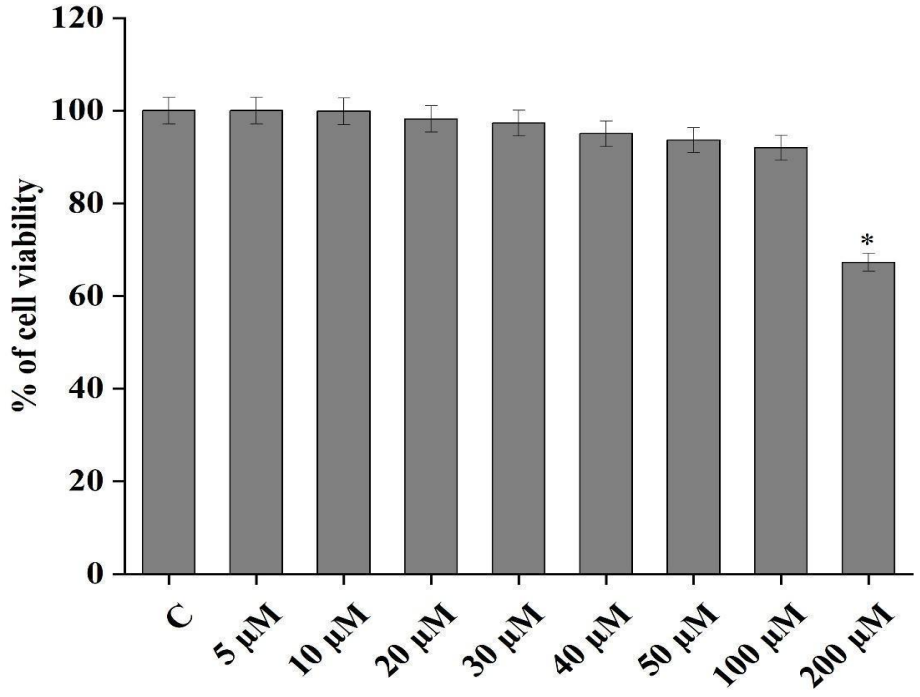

Figure 2.4 Effect of fenofibrate on the viability of HepG2 cells. HepG2 cells were treated with fenofibrate (5, 10, 20,30, 40, 50, 100, 200 μM) for 24 h, and cell viability was determined by MTT assay. Data are expressed as mean ± SEM where n = 6. * denotes significant difference from the control group ($p \leq 0.05$).

2.3.5 Effect of extracts on inhibition of lipid accumulation in HepG2 cells

Lipid accumulation was significantly increased in the OA group (36.34%; $p \leq 0.05$) when compared to control. Pretreatment with DI-HX and DI-EA does not show a significant reduction in lipid accumulation when compared to the OA group (3.41% with DI-HX-5 and 7.21% with DI-HX-10; $p \leq 0.05$; Figure.2.6a and b)(5.31% with DI-EA-5 and 11.01% with DI-EA-10; $p \leq 0.05$; Figure.2.6a and c) However, pretreatment with DI-HET caused a significant reduction of lipid content in a dose-dependent manner (22.40% with DI-HET-5 and 33.80% with DI-HET-10; $p \leq 0.05$; Figure.2.6a

and d) when compared to the OA group. Treatment with fenofibrate significantly reduced lipid accumulation by 37.59%.

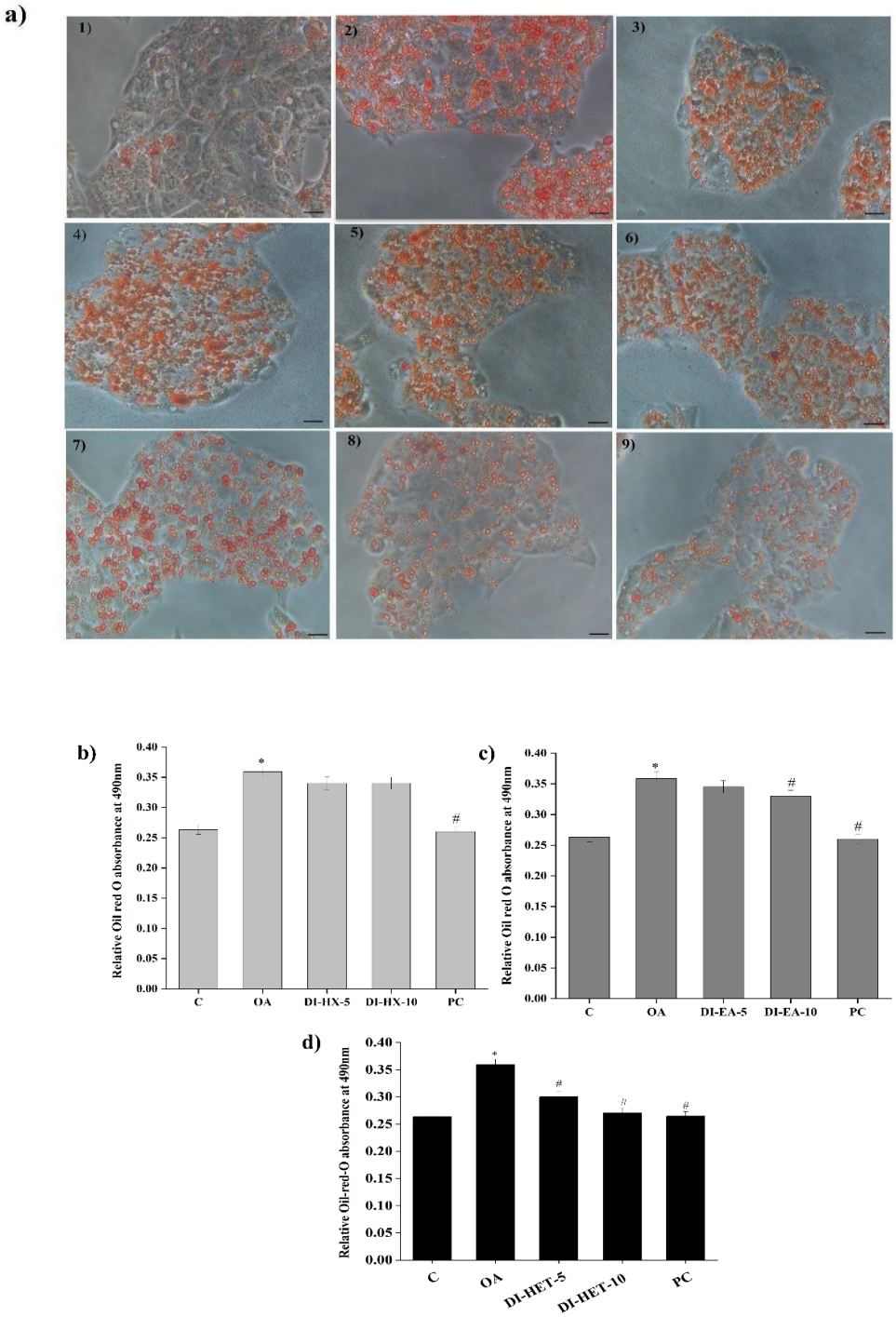

Figure.2.5 Effect of extracts on oleic acid-induced lipid accumulation in HepG2 cells: (A)The lipid accumulation was studied by Oil Red O staining. Representative phase-contrast microscopic images of Oil Red O stained HepG2 were presented, scale

bar 50 µm. (1) Control (C) (2) Oleic acid 100 µM (OA) (3) Oleic acid + 5 µg/mL extract (DI-HX-5) (4) Oleic acid + 10 µg/mL extract (DI-HX-10) (5) Oleic acid +5 µg/mL extract (DI-EA-5) (6) Oleic acid +10 µg/mL extract (DI-EA-10) (7) Oleic acid +5 µg/mL extract (DI-HET-5) (8) Oleic acid +10 µg/mL extract (DI-HET-10) (9) Oleic acid 100 µM + fenofibrate 20 µM (PC). b), c) & d) Absorbance was read at 490 nm after Oil-Red-O staining in different groups DI-HX, DI-EA & DI-HET. Data are expressed as mean ± SEM; where n=6. * denotes significant difference from the control group ($p \leq 0.05$) and # denotes significant difference from the OA treated group ($p \leq 0.05$).

2.3.6 DI-HET inhibits TG accumulation in HepG2 cells

To validate the result obtained from ORO staining, triglyceride accumulation in HepG2 cells was quantified with DI-HET treatment. Here also, the TG content was significantly decreased in a dose-dependent manner with both extracts (30.38% with DI-HET-5 and 41.18% with DI-HET-10; $p \leq 0.05$; Figure. 2.7) when compared to the OA group (45.86%). Fenofibrate-treated cells also showed a substantial reduction in TG levels by 42.71%.

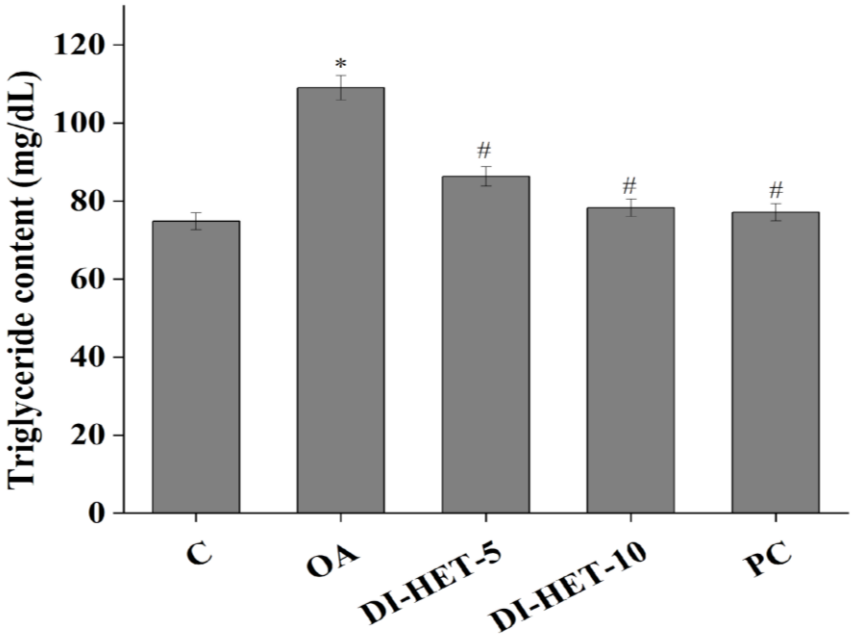

Figure 2.6 Effect of DI-HET on oleic acid-induced TG accumulation in HepG2 cells: Measurement of intracellular triglyceride content in HepG2 cells. Absorbance was spectrophotometrically measured at 530 nm. Data are expressed as mean ± SEM;

where n=6. * denotes significant difference from the control group ($p \leq 0.05$) and # denotes significant difference from the OA treated group ($p \leq 0.05$).

2.4 Discussion

One of the most common liver disorders in the world is NAFLD. The absence of FDA-approved medication and the increased prevalence rate of NAFLD demands novel drugs. Plant-based formulations are becoming more popular because of their low cost, few side effects, and pleiotropic biomedical properties. In this regard, there is a growing trend towards plant-based drug development. Several plant species are reported to have hepatoprotective functions. However, most of them are not scientifically validated. Hence, screening plants used in ethnomedicine will help to identify new phytoconstituent and their pharmacological activity to treat diseases like NAFLD.

D. indica, a common plant in Ayurveda and folk medicine, is relevant from the perspective of ethnopharmacology. Various plant parts of *D. indica* has many pharmacological effects, including those related to anticancer, antidiabetic, antihyperlipidemic, antileukemic, antioxidant, antimutagenic, antibacterial, antinociceptive, and antidiarrheal (Singh & Saha, 2019).

Leaves of *D. indica* have a wide range of therapeutic benefits. Studies have shown that the crude methanolic extract of *D. indica* leaves has been shown to have antibacterial and cytotoxic effects (Apu et al., 2010). *In vivo* study in diabetic mice shows that the oral administration of *D. indica* leaves extract significantly reduced blood sugar level, total lipid level, improved body weight, and liver and renal profile (S. Kumar et al., 2011). Research studies have demonstrated the anti-inflammatory property of the methanolic extract of *D. indica* leaves in mice (Yeshwante et al., 2009). *In vitro* evaluation of the antioxidant activity of *D. indica* Leaf extract also shows its potential in free radical scavenging (Shendge et al., 2011). The presence of biologically relevant bioactives in the *D. indica* leaves extracts are the reason for the therapeutic benefits.

According to scientific studies numerous flavonoids, triterpenoids (of the lupene type), phytosteroids, phenolics, alcohols, ketones, and an anthraquinone have been present in variously prepared extracts of this plant. Various phytochemical studies

have shown that several active ingredients, such as β-sitosterol, stigmasterol, betulin, betulinic acid, kaempferol, myricetin, quercetin, dillenetin, and rhamnetin in significant amounts. The two main phytoconstituent of these are betulin and betulinic acid (Singh & Saha, 2019).

Sequential extraction was done to analyze the phytoconstituent according to the polarity using hexane, ethyl acetate, and 70% hydroethanol. Hence from sequential extraction, three extracts DI-HX extract, DI-EA extract, and DI-HET extract, were obtained. All these three extracts were subjected to HPLC and LCMS/MS analysis for their qualitative and quantitative analysis. Partial characterization of plant extract through HPLC and LCMS/MS analysis revealed the presence of pharmacologically important phytoconstituents.

DI-HX extract contains twenty polyphenols that include vanillic acid, naringenin, tocopherol, syringic acid, p-coumaric acid, ferulic acid, catechin, cinnamic acid, epicatechin, caffeic acid, apigenin, myricetin, kaempferol, chrysin, genistein, hesperetin, epigallocatechin, gallic acid, luteolin, and quinine.

DI-EA extract contains twenty-two polyphenols that include naringenin, syringic acid, vanillic acid, ferulic acid, tocopherol, kaempferol, p-coumaric acid, catechin, epicatechin, ellagic acid, caffeic acid, luteolin, gallic acid, cinnamic acid, quercetin, morin, myricetin, hesperetin, daidzein, apigenin, chrysin, and genistein.

DI-HET extract contains twenty-two polyphenols that include naringenin, catechin, epicatechin, shikimic acid, syringic acid, vanillic acid, kaempferol, tocopherol, ferulic acid, cinnamic acid, gallic acid, luteolin, p-coumaric acid, myricetin, quercetin, hesperetin, apigenin, caffeic acid, chrysin, epigallocatechin, genistein, and quinine.

All the polyphenols present in the extract were consistent with the previous scientific reports. With literature support (Akter et al., 2022; Fu et al., 2015; M. M. Islam et al., 2013; Kaur et al., 2016; Meeprathom et al., 2018; Singh & Saha, 2019), the above-mentioned compounds were identified and quantified from leaves of *D. indica* extract using LCMS/MS.

Some scientific studies have reported that *D. indica* extract has hepatoprotective functions. Studies are found that hexane extract from *D. indica* seed shows hepatoprotective effects by decreasing the activity of serum enzymes, bilirubin, urea, creatinine, and lipid peroxidation (Reddy et al., 2010). Oral administration of *D. indica* methanolic leaves (DIME) extract in diabetic rats shows beneficial effects on blood glucose levels as well as improving kidney and liver functions and hyperlipidaemia due to diabetes (S. Kumar et al., 2011). The isolated compound from *D. indica* alcoholic extract improved lipid levels in diabetic rats (Kaur et al., 2016). Despite this, in traditional medicine, fruit decoction of *D. indica* was used to treat jaundice (Rai & Lalramnghinglova, 2010; Sharma et al., 2012).

To substantiate scientifically the hepatoprotective property of the extract of *D. indica* leaves, *in vitro* experiments were conducted to investigate its potential against NAFLD in an oleic acid-induced model in HepG2 cell lines. The OA-induced steatosis in HepG2 cells provides a unique model to study the changes in lipid metabolism in the hepatocytes (Cui et al., 2010). In humans, hepatic steatosis is linked to the buildup of too much oleic acid (OA), a monosaturated omega-9 fatty acid that is the byproduct of *de novo* fatty acid synthesis (Araya et al., 2004). HepG2 cells, a human hepatoblastoma cell line, exhibit characteristics of steatotic hepatocytes after being treated with OA.

DNL in the liver is a tightly regulated and complex metabolic pathway. Under normal physiological conditions, extra glucose is converted by DNL into fatty acids, which are then esterified to form storage triacylglycerols (TGs). Later on, these TGs provide energy through oxidation. This pathway is predominantly active in the liver and adipose tissue of humans. Dysregulation of lipogenesis is associated with diverse pathological conditions (Ameer et al., 2014). NAFLD is characterized by excess lipid accumulation in hepatocytes, and histologically steatosis can be defined as the deposition of lipid droplets greater than 5.5% in liver tissue (Bril et al., 2015). Enhanced lipogenesis during steatosis leads to triglyceride (TG) accumulation in hepatocytes. Therefore, intracellular TG level is a significant parameter that enhances NAFLD progression. Hence finding new compounds inhibiting DNL provides therapeutic benefits to tackle diseases like NAFLD.

Hence, the lipolytic effect of different extracts (DI-HX, DI-EA, and DI-HET) was studied in HepG2 cell lines using the Oil red O staining (ORO) method. ORO stain is a fat-soluble dye that specifically stains lipids in cells. ORO is transported from the dye solution to fat during staining, allowing the lipid droplets to be colored red (Du et al., 2023). Here we found that lipid accumulation was significantly reduced with DI-HET extract treatment in a concentration-dependent manner. While pretreatment with DI-HX and DI-EA extract showed no significant reduction in cell lipid accumulation. The spectrometrical analysis also showed the same result. Based on ORO staining method, the most effective DI-HET extract was selected for further studies. Therefore, TG accumulation with DI-HET treatment was quantified to validate the result obtained from the ORO staining method. Here also, the TG content was significantly decreased in a dose-dependent manner.

The major polyphenols present in DI-HET were naringenin, catechin, epicatechin, shikimic acid, syringic acid, vanillic acid, and kaempferol. These compounds have been extensively studied for their potential role in lipid metabolism. Naringenin, a flavonoid abundant in citrus plants, has demonstrated anti-obesity effects by inhibiting adipogenesis and promoting lipolysis during NAFLD (Rufino et al., 2021, Yang et al., 2022). Catechin and epicatechin, are bioactive compounds in green tea, have shown lipid-lowering properties by enhancing β-oxidation of fatty acid and reducing lipogenesis (Chen et al., 2020). Shikimic acid, is present in plants such as star anise, exhibits anti-hyperlipidemic effects by regulating lipid metabolism (Shahrajabian et al., 2019). Berries, fruits, and vegetables include syringic acid, vanillic acid, and kaempferol, which have been linked to better lipid profiles, reduced triglyceride levels, and stronger antioxidant defense (Davoodi et al., 2017; Ochiai et al., 2021; Shekari et al., 2021). These bioactive substances have the ability to regulate lipid metabolism, presenting potential therapeutic approaches to treat dyslipidemia and associated metabolic diseases.

In conclusion, the qualitative and quantitative analysis showed the robust presence of phytoconstituent in *D. indica* leaves extracts. As well as this study revealed that DI-HET extract was effective in lowering intracellular lipid when compared to DI-HX and DI-EA extract treatment. TG accumulation was also significantly reduced with DI-HET treatment. Most polyphenols in DI-HET are pharmacologically active

compounds, especially liver function-promoting ones. These include naringenin, catechin, epicatechin, shikimic acid, syringic acid, vanillic acid, and kaempferol, with traces of other compounds. Hence, DI-HET was selected for further detailed *in vitro* experiments to investigate its potential against NAFLD in an oleic acid-induced model in HepG2 cells.

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Chapter 3

Evaluation of DI-HET against oleic acid-induced lipogenesis in HepG2 cell lines

3.1 Introduction

NAFLD is characterized by the surplus fat in the hepatocytes of people with little or no consumption of alcohol. This causes serious stages of the disease (Papatheodoridi & Cholongitas, 2018). This is associated with various conditions like metabolic abnormalities, insulin resistance, steatosis, oxidative stress, inflammation, fibrosis, etc. (Cobbina & Akhlaghi, 2017). Cells in NAFLD are distinguished by an excessive buildup of triglycerides and cholesterol in lipid droplets (Gluchowski et al., 2017). No effective treatment or approved drug therapy for NAFLD is available now. It is difficult to establish a treatment for people with NAFLD due to the conditions like complex aetiology, complicated diagnosis, wide range of stages, and associated diseases. However, herbal medicine has thus far emerged as a substitute strategy for the prevention and/or treatment of NAFLD. Clinical studies have already proven the effectiveness of some natural remedies for treating NAFLD (Pan et al., 2013), including curcumin (Pan et al., 2013), resveratrol (Faghihzadeh et al., 2014), and berberine (Yan et al., 2015), which have been shown to improve NAFLD parameters.

Current drug discovery is encountering serious challenges due to low success rate and rising costs. This scenario has compelled us to come out with a novel approach to consolidated drug discovery, where Ayurvedic/traditional knowledge can synergize with drug discovery. Starting step in new drug discovery is the identification of new chemical entities. This can be obtained from natural sources through activity-based extraction and fractionation. The starting point for plant-based new drug discovery is the identification of the right plants. This is done by utilizing Ayurvedic insight, traditional knowledge, and elaborate literature search. The integration of traditional practice with drug discovery brings a fundamental change in extraction methods. Bioassay-based extraction and fractionation of the selected plant may lead to

standardized extract or druggable compound as the new drug. This integrated approach would save cost and time, coupled with an enhanced success rate in drug discovery.

Recently, the effect of obeticholic acid, a farnesoid X nuclear receptor ligand, has been tested in adult patients and found that it eased the liver histology suggesting the potential efficacy in the treatment of NAFLD (Sumida et al., 2020). However, the details of the limited benefits and safety of obeticholic acid in long-term application are still unknown. Lifestyle modifications like an increase in physical activity, weight reduction, consumption of antioxidants, restriction of high-energy food, etc. are recommended for control and management. These actions have only a limited impact on the incidence and severity of NAFLD at the population level and may not be effective for complete recovery. So, it is essential to have effective therapy for NAFLD. Although there are advances in conventional medicine, plant-derived traditional medicines are easily accessible, affordable, and do not require stringent synthesis. Thus, indigenous medicines seem highly attractive for the effective management of diseases including NAFLD. Herbal medicines, described as entire plants and unpurified plant extracts, have been traditionally used in various countries for liver diseases (Okaiyeto et al., 2018). There are reports about various extracts and natural compounds with liver function-promoting properties (Mohamed Saleem, 2010). Plant-based formulations for NAFLD were evaluated generally in various signaling pathways relevant to hepatic lipogenesis, beta-oxidation of fatty acids, antioxidant potential, anti-inflammatory potential, cellular lipid transport, etc. (Bagherniya et al., 2018).

In this study, the potential of *D. indica*, an edible plant was investigated against NAFLD in an *in vitro* model using HepG2 cells. Studies have revealed that *D. indica* plant extract exhibits lipid-lowering properties and has a traditional application in treating jaundice. Furthermore, the extract has demonstrated cardioprotective and hepatoprotective effects, along with potential benefits in terms of its anticancer, antidiabetic, antioxidant, and anti-inflammatory properties (Padhya et al., 2008, Reddy et al., 2010, Tene et al., 2021).

Based on the prior investigation in HepG2, employing ORO staining and examining TG accumulation, DI-HET extract was effective in reducing intracellular

lipid levels and TG accumulation. Hence the present chapter deals with the molecular mechanism through which DI-HET extract exerts its effect on lipid metabolism. An *in vitro* experimental model of oleic acid-induced NAFLD in HepG2 cells was employed in this study and studied the SIRT-1/p-LKB-1/AMPK signaling pathways in lipid metabolism.

3.2 Materials and methods

3.2.1 Chemicals

Minimum essential medium eagle (MEME) was from HiMedia (Mumbai, India). Fetal bovine serum (FBS), penicillin-streptomycin antibiotics, trypsin - ethylenediaminetetraacetate (EDTA), Hanks balanced saline solution (HBSS), and phosphate buffer saline (PBS) were bought from Gibco, USA. 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), oleic acid, dimethyl sulfoxide (DMSO), 2, 7- dichlorodihydrofluorescein diacetate (DCFH-DA), skimmed milk powder, RIPA buffer, protease inhibitor cocktail, and Oil Red O stain were purchased from Sigma-Aldrich Co. USA. The triglyceride and oxygen consumption assay kits were from Cayman, USA. MitoSOX Red was from Carlsbad, USA. BCA protein assay kit was from Bio-Rad Laboratories Pvt Ltd, and chemiluminescence detection kit was from Takara Bio Inc. USA. All primary and secondary antibodies used in western blotting were from Santa Cruz Biotechnology, USA. All other chemicals and solvents used were of analytical grade.

3.2.2 Cell culture and treatments

Hepatocellular carcinoma cells (HepG2) from National Centre for Cell Sciences (NCCS, Pune, India) were cultured and maintained in modified eagle medium (MEME) supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin solution. The cells were cultured in a humidified atmosphere with 5% CO2 and 37 ◦C. The experimental group consists of.

(a) Control group (C): HepG2 cells without any treatment are designated as C.

(b) OA group: HepG2 cells were incubated with 100 μM of OA for 24 h and indicated as OA.

(c) DI 5 group: HepG2 cells were pretreated with 5 μg/mL of extract for 2 h and followed by 100 μM of OA for 24 h designated as DI 5.

(d) DI 10 group: HepG2 cells were pretreated with 10 μg/mL of extract for 2 h and followed by 100 μM of OA for 24 h designated as DI 10.

(e) PC group: Cells were pretreated with fenofibrate (20 μM) for 2 h followed by OA (100 μM) for 24 h designated as PC.

3.2.3 Studies on lipid metabolism

After respective treatments, cells were lysed in RIPA buffer with the protease inhibitor cocktail. After incubation, cell suspensions were centrifuged at 12,000 RPM for 20 min at 4 ◦C. The supernatant was collected and used for further immunoblot analysis. The protein concentration was measured using the bicinchoninic acid kit. An equal amount of proteins were separated by 8 or 10% SDS-PAGE and transferred to PVDF. After blocking in TBS-Tween 0.1% containing 5% nonfat skimmed milk, membranes were incubated with primary antibodies overnight at 4 ◦C. AMPK function as a crucial regulator protein involved in lipid metabolism by directly phosphorylating proteins or altering the transcription of certain genes. Activation of AMPK leads to lipolysis and inhibition of lipogenic proteins. Hence, alterations of various proteins in lipid metabolism such as acetyl-CoA carboxylase (ACC), phosphorylated ACC (p-ACC), fatty acid synthase (FAS), adenosine monophosphate-activated protein kinase (AMPK), phosphorylated AMP-activated kinase (p-AMPK), apolipoprotein B100 (apo B100) 3-hydroxy-3- methyl-glutaryl-coenzyme A reductase (HMGCR), peroxisome proliferator-activated receptor- coactivator-1α (PGC-1α), peroxisome proliferator-activated receptor-α (PPAR-α), sirtuin-1 (SIRT-1), sterol regulatory element-binding protein-1C (SREBP-IC), sterol regulatory element-binding protein-2 (SREBP-2), cluster of differentiation-36 (CD36), peroxisomal acyl-coenzyme A oxidase-1 (ACOX-1), liver X receptor-α (LXR-α), carnitine palmitoyltransferase (CPT-1) and phosphorylated liver kinase B-1(p-LKB-1) were studied. After washing with TBS-Tween 0.1%, the membranes were incubated with HRP -conjugated corresponding secondary antibodies for 2–4 h at room temperature. Again, it was washed three times with TBS-Tween 0.1%. The membranes were developed using Western Blot Hyper

HRP Substrate, and the protein bands were analyzed (Bio-Rad ChemiDoc MP imaging systems, USA) (Mahmood & Yang, 2012).

3.2.4 Regulation of cholesterol biosynthesis by staining and western blot analysis

The cholesterol accumulation was measured using a cholesterol detection kit (Biovision). This assay is based on the staining of the Filipin III interaction with cholesterol. Interaction with cholesterol alters Filipin absorption and fluorescence spectra. This assay provides a simple and easy way to study cell cholesterol localization.

3.2.5 Statistical analysis

Statistical analysis was done using the SPSS statistical program. The results were represented as mean ± SEM. The significant differences among the treatments were evaluated using one-way analysis of variance (ANOVA). It is followed by Duncan's multiple range to determine which mean values were significantly different at $p \leq 0.05$. Statistical significance was indicated as * for $p \leq 0.05$ Vs C and # for $p \leq 0.05$ Vs OA.

3.3 Results

3.3.1 Activation of AMPK through SIRT-1 and p-LKB-1

There was a significant decrease in SIRT-1 expression with OA treatment (38.85%; $p \leq 0.05$) compared to the control group. Pretreatment with DI-HET significantly increased the expression of SIRT-1(32.51% with DI 5 and 54.16% with DI 10; $p \leq 0.05$; Figures. 3.1. a & b) when compared to the OA group. The expression of SIRT-1 was enhanced with fenofibrate treatment by 35.94% (Figures. 3.1.a & b). The expression of the phosphorylated form of LKB-1 was significantly downregulated with OA treatment (15.38%; Figures. 3.1.a & c compared to the control group. Pretreatment with DI-HET significantly increased the expression of p-LKB-1 (29.73% with DI 5 and 51.86% with DI 10; Figures. 3.1.a & c) compared to the OA group.Western blot showed phosphorylation of AMPK was inhibited significantly in the OA group (13.33%, $p \leq 0.05$), and pretreatment with DI-HET extract significantly increased p-AMPK (14.63% with DI 5 and 30.15% with DI 10; $p \leq 0.05$; Figures. 3.1.a & d). Fenofibrate treatment also significantly increased the expression of p-AMPK (8.10%; $p \leq 0.05$) compared to the OA group.

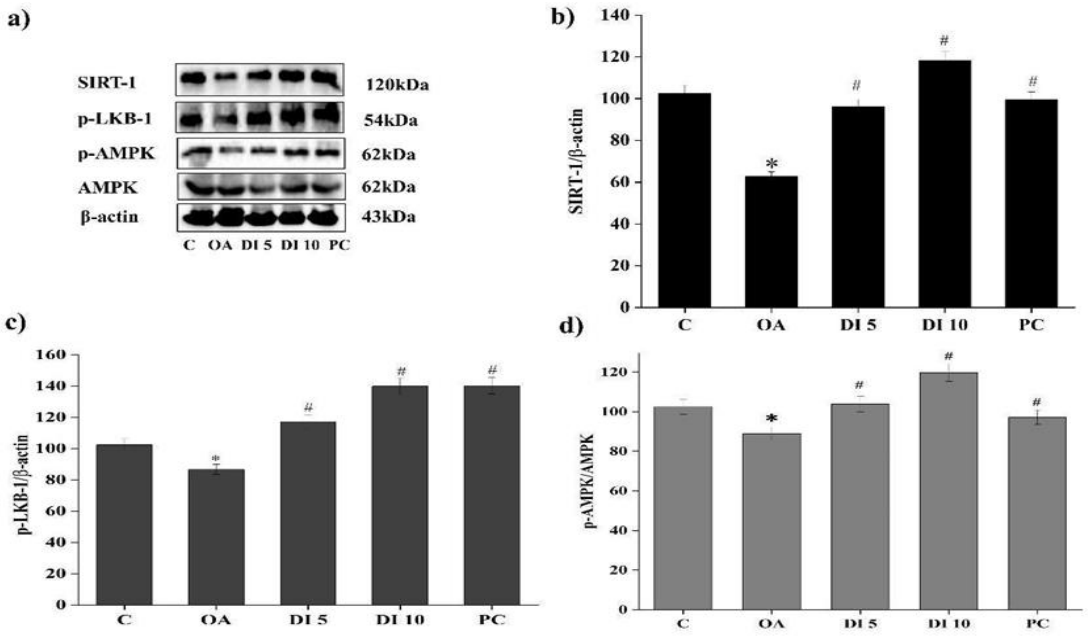

Figure 3.1. Effect of DI-HET on SIRT-1, p-LKB-1, and p-AMPK/AMPK: (a) The protein expression in different experimental groups. (b-d) The relative intensity of each band was quantified with β-actin. Control (C); Oleic acid 100 µM (OA); Oleic acid + 5 µg/mL extract (DI 5); Oleic acid + 10 µg/mL extract (DI 10); Oleic acid 100 µM + fenofibrate 20 µM (PC). Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the control group ($p \leq 0.05$) and # denotes significant difference from the OA treated group ($p \leq 0.05$).

3.3.2 Effect of DI-HET on lipogenic proteins like LXR-α, SREBP-1C and FAS

Upregulated LXR-α (24.4%; $p \leq 0.05$; Figures. 3.2.a & b) and SREBP-1C (39.31%; $p \leq 0.05$; 3.2.a and c) expression in the OA group was reversed with pretreatment with DI-HET. The expression of both LXR-α (27.82% with DI 5 and 35.65% with DI 10; $p \leq 0.05$; Figures. 3.2.a & b) and SREBP-1C were down-regulated with DI-HET (16.4% with DI 5 and 52.59% with DI 10%; $p \leq 0.05$; Figures. 3.2.a & c). Fenofibrate treatment also decreased the expression of LXR-α and SREBP-1C by 47.03% and 48.89%, respectively ($p \leq 0.05$).

Consistent with the suppression of SREBP1-C, the expression of FAS, the target protein was also downregulated with DI-HET pretreatment (12.84% with DI 5 and 32.47% with DI 10; $p \leq 0.05$ Figures. 3.2.a & d) when compared to the OA group (51.95%; $p \leq 0.05$). The expression of FAS was also significantly reduced (23.84%; $p \leq 0.05$) with fenofibrate treatment compared to the OA group.

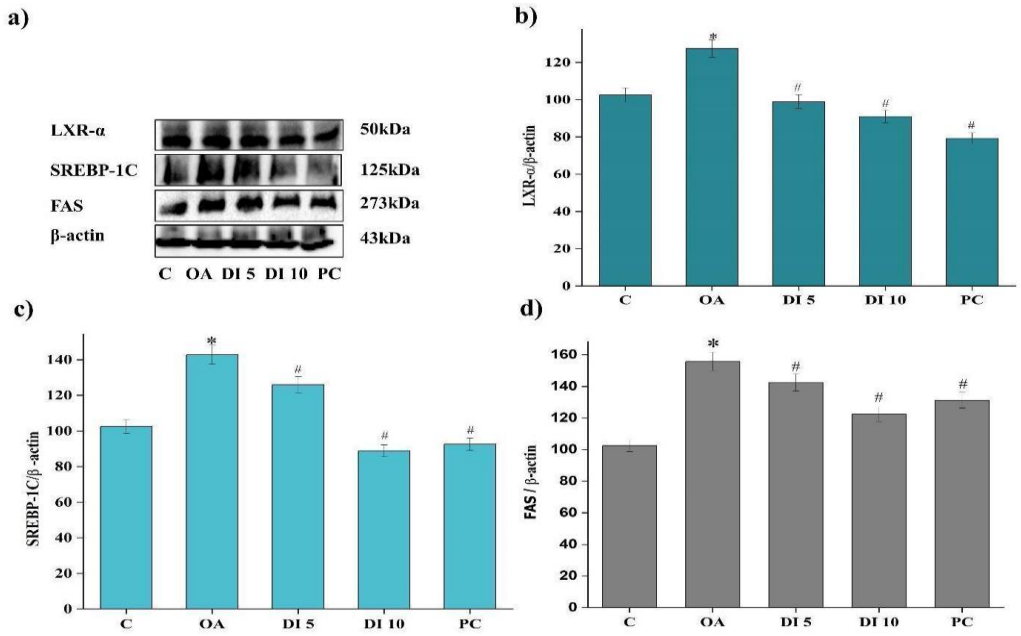

Figure 3.2. Effect of DI-HET LXR-α, SREBP-1C, and FAS: (a) The protein expression in different experimental groups. (b-d) The relative intensity of each band was quantified with β-actin. Control (C); Oleic acid 100 µM (OA); Oleic acid + 5 µg/mL extract (DI 5); Oleic acid + 10 µg/mL extract (DI 10); Oleic acid 100 µM + fenofibrate 20 µM (PC). Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the control group ($p \leq 0.05$) and # denotes significant difference from the OA treated group ($p \leq 0.05$).

3.3.3 Regulation of p-ACC, ACC, and CPT-1 with DI-HET pretreatment

There was a significant downregulation of the phosphorylated form of ACC in the OA group by 48.73% ($p \leq 0.05$) compared to the control group. The expression of the phosphorylated form of ACC was significantly increased with DI-HET treatment (35.63% with DI 5 and 36.74% with DI 10; $p \leq 0.05$; Figures. 3.3.a & b) when compared to the OA group. Fenofibrate treatment also increased the expression of phosphorylated forms of ACC by 17.10% ($p \leq 0.05$).

The expression of CPT-1 was reduced with OA treatment (25.63%; $p \leq 0.05$ Figures. 3.3.a & c) compared to the control group. Pretreatment with DI-HET significantly increased the expression of CPT-1(28.35% with DI 5 and 27.71% with DI 10; $p \leq 0.05$; Figures. 3.3.a & c) when compared to the reduction caused by OA group. Fenofibrate treatment also upregulated the expression of CPT-1 by 29.16% ($p \leq 0.05$).

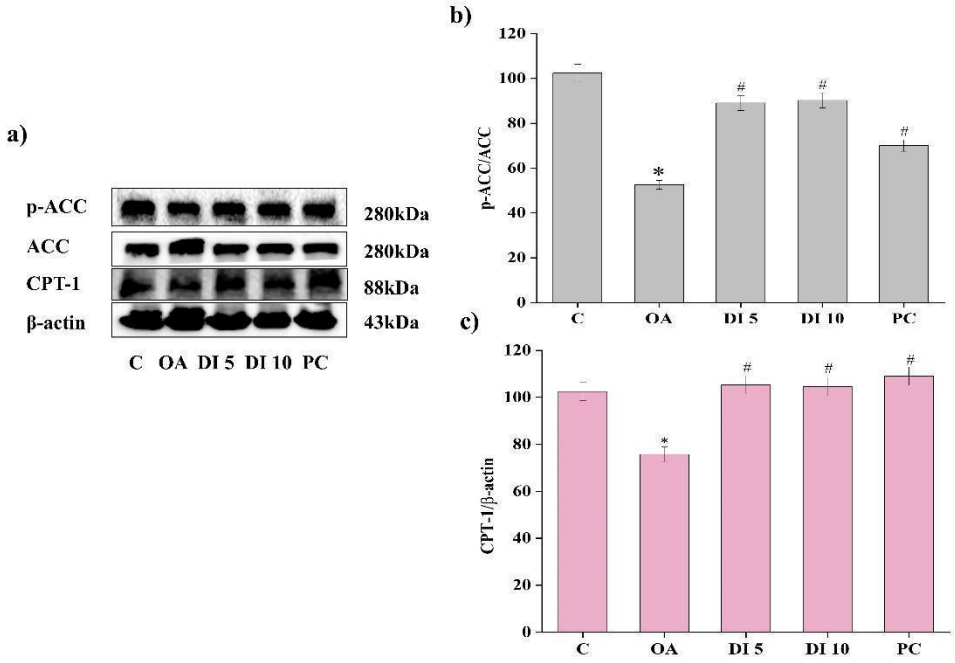

Figure 3.3. Effect of DI-HET p-ACC/ACC and CPT-1: (a) The protein expression in different experimental groups. (b-c) The relative intensity of each band was quantified with β-actin. Control (C); Oleic acid 100 µM (OA); Oleic acid + 5 µg/mL extract (DI 5); Oleic acid + 10 µg/mL extract (DI 10); Oleic acid 100 µM + fenofibrate 20 µM (PC). Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the control group ($p \leq 0.05$) and # denotes significant difference from the OA treated group ($p \leq 0.05$).

3.3.4 Pretreatment with DI-HET regulates CD-36, ACOX-1 and apoB 100

The expression of CD36 was found increased with OA treatment (26.84%; $p \leq 0.05$) when compared to the control group. The expression of CD36 was significantly reduced with DI-HET pretreatment (31.54% with DI 5 and 59.59% with DI 10; $p \leq 0.05$; Figures. 3.4.a & b) when compared to the OA group. With fenofibrate treatment, CD36 expression has been reduced by 64.85%.

The level of ACOX-1 was significantly increased with OA treatment (41%; $p \leq 0.05$) compared to the control group. The expression of ACOX-1 was significantly reduced

with DI-HET pretreatment (7.58% with DI 5 and 36.04% with DI 10; $p \leq 0.05$; Figures. 3.4.a & c) when compared to the OA group ACOX-1 expression was also reduced with fenofibrate treatment by 25.88% ($p \leq 0.05$).

There was a significant increase in the level of apoB 100 in the OA treated group (23.53%; $p \leq 0.05$ Figures. 3.4.a & d) when compared to control. Pretreatment with DI-HET significantly decreased the expression of apoB 100 (81.92% with DI 5 and 99.2% with DI 10; $p \leq 0.05$; Figures. 3.3.a & d) when compared to the OA treated group. Fenofibrate treatment also downregulated the expression of apoB 100 significantly by 38.6% ($p \leq 0.05$).

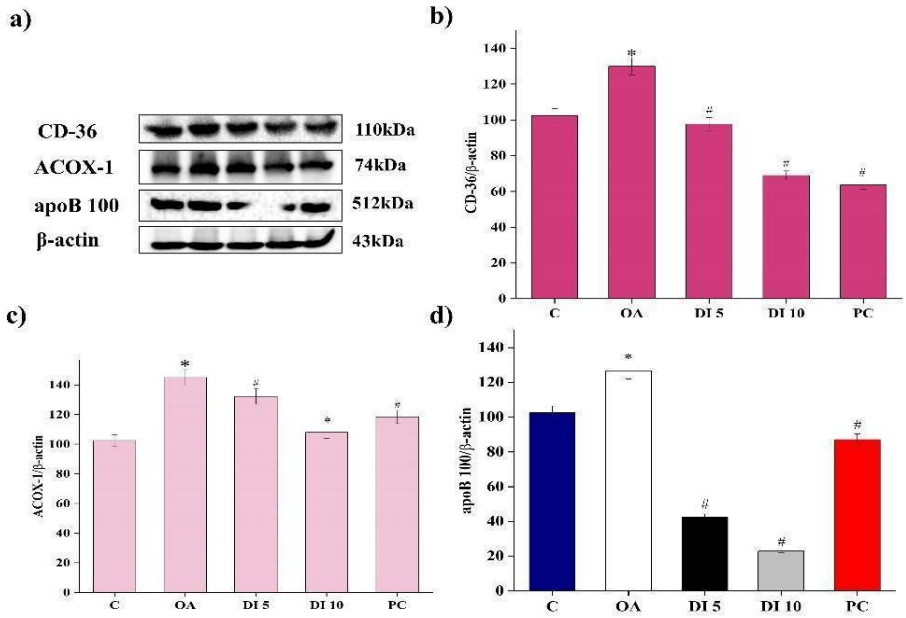

Figure 3.4. Effect of DI-HET CD36, ACOX-1, and apoB 100: (a) The protein expression in different experimental groups (b-d). The relative intensity of each band was quantified with β-actin. Control (C); Oleic acid 100 µM (OA); Oleic acid + 5 µg/mL extract (DI 5); Oleic acid + 10 µg/mL extract (DI 10); Oleic acid 100 µM + fenofibrate 20 µM (PC). Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the control group ($p \leq 0.05$) and # denotes significant difference from the OA treated group ($p \leq 0.05$).

3.3.5 Upregulation of transcription factors like PPAR-α and PGC-1α involved in lipid metabolism

The expression of PGC-1α was decreased in the OA group (43.48 %; $p \leq 0.05$) when compared to control (Figures. 3.5.a & b). There was an increased expression of PGC-1α with extract (122.03% with DI 5 and 133.28 % DI 10). Fenofibrate treatment also increased the expression of PGC-1α (76.09%).

Consistent with the expression of PGC-1α, the protein expression of PPAR-α was also decreased in the OA group (40%; $p \leq 0.05$) when compared to control (Figures. 3.5.a & c). There was an increased expression of PPAR-α with extract (33.31% with DI 5 and 34.37% DI 10). Fenofibrate treatment also increased the expression of PPAR-α (24.46%).

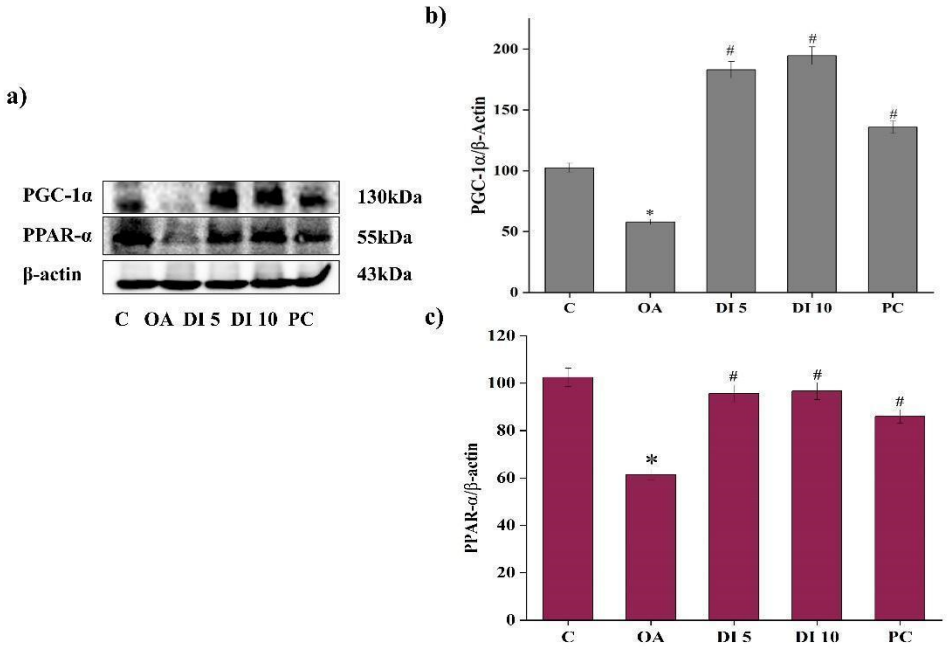

Figure 3.5 Effect of DI-HET PGC-1α and PPAR-α: (a) The protein expression in different experimental groups. (b-c) The relative intensity of each band was quantified with β-actin. Control (C); Oleic acid 100 µM (OA); Oleic acid + 5 µg/mL extract (DI 5); Oleic acid + 10 µg/mL extract (DI 10); Oleic acid 100 µM + fenofibrate 20 µM (PC). Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the control group ($p \leq 0.05$) and # denotes significant difference from the OA treated group ($p \leq 0.05$).

3.3.6 Effect of DI-HET in cholesterol biosynthesis

Treatment with OA upregulated the expression of SREBP-2 by 11.70% ($p \leq 0.05$). Pretreatment with DI-HET caused a significant drop in the expression of SREBP-2 (10.42% with DI 5 and 18.13% with DI 10; $p \leq 0.05$; Figures. 3.6.a & b) and with fenofibrate treatment, SREBP-2 expression was reduced by 56.43%. Consistent with the reduction in SREBP-2 the expression of HMGCR was also reduced with DI-HET treatment (24.43% with DI 5 and 39.28% with DI 10; $p \leq 0.05$; Figures. 3.6.a & c) when compared with an increase seen with OA (23.23%). Treatment with fenofibrate also caused a significant reduction in HMGCR expression by 55.18%.

The cholesterol detection study showed increased cholesterol with OA treatment (137.4%; $p \leq 0.05$) compared to the control group. There was a reduction in cholesterol accumulation with DI-HET treatment (87.4% with DI 5 and 107.4% DI 10; $p \leq 0.05$) compared to the OA group. Pretreatment with fenofibrate (112.4%; $p \leq 0.05$) and cholesterol inhibitor (U-18666A) (187.4%; $p \leq 0.05$) also reduces cholesterol in HepG2 cells (Figures. 3.7.a & b).

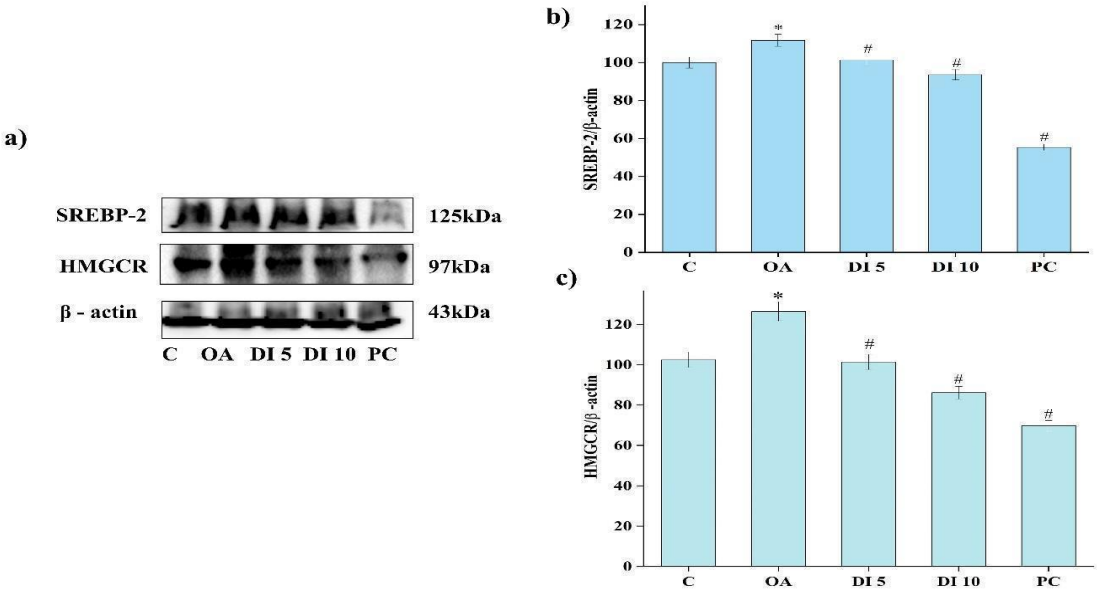

Figure.3.6. Effect of DI-HET on SREBP-2, and HMGCR: (a) The protein expression in different experimental groups. (b-c) The relative intensity of each band was quantified with β-actin. Control (C); Oleic acid 100 µM (OA); Oleic acid + 5 µg/mL extract (DI 5); Oleic acid +10 µg/mL extract (DI 10); Oleic acid 100 µM + fenofibrate 20 µM (PC). Data are expressed

as mean ± SEM; where n = 6. * denotes significant difference from the control group ($p \leq 0.05$) and # denotes significant difference from the OA treated group ($p \leq 0.05$).

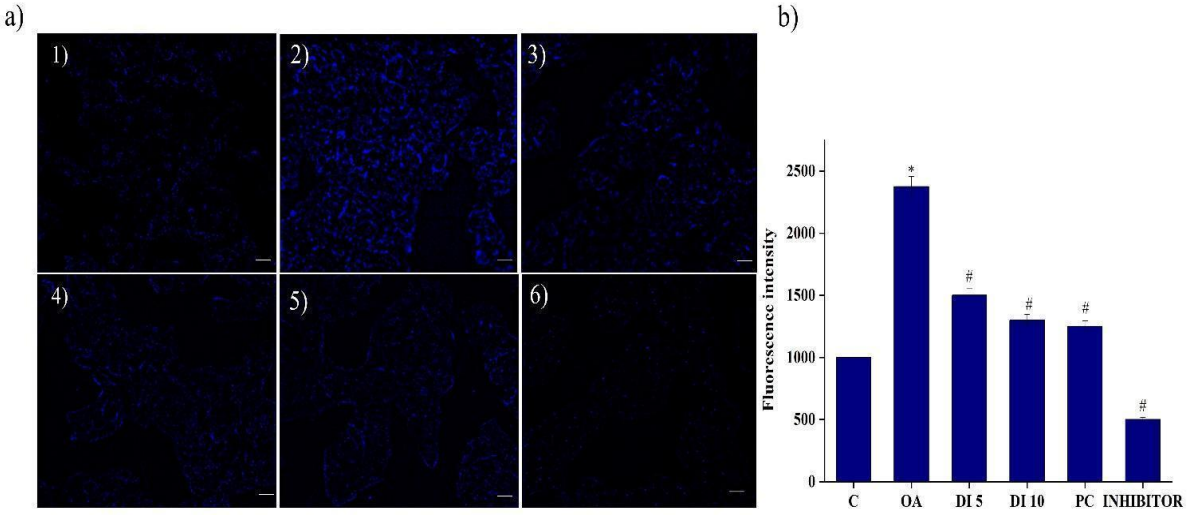

Figure.3.7. Effect of DI-HET on cholesterol biosynthesis in HepG2 cells: (a)The aberrant cholesterol accumulation was assessed by cholesterol detection kit. Representative phase-contrast microscopic images of stained HepG2 cells were presented, scale bar 20 µm. b) Bar diagram showing fluorescence intensity of each group (1) Control (C) (2) Oleic acid 100 µM (OA) (3) Oleic acid + 5 µg/mL extract (DI 5) (4) Oleic acid +10 µg/mL extract (DI 10) (5) Oleic acid 100 µM + fenofibrate 20 µM (PC) (6) (5) Oleic acid 100 µM + inhibitor. B) Fluorescence intensity was measured in control and treated groups. Values are expressed as mean ± SEM where n = 6. * indicates significantly different from the control group. # indicates significantly different from the OA induced group ($p \leq 0.05$).

3.4 Discussion

NAFLD, one of the most prevalent liver diseases globally, presents a complex aetiology that impacts hepatocytes, adipose tissue, the stomach, and muscular tissue. With the absence of an approved, effective treatment, the demand for innovative drugs to combat NAFLD has surged, leading to extensive research in laboratories worldwide. In this pursuit, medicinal plants have garnered attention as potential sources for drug discovery in NAFLD treatment. Encouragingly, clinical studies have revealed the effectiveness of phytocompounds against NAFLD, offering hope amidst this challenging drug development (Cicero et al., 2018).

Here in, various biochemical pathways involved in the OA-induced lipogenesis in HepG2 cells were studied to evaluate the potential of hydroethanolic extract of *D. indica* against NAFLD. OA incubation caused the build-up of triglyceride in HepG2 cells via surplus lipogenesis. It is through downregulating p-AMPK and associated downstream pathways such as p-ACC, CPT -1, PGC-1α, and PPAR-α. On the other hand, it also upregulated lipogenesis, promoting enzymes and transporters like CD36, ACOX-1, FAS, HMGCR, SREBP-1C, SREBP-2, apoB 100, and LXR-α.

AMPK is a vital energy detector of intracellular energy metabolism (Grahame Hardie, 2016). It regulates cellular triglyceride and cholesterol production. The phosphorylation of AMPK abates free fatty acid-mediated *de novo* lipogenesis genes and hepatic lipid build-up. Based on this, AMPK phosphorylation has been studied as an important biochemical pathway customarily in hepatic lipid metabolism to assess the potential of many herbal medicines (Leng et al., 2018; Viollet et al., 2006). The extract has increased phosphorylation of AMPK, revealing its potential to reduce lipid accumulation via promoting fatty acid oxidation in HepG2 cells. AMPK activation also occurs by upstream kinase such as LKB-1 and is also found increased with the extract. LKB-1 is a primary upstream kinase of AMPK, a necessary element in cell metabolism that is required for maintaining energy homeostasis. LKB-1 regulates lipid metabolism in its uptake, *de novo* synthesis, and fatty acid β-oxidation (Kwon et al., 2018).

SIRT-1 is a regulator of LKB-1 that is also found to increase with extract, which again confirms AMPK phosphorylation. The role of SREBP-1C for the regulation of lipogenesis in the liver has been well established by several lines of evidence (Hao et al., 2016). It is known that activated AMPK also inhibits SREBP-1C. This, in turn, will downregulate the expression of ACC and FAS (Horton et al., 2002; Shimano, 2001). This will result in increased mitochondrial β-oxidation and decreased fatty acid synthesis. The extract was found to decrease SREBP-1C. ACC is an enzyme involved in fatty acid biosynthesis. Phosphorylation of ACC causes the downregulation of fatty acid biosynthesis in cells (Randy et al., 2016). The same has been found enhanced with extract preventing biosynthesis of fat. In hepatocytes, FAS is considered as a housekeeping protein, creating fat for the storage of energy when resources are abundant. (Jensen-Urstad & Semenkovich, 2012). Based on this it is an attractive

therapeutic target for treating fatty liver and dyslipidemia. Interestingly FAS was found to decrease with extract supporting its benefits against NAFLD. From these results, there is a clue for its liver function-promoting properties via phosphorylation of AMPK during acute lipogenesis.

Hereafter cholesterol synthesis pathway was studied. For this alteration of SREBP-2 and HMGCR with extract were seen at the protein level. SREBP-2 regulates genes involved in cholesterol biosynthesis and homeostasis. SREBP-2 activates the transcription of mevalonate pathway genes and HMGCR enzymes (Brown & Goldstein, 1997). Both SREBP-2 and HMGCR were found to have decreased with extract revealing its potential to prevent hypercholesterolemia. Hypercholesterolemia is a serious risk factor for NAFLD. Cholesterol detection using the staining method also showed reduced cholesterol accumulation consistent with the downregulation of critical proteins involved in cholesterol synthesis, like SREBP-2 and HMGCR.

Hereafter the fatty acid translocase protein CD36 regulation was studied, CD36 aid in the transport of long-chain fatty acids and that controlled by PPARγ, liver X receptor-α (LXR-α), pregnane X receptor (Rada et al., 2020) was studied. In adolescents and adults of both genders, diagnosed with hepatic steatosis, the CD36 gene, and protein expression were increased compared to healthy controls (Ipsen et al., 2018). Interestingly enough, the extract was found to decrease its activity supporting its application for NAFLD.

LXRs play a critical role in cholesterol homeostasis and bile acid metabolism (Zhu et al., 2012). This establishes LXR-α as a master lipogenic transcription factor, as it directly regulates both SREBP-1C and ChREBP to enhance hepatic fatty acid synthesis. LXR-α is found to decrease with extract paving the way for the inhibition of fatty acid synthesis.

ACOX-1 is a rate-limiting enzyme for fatty acid β-oxidation in the peroxisome, involved in metabolism, progression of steatosis, and gradual hepatocellular damage with time (Chen et al., 2018). ACOX-1 is reported in a very high amount in patients with NAFLD compared to controls (Ipsen et al., 2018). However, the peroxisomes generate ROS as they oxidize fatty acids, and likewise, the

peroxisomes may induce oxidative stress and promote disease progression (Islam et al., 2020).

Apolipoprotein (apo) B is a large, amphipathic glycoprotein crucial to the metabolism of human lipoproteins. The two apoB variants, apo B-48 and apo B-100 are generated from the APOB gene through a specific posttranscriptional editing procedure. Producing chylomicrons in the small intestine apoB-48 is necessary, while apo B-100 helps in making VLDL in the liver. Besides, apo B-100 also acts as a ligand for LDL-receptor-mediated endocytosis of LDL particles (Whitfield et al., 2004). Scientific studies have demonstrated the plasma level of apoB-100 is elevated in NAFLD patients. It also increases the risk of atherosclerosis, CVDs, and the progression of NAFLD (Deprince et al., 2020). Treatment with extract reduced the expression of apo B-100 while its expression was upregulated with OA.

PGC-1α is a powerful coactivator of numerous transcription factors affecting overall body energy metabolism. PGC-1α coordinates an increase in the rate of respiration and biogenesis in mitochondria as well as in the uptake and use of substrates for energy production. PGC-1α directly coactivates numerous transcription factors, including nuclear receptors like the PPARs, in order to carry out such a broad range of signaling pathways (Cantó & Auwerx, 2009). The expression of PGC-1α was downregulated with OA treatment while reversed with the extract. PPAR-α is a ligand-activated transcriptional factor belonging to the nuclear receptor family. PPAR-α regulates protein expression in fatty acid beta-oxidation and energy homeostasis (Wang et al., 2020). PPAR ligands have been researched as potential target for NAFLD due to their significant role in the transcriptional control of glucose and lipid metabolism. The extract was found to upregulate its expression against OA-induced inhibition. It is worth mentioning that DI 10 was found more effective than DI 5 though both extracts were beneficial. Similarly, fenofibrate was also found effective in this *in vitro* study. From the overall data, DI-HET extract was effective against OA-induced NAFLD in HepG2 cells. This is accomplished via activation of the SIRT-1/p-LKB-1/AMPK pathway, promotion of mitochondrial function, and inhibition of lipogenesis.

The robust presence of pharmacologically active phytochemicals in the extract may be the reason for the protective role against OA-induced NAFLD in HepG2 cells.

The extract consists of polyphenols like naringenin, catechin, epicatechin, shikimic acid, syringic acid, vanillic acid, and kaempferol with traces of other compounds. Naringenin acts as an antidyslipidemic by regulating hepatocyte TG accumulation by activating PPAR-α, SIRT-1, and LKB-1 (Goldwasser et al., 2010; Hua et al., 2021). Catechin is found to have robust antioxidant activity and antidyslipidemic activity through the inhibition of cholesterol and triglyceride synthesis by the activation of LKB-1/AMPK (Hui et al., 2020; Murase et al., 2009). Shikimic acid is found to attenuate mRNA expression of *de novo* lipogenesis-related genes such as FAS, SREBP-1C, and LXR- α in the HepG2 and 3T3L-1 cells(Kim et al., 2019). In addition, shikimic acid was found to activate AMPK and p-ACC (Kim et al., 2019). Syringic acid is a potent antioxidant and antidyslipidemic in hepatocytes (Vo et al., 2020). It also decreases hepatic lipogenic enzymes and elevates fatty acid oxidation enzymes (Ham et al., 2016). Vanillic acid is found to activate AMPK to mediate its therapeutic activity against NAFLD (Shekari et al., 2021). Kaempferol decreases the expression of LXR-α and also has antioxidant properties (Xiang et al., 2021). It also reduces TG and cholesterol and activates PPAR-α in hepatocytes (Chang et al., 2011). In addition to the above-mentioned phytochemicals, the presence of pharmacologically important bioactives with comparatively lesser concentrations was also found. These bioactives were also expected to contribute to the anti-NAFLD properties of DI-HET.

From the overall data, *D. indica* hydroethanolic extract was found to be effective against OA-induced NAFLD in HepG2 cells. This is accomplished via activation of the SIRT-1/p-LKB-1/AMPK pathway and inhibition of lipogenesis. Also found that treatment with extract reduces the accumulation of cholesterol by regulating SREBP-2 and HMGCR. Various bioactives compounds such as naringenin, catechin, epicatechin, shikimic acid, syringic acid, vanillic acid, kaempferol, and traces of other compounds found in the extract are expected to contribute to its beneficial effects.

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Chapter 4

Protective role of DI-HET extract in OA-induced NAFLD via antioxidant, anti-inflammatory, and autophagy regulation.

4.1 Introduction

The prevalence of chronic liver ailments like NAFLD is characterized by the buildup of fat in the liver. It has many associated complications including insulin resistance, high blood pressure, dyslipidemia, diabetes, and heart disease. Uncertainty exists regarding the molecular mechanisms underlying the development and progression of NAFLD. Reactive oxygen species (ROS)-induced oxidative stress (OS) and inflammation are probably essential mechanisms that can cause hepatic cell death, tissue damage, and disease progression. In NAFLD, the overproduction of ROS primarily stems from mitochondrial abnormalities, down-regulation of multiple antioxidant enzymes, leukocyte infiltration, and hepatic inflammation. In NAFLD, excessive ROS generation reduces the effectiveness of other antioxidant defense mechanisms and increases oxidative damage. Oxidative stress in NAFLD leads to aberrant cellular activities and increased cell death, highlighting an imbalance between the cellular antioxidant system's availability and the uncontrolled generation of ROS (Farzanegi et al., 2019). ROS have physiological roles at low concentrations, but at elevated levels, they can interact with cellular components. Free radicals can harm DNA, resulting in chromosome instability, altered gene expression, genetic mutations linked to cell death, and liver damage, which will cause more severity to fatty liver conditions. They can also cause the oxidation of proteins and lipids, changing the function of enzymes, structural proteins, and cell membranes (Dornas & Schuppan, 2020).

An important step in the development of NAFLD is hepatic inflammation. NAFLD is a proinflammatory condition that promotes the development of NASH. In reality, while “pure” steatosis does not have a negative impact on the course of NAFLD, inflammation and its principal complication, fibrosis, are crucial factors in determining the prognosis of the condition over the long run (Padwal et al., 2003). When the

liver is overloaded with lipids, the kinds of fats that build up and how the liver cells respond to this lipid burden may lead to adaptation with the emergence of isolated hepatic steatosis or may result in cell death by various diverse molecular processes. The latter causes hepatocytes to generate stressed signals, also known as danger signals, which activate infection-free inflammatory pathways over time, leading to chronic damage and an aberrant wound-healing response with fibrosis. In NASH, inflammation arises as a secondary effect of the hepatic lipotoxic attack. The increased production of inflammatory substances from both extrahepatic locations (such as adipose tissue and the gut) and the liver itself is associated with inflammation. Kupffer cells and hepatocytes contribute to this inflammation through their activation. (Bessone et al., 2019). Recent studies have shown that proinflammatory mediators like NF-κB, TNF-α, and interleukins are produced in large amounts and are upregulated in NAFLD patients. Rats with NAFLD exhibited elevated levels of TNF-α in their livers, while the anti-inflammatory cytokine IL-10 was significantly decreased (Hajighasem et al., 2018).

An important cellular function that links the control of autophagy in NAFLD and other metabolic diseases is inflammation. The “self-eating” process of autophagy keeps the cells’ homeostasis in check (Schneider & Cuervo, 2014). Recent scientific and clinical research shows several human disorders, including liver disease, cardiovascular and cerebrovascular diseases, neurodegenerative diseases, neoplastic diseases, and malignancies, have been linked to autophagic anomalies. Pharmacological regulation of autophagy may provide potential therapeutic approaches to a wide spectrum of illnesses. Initially, the role of autophagy in the pathophysiology of NAFLD was highlighted by its involvement in the breakdown of intracellular lipids in hepatocytes, which can potentially control the development of hepatic steatosis. However, subsequent research has unveiled additional significant roles of autophagy. These include regulating insulin sensitivity, hepatocellular damage, innate immunity, fibrosis, and even carcinogenesis in various hepatic cell types such as hepatocytes, macrophages, and stellate cells. According to these findings, autophagy may have a variety of mechanistic roles in the emergence of NASH, its progression to NAFLD, and its consequences (Czaja, 2016).

Hence in this study, efforts were made to check the antioxidant properties of DI-HET and the molecular mechanism by which its antioxidant potential controls inflammation. In addition, the effect of the extract on autophagy was also studied.

4.2 Materials and methods

4.2.1 Chemicals

Minimum essential medium eagle (MEME) was from HiMedia (Mumbai, India). Fetal bovine serum (FBS), penicillin-streptomycin antibiotics, trypsin - ethylenediaminetetraacetate (EDTA), Hanks balanced saline solution (HBSS), and phosphate buffer saline (PBS) were bought from Gibco, USA. 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), oleic acid, dimethyl sulfoxide (DMSO), 2, 7- dichlorodihydrofluorescein diacetate (DCFH-DA), RIPA buffer, protease inhibitor cocktail, and skimmed milk powder were purchased from Sigma-Aldrich Co. USA. The triglyceride and oxygen consumption assay kits were from the Cayman, USA. MitoSOX red was from Carlsbad, USA. BCA protein assay kit was from Bio-Rad Laboratories Pvt Ltd, and the chemiluminescence detection kit was from Takara Bio Inc. USA. Beclin1 and LC3 were from Immunotag, Geno Technology Inc., USA. All other primary and secondary antibodies used in western blotting were from Santa Cruz Biotechnology, USA. All other chemicals and solvents used were of analytical grade.

4.2.2 Cell culture and treatments

Hepatocellular carcinoma cells (HepG2) obtained from the National Centre for Cell Sciences (NCCS, Pune, India) were cultured and maintained in a humidified atmosphere at 37°C with 5% CO2. The cells were cultured in modified eagle medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin solution. Cultures were utilized when they reached 80% confluency. The experimental group consists of.

(a) Control group: HepG2 cells without any treatment are designated as C.

(b) OA group: HepG2 cells were incubated with 100 μM of OA for 24 h and indicated as OA.

(c) DI 5 group: HepG2 cells were pretreated with 5 μg/mL of extract for 2 h and followed by 100 μM of OA for 24 h designated as DI 5.

(d) DI 10 group: HepG2 cells were pretreated with 10 μg/mL of extract for 2 h and followed by 100 μM of OA for 24 h designated as DI 10.

(e) PC group: Cells were pretreated with fenofibrate (20 μM) for 2 h followed by OA (100 μM) for 24 h designated as PC.

4.2.3 Determination of antioxidant potential of DI-HET

4.2.3.1 Determination of DPPH radical scavenging activity

The assessment of the extract's ability to scavenge free radicals was performed using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) technique (Shimada et al., 2002). When DPPH interacts with an antioxidant that has the ability to donate hydrogen, it gets reduced and colour changes from violet to yellow. The radical scavenging activity is proportional to the colour change. 100μL of DPPH and 100μL of extract were present in a 200μL reaction mixture at varying concentrations (20–300μg). Instead of a sample, 70% hydroethanol was used in the control. Antioxidants in the sample reduce the DPPH and the subsequent drop in absorbance at 517 nm was compared to the reagent blank (70% hydroethanol alone). Gallic acid at various concentrations (10-20μg) was used as the standard.

The percentage inhibition of DPPH by the extract was calculated using the following method:

% inhibition = [(A0-A1)/ A0 × 100]

The calculation of the percentage inhibition of DPPH by the extract is based on the absorbance values. A0 represents the absorbance of the control, while A1 represents the absorbance in the presence of the extract or standard.

4.2.3.2 ABTS radical scavenging activity

The capability of extracts and standard compound to scavenge ABTS radical was studied using ABTS scavenging assay (Re et al., 1999). In the presence of potassium persulphate, ABTS is oxidised to ABTS+ (coloured radical cation). Antioxidants in extract scavenge ABTS+, which reduces dark green colour to

colourless and absorbance was measured at 734 nm. A 1:1 volume ratio of 7mM ABTS and 2.45mM potassium persulfate was used to create the ABTS stock solution, which was then incubated for 12 h at room temperature and in the dark. The ABTS solution was diluted prior to use to achieve an absorbance of 0.675–0.725 at 734 nm. Samples were obtained at various concentrations (between 40 and 300 μg/mL). 50 μL of extract and 150 μL of ABTS were both present in the 200 μL reaction mixture. Instead of sample, 70% ethanol water was utilised in the control. A after 6 minutes of incubation at room temperature the absorbance was measured at 734 nm against a blank (70% hydroethanol). Ascorbic acid was used as standard, and the percentage of inhibition was determined.

The percentage inhibition of ABTS by the extract was calculated using the following method:

% inhibition = [(A0-A1)/ A0 × 100]

The calculation of the percentage inhibition of ABTS by the extract is based on the absorbance values. A0 represents the absorbance of the control, while A1 represents the absorbance in the presence of the extract or standard.

4.2.3.3 Superoxide radical scavenging activity assay

With a minor modification, the Liu et al. method was used to measure the superoxide anion's scavenging activity (Liu et al., 1997). The production of superoxide anions occurred in the reaction between PMS, NADH, and oxygen in a non-enzymatic phenazine methosulphate-nicotinamide adenine dinucleotide (PMS-NADH) system. It was tested using nitroblue tetrazolium (NBT), which produces a chromogenic product that can be measured at 560 nm. Using Tris HCL buffer, different quantities of ascorbic acid (25-100 μg/mL) and DI-HET extracts (40-300 g/ml) were prepared up to 1 mL, and to this 250 μL of NBT, 250 μL NADH, and 250 μL PMS were added. Using a multimode reader, the absorbance was measured at 560 nm in comparison to a reagent blank (pure Tris HCL buffer). DI-HET extracts were replaced with Tris HCL buffer in the control.

Percentage inhibition was calculated as:

% inhibition = [(A0-A1)/ A0 × 100]

Where A0 is the absorbance of the control and A1 is the absorbance in the presence of extract/ standard.

4.2.3.4 Total Reducing Power (TRP)

The Oyaizu method (Oyaizu, 1986) was used to determine the extract's reductive potential. The theory is based on the antioxidants in the sample reducing potassium ferricyanide to ferrous cyanide, which then interacts with ferric chloride to generate a ferric ferrous complex, which is assessed by the appearance of Prussian blue at 700 nm. When the reducing power increases along with the antioxidant content, the absorbance also rises.

200 µL of phosphate buffer (0.2 mM, pH-6.6) and 200 µL of 1% potassium ferricyanide were combined with 50 µL of the sample in various concentrations (ranging from 40 to 300 µg). During 30 minutes, the reaction system was sealed and heated to 50 °C in a water bath. 250 µL of 10% trichloroacetic acid (TCA) was added to the assay apparatus after incubation. The reaction mixture was then centrifuged for 10 minutes at room temperature at 1000 RPM. 500 µL of the sample was taken from the aforementioned mixture and combined with 500 µL of distilled water and 100 µL of 0.1% ferric chloride (Fecl3). Using a multimode reader, the colour generated was read at 700 nm against a blank (phosphate buffer). Gallic acid at different concentrations (40-300 µg) was used as standard.

4.2.3.5 Estimation of intracellular ROS generation

ROS generation in HepG2 cells from different groups was assessed by employing DCFH-DA (Wang and Joseph, 1999). Fluorescent images were captured with a fluorescent microscope (Olympus IX 83), and fluorescence was read at 488 nm excitation and 525 nm emission (Infinite® M200 PRO, Tecan Group Ltd, Switzerland).

4.2.3.6 Assessment of mitochondrial superoxide formation

Changes in mitochondrial superoxide production were monitored using MitoSOX^{TM} red, and fluorescent images of cells were captured with a fluorescent microscope (Olympus IX 83) at an excitation/emission range of 514/580 nm (Wojtala et al., 2014).

4.2.4 Oxygen consumption assay

The oxygen consumption rate was assessed by using a cell-based assay kit (Cayman, USA). With the exception of the blank, all other wells were added phosphorescent oxygen probes. After the wells had been coated with HS mineral oil. Fluorescence was measured for 120 min at an excitation wavelength of 380 nm and an emission wavelength of 650 nm (Sruthi & Raghu, 2022).

4.2.5 Western Blot analysis

Following the respective treatments, cells were lysed in RIPA buffer supplemented with a protease inhibitor cocktail. Subsequently, the cell suspensions were incubated and then centrifuged at 12,000 RPM for 20 min at 4°C. The supernatant was collected and used for further immunoblot analysis. The protein concentration was measured by using the bicinchoninic acid kit. Proteins, in equal amounts, were separated by 8% or 10% SDS-PAGE and subsequently transferred to PVDF membranes. Following the transfer, the membranes were blocked in TBS-Tween 0.1% solution containing 5% nonfat skimmed milk. Primary antibodies were then incubated with the membranes overnight at 4°C. The alterations of various proteins such as tumour necrosis factor-α (TNF-α), toll-like receptor-4 (TLR-4), NLR family pyrin domain containing 3 (NLRP3), Nuclear factor kappa B (NF-κB), phosphorylated Jun N-terminal kinase (p-JNK), Jun N-terminal kinase (JNK), Beclin-1, and LC-3 were analyzed. Following the washing step with TBS-Tween 0.1%, the membranes were incubated with the corresponding HRP-conjugated secondary antibodies for 2-4 hours at room temperature. The membranes were then washed three times with TBS-Tween 0.1%. Subsequently, the membranes were developed using Western Blot Hyper HRP Substrate, and the protein bands were analyzed (Bio-Rad ChemiDoc MP imaging systems, USA) (Mahmood and Yang, 2012).

4.2.6 Immunofluorescence

Cells were seeded in 96 well plates and subjected to various treatments. Cells were then washed three times with PBS and followed by fixation with 100µl of 4% paraformaldehyde in PBS for 20 min. After fixation of cells, with gentle agitation, the cells were permeabilized at room temperature for 15 minutes using 0.25% Triton X-

100 in PBS. 10% natural goat serum was used for blocking of cells for 1 hour before primary antibodies (4 °C, overnight) and secondary antibodies were added (1 h, at room temperature). Using fluorescently labeled anti-rabbit and anti-mouse Alexa flour secondary antibodies (cell signaling) were used to detect the primary antibodies. Nuclei were counterstained with DAPI (1 mg/mL in PBS) and visualized with a fluorescence microscope (Olympus IX 83).

4.2.7 Statistical analysis

Statistical analysis was done using the SPSS statistical program. The results were represented as mean ± SEM. The significant differences among the treatments were evaluated using one-way analysis of variance (ANOVA). It is followed by Duncan's multiple range to determine which mean values were significantly different at $p \leq 0.05$. Statistical significance was indicated as * for $p \leq 0.05$ Vs C and # for $p \leq 0.05$ Vs OA.

4.3 Results

4.3.1 Determination of antioxidant potential of DI-HET

Table 4.1 shows the free radical scavenging property of DI-HET extracts and standards in terms of its ability to scavenge DPPH, ABTS, and superoxide. The standards used for the study are gallic acid (for DPPH) and ascorbic acid (for ABTS and superoxide) respectively. The free radical scavenging ability was expressed in terms of IC_{50} value and estimated IC_{50} values stand for the effective concentration of antioxidants required to scavenge 50% of radicals in the reaction mixture and lower the IC_{50} value higher the antioxidant potential. DI-HET extract shows significant radical scavenging potential with IC_{50} value 58.95 ± 1.02 µg/ml for DPPH (Figure.4.1 & Table.4.1), IC_{50} value 143.83 ± 2.49 µg/ml for ABTS (Figure.4.2 & Table.4.1) and IC_{50} value of 162.17 ± 2.80 µg/ml for superoxide radical scavenging activity (Figure.4.3 & Table.4.1). Figure.4.4 shows the total reducing power (TPR) of DI-HET extract which is compared with gallic acid as standard. The principle of TRP is that absorbance of the reaction mixture increases with its antioxidant potential. Here we found that the TRP is increased with the increasing concentration of DI-HET (Figure.4.4).

4.3.1.1 DPPH scavenging activity of DI-HET

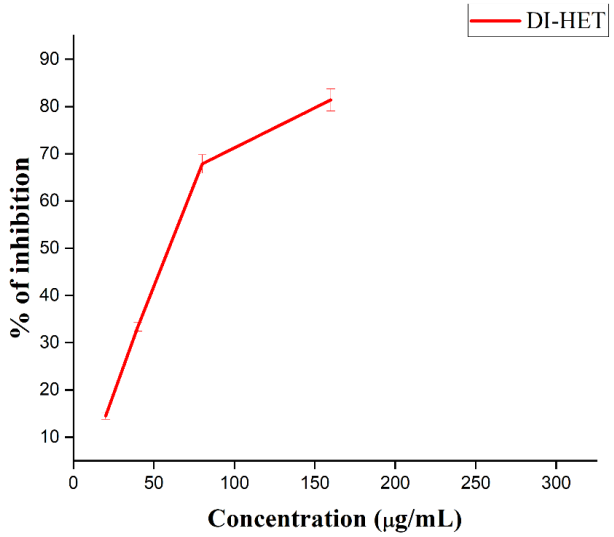

Figure.4.1. DPPH scavenging potential of DI-HET: Estimated IC_{50} value of DPPH scavenging activity of DI-HET is 58.95µg/mL. Estimated IC_{50} value of DPPH scavenging activity of standard (gallic acid) is 9.06 µg/mL. Values are expressed as mean ± SEM (n=6).

4.3.1.2 ABTS scavenging activity of DI-HET

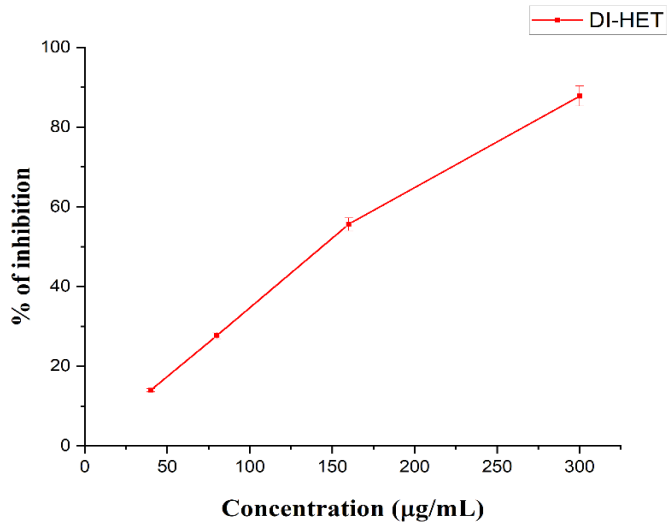

Figure.4.2. ABTS scavenging potential of DI-HET: Estimated IC_{50} value of ABTS scavenging activity of DI-HET is 143.83 µg/mL. Estimated IC_{50} value of ABTS scavenging activity of standard (Ascorbic acid) is 27.1 µg/mL. Values are expressed as mean ± SEM (n=6).

4.3.1.3 Superoxide scavenging activity of DI-HET

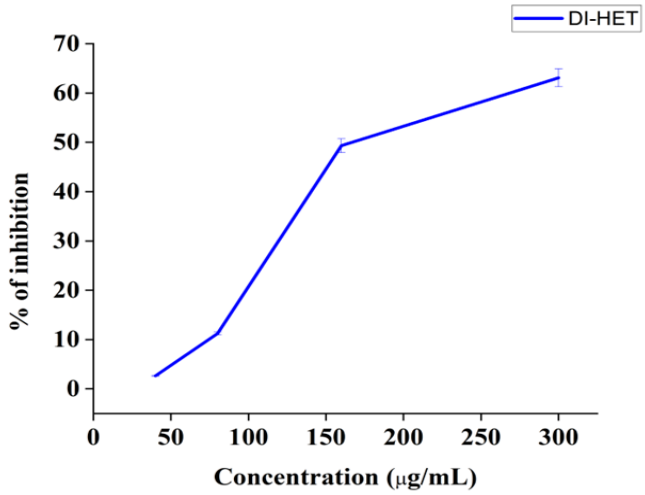

Figure.4.3. Superoxide radical scavenging potential of DI-HET: Estimated IC_{50} value of superoxide radical scavenging activity of DI-HET is 162.17 µg/mL. Estimated IC_{50} value of superoxide radical scavenging activity of standard (Ascorbic acid) is 88.01 µg/mL. Values are expressed as mean ± SEM (n=6).

4.3.1.4 Total Reducing Power of DI-HET

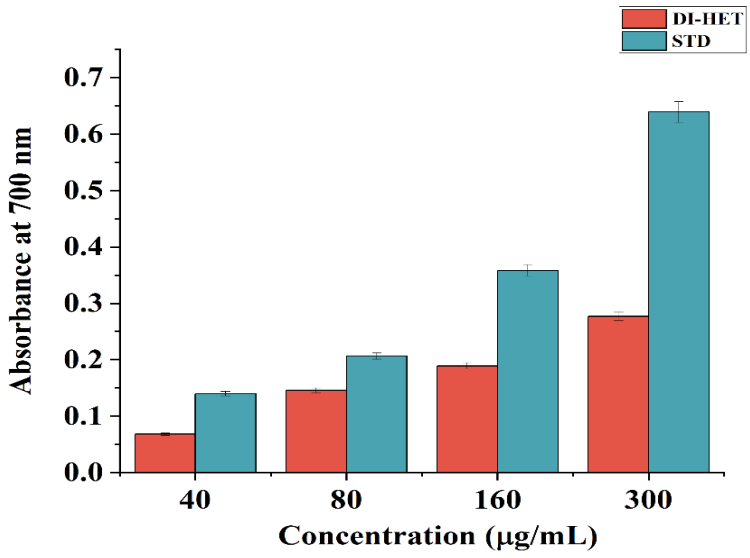

Figure.4.4. Total reducing power of DI-HET and standard (Gallic acid). Values are expressed as mean ± SEM (n=6).

IC_{50} (µg/ml)			
Sample	**DPPH**	**ABTS**	**Superoxide scavenging activity**
DI-HET	58.95 ± 1.02	143.83 ± 2.49	162.17 ± 2.80
Gallic acid	9.06 ± 0.156	---	---
Ascorbic acid	---	27.1 ± 0.46	88.01 ± 1.52

Table.4.1 Estimated IC_{50} value of DPPH, ABTS and superoxide radical scavenging activity of DI-HET extract and standards. Values are expressed as mean ± SEM (n=6).

4.3.1.5 Effect of DI-HET on intracellular ROS generation

OA caused the surplus generation of ROS (24.14%; $p \leq 0.05$; Figures. 4.5.a & b) when compared to the control group. Pretreatment with DI-HET significantly reduced ROS generation in HepG2 cells (15.49% with DI 5 and 16.95% with DI 10; $p \leq 0.05$; Figures. 4.5. a & b). Fenofibrate also reduced ROS generation by 19% ($p \leq 0.05$) Results are as evident as in the (Figures. 4.5.a & b).

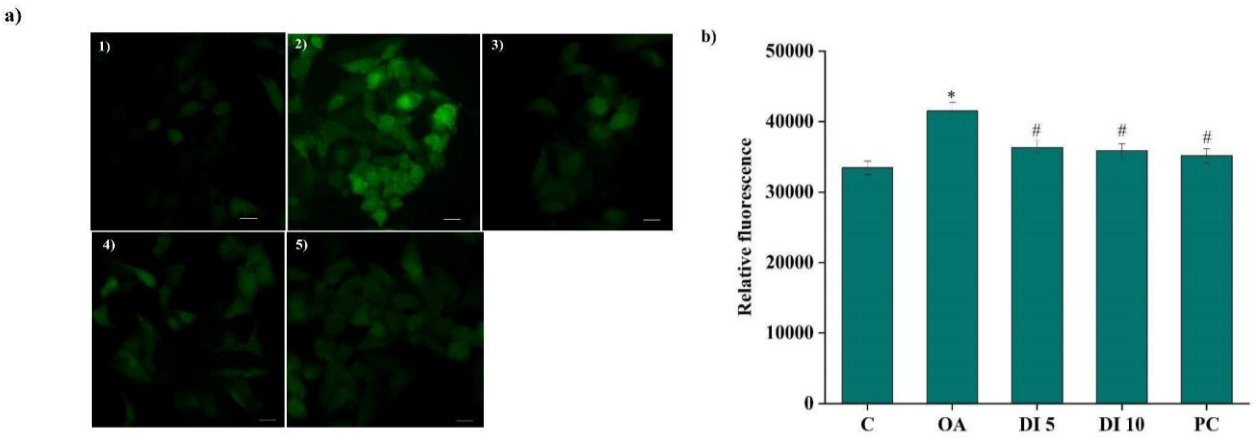

Figure.4.5. Effect of DI-HET on oleic acid-induced ROS generation in HepG2 cells. a) ROS generation in various groups (1) Control (C) (2) Oleic acid 100 µM (OA) (3) Oleic acid + 5 µg/mL extract (DI 5) (4) Oleic acid + 10 µg/mL extract (DI 10) (5) Oleic acid + fenofibrate 20 µM (PC). b) Relative fluorescence intensity of each group. Data are expressed as mean ± SEM; where n = 6. * denotes significant

difference from the control group ($p \leq 0.05$) and # denotes significant difference from the OA treated group ($p \leq 0.05$).

4.3.1.6 Effect of DI-HET on mitochondrial superoxide formation

Mitochondrial superoxide generation was significantly increased in the OA group (57.39%; $p \leq 0.05$: Figures. 4.6.a & b) when compared to the control group. With both extracts of DI-HET, surplus generation of superoxide generation was found to be inhibited (23.25% with DI 5 and 56.72% with DI 10; Figures. 4.6.a & b). Similarly, a significant reduction (54.69%) in superoxide generation was also observed with fenofibrate (Figures. 4.6.a & b).

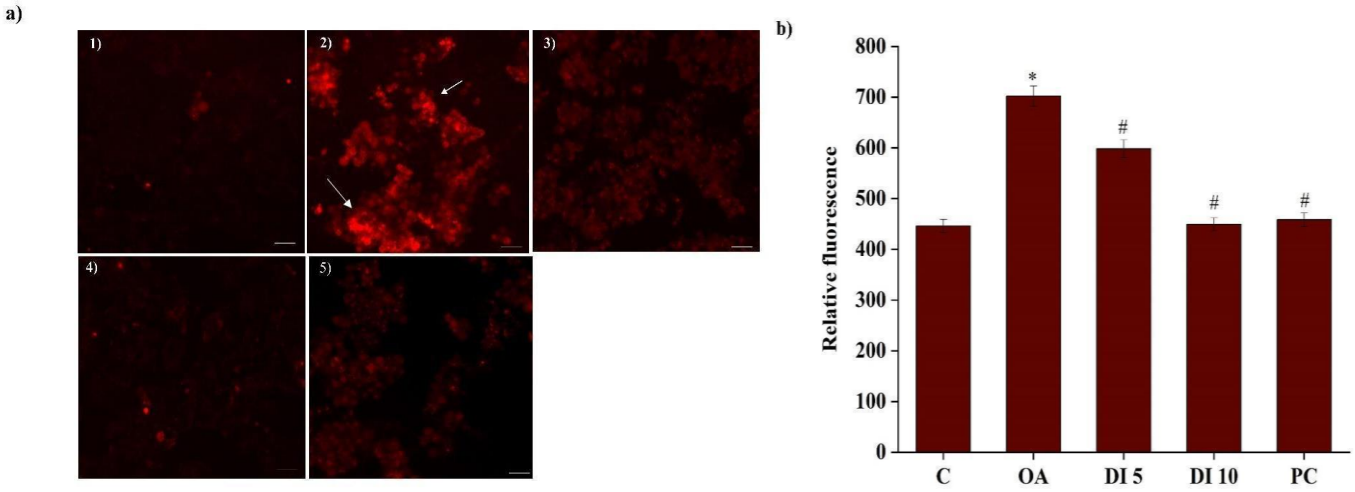

Figure.4.6. Effect of DI-HET on oleic acid-induced mitochondrial superoxide generation in HepG2 cells: a) The fluorescent microscopic images of cells stained with MitoSOX™ Red indicator, scale bar 20 µm. (1) Control (C) (2) Oleic acid 100 µM (OA) (3) Oleic acid + 5 µg/mL extract (DI 5) (4) Oleic acid + 10 µg/mL extract (DI 10) (5) Oleic acid 100 µM + fenofibrate 20 µM (PC). (b) Fluorescence intensity emitted by MitoSOX™ in control and treated cells. Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the control group ($p \leq 0.05$) and # denotes significant difference from the OA treated group ($p \leq 0.05$).

4.3.2 Effect of DI-HET on oxygen consumption rate

The oxygen consumption rate was found decreased (38.64% $p \leq 0.05$; Figure. 4.7) in the OA group compared to control. However, extracts improved oxygen consumption rate significantly (17.39% with DI 5 and 19.96% with DI 10; Figure. 4.7)

compared to the OA group. Fenofibrate also caused an improvement in oxygen consumption rate by 9.52% (Figure. 4.7).

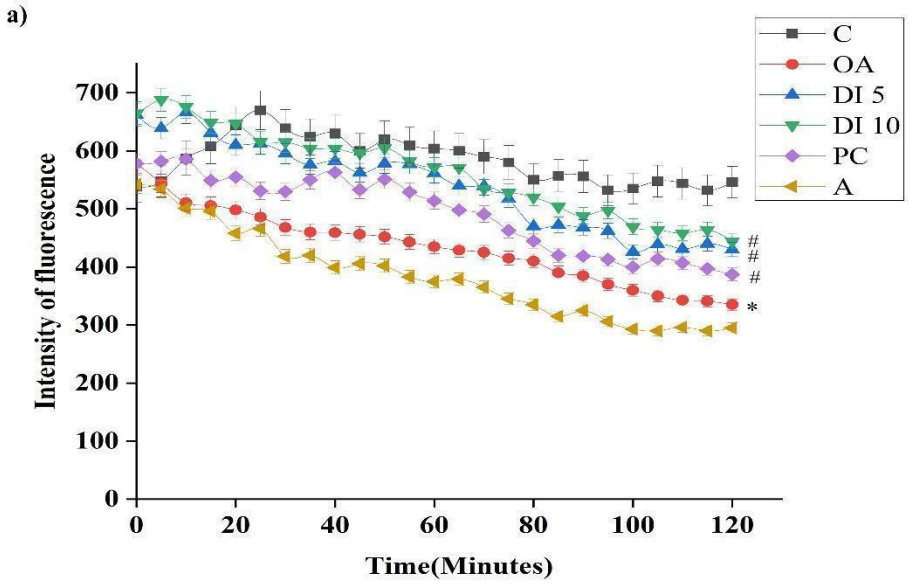

Figure.4.7. Effect of DI-HET on oxygen consumption rate: Control (C), Oleic acid 100 µM (OA), Oleic acid + 5 µg/mL extract (DI 5), Oleic acid + 10 µg/mL extract (DI 10), Oleic acid + fenofibrate 20 µM (PC) and Antimycin A (A). Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the control group ($p \leq 0.05$) and # denotes significant difference from the OA treated group ($p \leq 0.05$).

4.3.3 Effect of DI-HET on inflammation

4.3.3.1 DI-HET downregulates TNF-α, TLR-4, and NLRP3

Upregulated TNF-α (49.71%; $p \leq 0.05$; Figures. 4.8.a & b) and TLR-4 (37.28%; $p \leq 0.05$; 4.8.a and c) expression in the OA group was reversed with pretreatment with DI-HET. The expression of both TNF-α (8.31% with DI 5 and 49.37% with DI 10; $p \leq 0.05$; Figures. 4.8.a & b) and TLR-4 were down-regulated with DI-HET (23.28% with DI 5 and 18.63% with DI 10%; $p \leq 0.05$; Figures. 4.8.a & c). Fenofibrate treatment also decreased the expression of TNF-α and TLR-4 by 45.36% and 10.37% respectively ($p \leq 0.05$).

OA treatment showed an upregulation of NLRP3 expression by 34.61% ($p \leq 0.05$). The expression of NLRP3 was downregulated with DI-HET pretreatment (31.39% with DI 5 and 24.16% with DI 10; $p \leq 0.05$ Figures. 4.8.a & d) when compared to the OA group. The expression of NLRP3 was also significantly reduced with fenofibrate treatment (51.61%; $p \leq 0.05$) when compared to the OA.

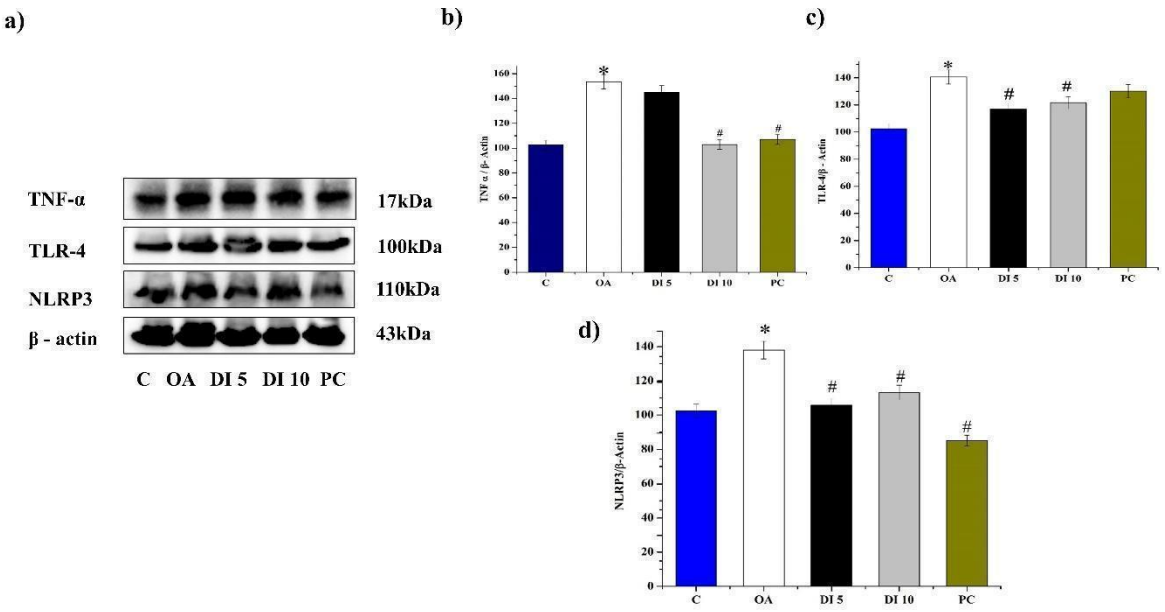

Figure.4.8. DI-HET plant extract downregulates the expression of TNF-α, TLR-4 and NLRP3: (a) The expression of TNF-α, TLR-4, and NLRP3 in OA-induced condition and in the treated group. (b),(c) & (d) The relative intensity of each band was quantified with β-actin. Values are expressed as mean ± SEM where n = 6. * indicates significantly different from the control group. # indicates significant difference from the OA induced group ($p \leq 0.05$).

4.3.3.2 DI-HET downregulates NF-κB

We also analyzed NF-κB expression. Western blot analysis showed upregulation of the expression of NF-κB in the OA group (29.02%; $p \leq 0.05$) when compared to the control group. However, treatment with DI-HET reduced expression of NF-κB (29.11% with DI 5 and 49.25% with DI 10; $p \leq 0.05$; Figures. 4.9.a & b). The expression of NF-κB was also significantly reduced with fenofibrate treatment (78.60%; $p \leq 0.05$) when compared to the OA (29.02%; $p \leq 0.05$). Immunostaining also showed the same results (Figure. 4.9.c).

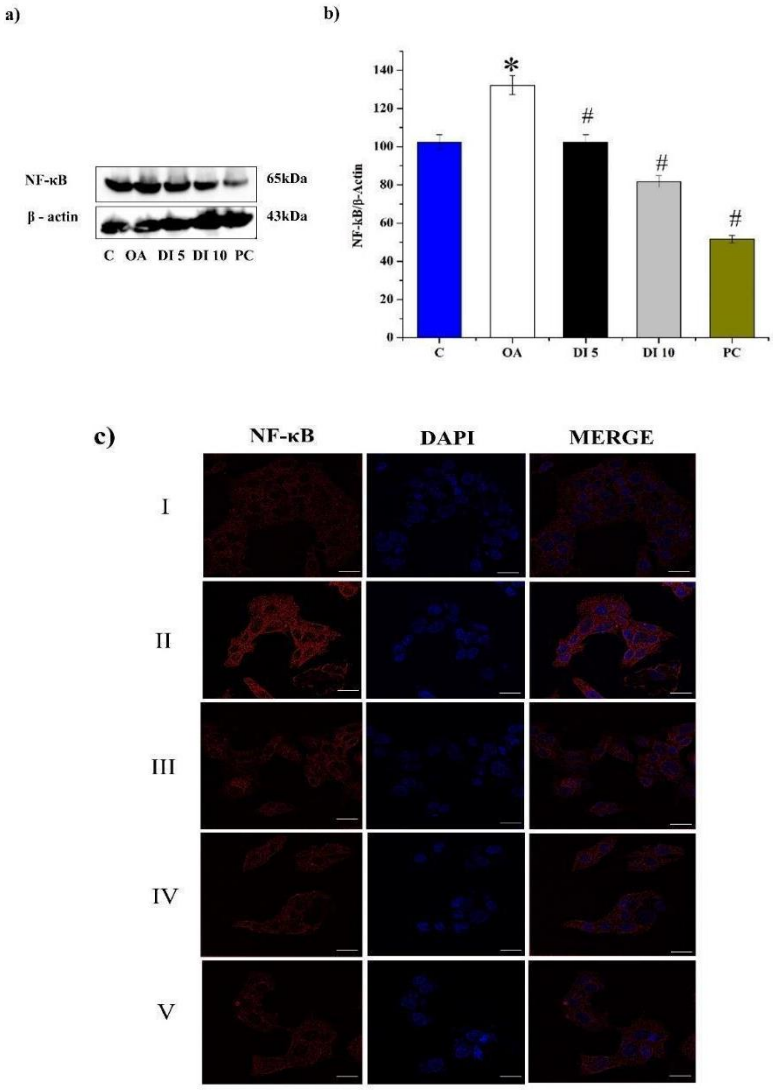

Figure.4.9. Effect of DI-HET on oleic acid-mediated NF-κB translocation: (a)The expression of NF-κB in OA-induced condition and in the treated group. (b)The relative intensity of each band was quantified with β-actin. (c) Immunofluorescence staining showing NF-κB distribution in OA-induced conditions in HepG2 cells. The cells were stained by DAPI (blue) and anti- NF-κB antibody (red). (i) Control cells; (ii) Oleic acid 100 µM (OA); (iii) Cells treated with OA + 5 µg/ml (DI 5); (iv) Cells treated with OA + 10 µg/ml (DI 10) (v) Cells treated with OA + fenofibrate 20 µM (PC). Original magnification 40X. Scale bar corresponds to 20 µm. Values are expressed as mean ± SEM where n = 6. * indicates significantly different from the control group. # indicates significantly different from the OA-induced group ($p \leq 0.05$).

4.3.3.3 Effect of DI-HET on p-JNK

The OA group showed significant upregulation in the expression of p-JNK by 35.46% ($p \leq 0.05$) compared to the control group. Pre-treatment with DI-HET downregulated the expression of p-JNK (13.49 % with DI 5 and 22.16 % with DI 10; $p \leq 0.05$; Figures. 4.10. a & b). Immunostaining also showed the same results (Figure. 4.10.c).

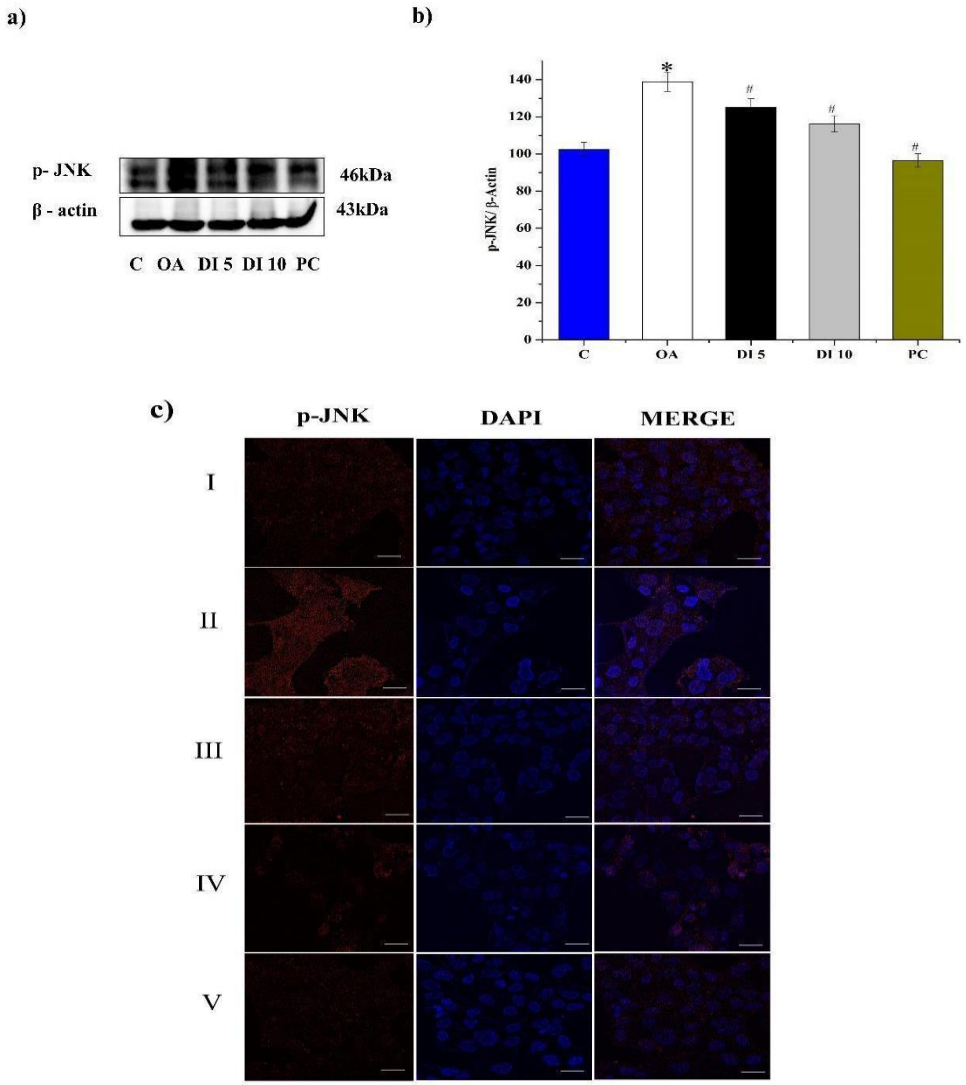

Figure 4.10. Effect of DI-HET on oleic acid-mediated p-JNK expression: (a)The expression of p-JNK in OA-induced condition and in the treated group. (b)The relative intensity of each band was quantified with β-actin. (c) Immunofluorescence staining showing p-JNK in OA-induced p-JNK expression in HepG2 cells. The cells were stained by DAPI (blue) and anti-p-JNK antibody (red). (i) Control cells; (ii) Oleic acid 100 µM (OA); (iii) Cells treated with OA

+ 5 µg/ml (DI 5); (iv) Cells treated with OA + 10 µg/ml (DI 10) (v) Cells treated with OA + fenofibrate 20 µM (PC). Original magnification 40X. Scale bar corresponds to 20 µm. Values are expressed as mean ± SEM where n = 6. * indicates significantly different from the control group. # indicates significantly different from the OA-induced group ($p \leq 0.05$).

4.3.4 Regulation of autophagy with DI-HET pre-treatment

The OA group showed significant upregulation in the expression of Beclin1 by 53.08 % ($p \leq 0.05$) compared to the control group. Pre-treatment with DI-HET downregulated the expression of Beclin1 (32.12% with DI 5 and 38.83% with DI 10; $p \leq 0.05$; Figures. 4.11. a & b) compared to the OA group. Treatment with fenofibrate also significantly reduced the expression of Beclin1 (97.44% $p \leq 0.05$).

The OA group showed significant upregulation in the expression of LC3 by 26.07% ($p \leq 0.05$) compared to the control group. The expression of LC3 was downregulated with DI-HET pretreatment (33.78% with DI 5 and 50.65% with DI 10; $p \leq 0.05$ Figures. 4.11.a & c) when compared to the OA group. The expression of LC3 was also significantly reduced with fenofibrate treatment (75.72%; $p \leq 0.05$) when compared to the OA group.

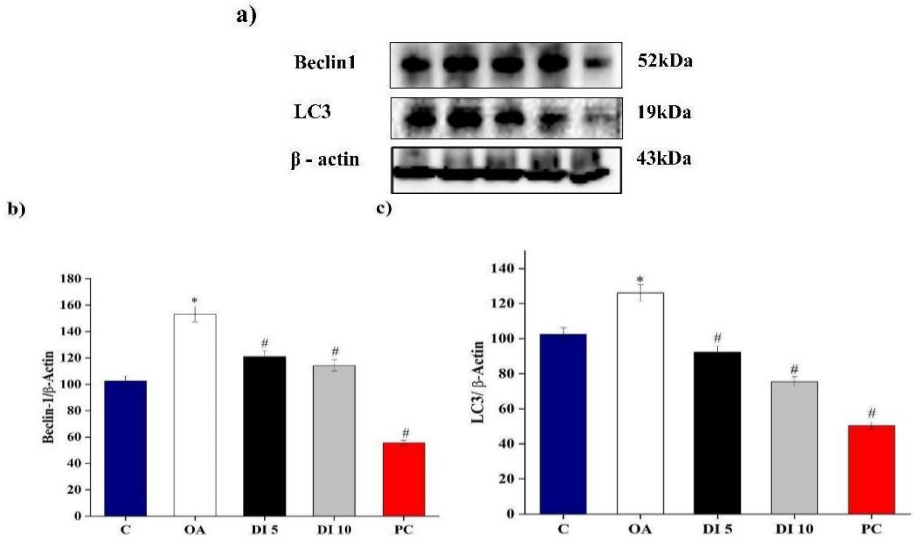

Figure.4.11. DI-HET plant extract downregulates the expression of Beclin1 and LC3: (a) The expression of Beclin1 and LC3 in OA induced condition and in the treated group. (b) & (c). The relative intensity of each band was quantified with β-Actin. Values are expressed as mean ± SEM where n = 6. * indicates significantly different from the control group. # indicates significantly different from the OA-induced group ($p \leq 0.05$).

4.4 Discussion

Numerous disorders, including NAFLD, have been linked to the formation of free radicals. Therefore, substances that can neutralize free radicals have a great potential to treat diseases like NAFLD. Thus, antioxidants are crucial in defending against reactive oxygen species damage in the human body. Due to the damage by ROS, peroxidation of lipids will occur, which then promotes the glycation of proteins, the inactivation of enzymes, changes in the structure and function of collagen, basement membranes, and other membranes, and contributes to the progression of NAFLD. Hepatic steatosis causes both oxidative stress and a decline in antioxidant status, which can exacerbate the harmful effects of free radicals and the advancement of fibrosis.

Oxidative stress is characterized by an imbalance between the amounts of antioxidants and pro-oxidants in cells, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), leading to cellular deterioration and cell death in most cases. Cells maintain a baseline amount of ROS in a normal physiological state to support the balanced redox signaling necessary for many functions, including cellular metabolism, differentiation, survival, immunity, regulation of transcription factors, and epigenetic state (Chen et al., 2020). Superoxide dismutase (SOD), an antioxidant enzyme, converts the superoxide radical into hydrogen peroxide (H_2O_2) in response to oxidative stress. Glutathione peroxidase (Gpx) or catalase enzyme activates the subsequent conversion of H_2O_2 to oxygen (O_2) and water (H_2O) (Ore & Akinloye, 2019). ROS generally occur in the mitochondria, peroxisomes, and endoplasmic reticulum (ER) of cells, although they can also be produced in the cytoplasm. These organelles are affected by high amounts of ROS, which amplifies oxidative stress and perpetuates the vicious cycle. Clinical and experimental research has demonstrated that the antioxidant pathways are altered as NAFLD progresses.

Hence, the extract's antioxidant property using cell-free assay was studied. It was found that the extract showed strong antioxidant properties. To substantiate that, intracellular ROS generation was studied in *in-vitro* model. ROS generation was significantly reduced with DI-HET extract treatment.

Mitochondria play a significant role in the energy supply to cells and are critical players in cell injury and death. And also, mitochondria are the greatest source

of ROS generation (Chen et al., 2020). The oxygen consumption rate is an essential indicator of normal cellular physiological function. Impaired oxygen consumption is associated with unhealthy mitochondria or diseased conditions of cells. Increased generation of reactive oxygen species (ROS) and utilization of cytochrome and peroxisome-mediated oxidation are important features of NAFLD, contributing to mitochondrial dysfunction. (Ipsen et al., 2018). During situations of energy imbalance, such as fat buildup, mitochondrial ROS generation substantially increases with the alteration in the mitochondrial respiratory chain. Inducing oxidative stress, further leads to damage in mitochondrial membranes, compromising cellular respiration and metabolism. As a result, liver function is impaired through both direct and indirect cellular damage. (Muriel et al., 2017). Lipogenesis induces oxidative stress and alters the mitochondrial innate antioxidant defense system (Chen et al., 2020). The extract was found to reduce oxidative stress in mitochondria in HepG2 cells treated with OA and increased oxygen consumption rate. This is considered an additional beneficial activity of the extract in strengthening its potential against NAFLD.

The progression of NAFLD is accelerated by inflammation. The primary process through which body tissues react with damage is inflammation (Stankov, 2012). During the progression of NAFLD, lipid accumulation occurs in the hepatocytes as macrovesicular fat that consists of ceramides, fatty acids, diacylglycerols, cholesterol, and phospholipids. Hence, this lipid buildup in the hepatocytes leads to lipotoxicity, leading to cellular stress, apoptosis, and cell death. The apoptotic hepatocytes release extracellular vesicles and proinflammatory cytokines, which start the inflammatory process (Katsarou et al., 2020). Therefore, the pathology of NAFLD is influenced by lipotoxicity caused by saturated fatty acids (SFAs).

To validate the effect of DI-HET extract on inflammation during OA-induced NAFLD, major proteins involved in inflammation, like TLR-4, TNF-α, NF-κB, p-JNK, and NLRP3 were studied. TLR-4 plays a significant role in activating innate immune response. The TLR4 signaling pathway is expressed by hepatocytes (Shen et al., 2018). There has been extensive research on the role of TLR4 in the pathophysiology of NAFLD. TLR-4 is activated by several factors, like lipopolysaccharides, saturated fatty acids, heat shock proteins, and DAMPs secreted by damaged cells (Guo & Friedman, 2010; Seki et al., 2007). Scientific studies have reported that TLR-4 specific knockout

in mice fed with diet-induced NAFLD causes the reversal of NAFLD pathogenesis (J. Liu et al., 2014). Lipotoxicity leads to the binding of free fatty acids to the TLR-4, which triggers rapid TLR-4 signaling that activates innate immune reactions with NF-κB through the adaptor molecule myeloid differentiation factor 88 (Myd88) (Akira et al., 2006; Spruss et al., 2009; Verstrepen et al., 2008). This in turn activates the secretion of several proinflammatory cytokines like TNF-α, IL-12, and IL-6 (Zhan & An, 2010). During the proinflammatory state Kupffer cells (KCs) in liver tissue also release TNF- α, which aids in the progression of NAFLD. Hence TNF-α secretion leads to insulin resistance in the liver that causes further hepatic steatosis, it promotes hepatic cell death (Zhan & An, 2010). Therefore, studies report that TNF-α targeting therapy may be advantageous in the treatment of NAFLD (Poonam Mishra & Zobair M. Younossi, 2007).

TLR-4 expression was studied and observed that treatment with OA increased TLR-4 expression, and pretreatment with DI-HET extract showed reduced expression of TLR-4. Consistent with the reduced expression of TLR-4, the protein expression of NF-κB and TNF-α was reduced with plant extract pretreatment. Hence inflammation through TLR-4 activation is reduced with plant extract showing the anti-inflammatory potential of *D.indica*.

Another major protein involved in inflammation is NLRP3. NLRP3 inflammasomes is a set of cytosolic protein complexes that are generated to mediate host immunological reactions to microbial infection and cellular damage. Inflammasomes activate caspase-1, which causes the maturation of IL-1β and IL-18 which triggers pyroptosis, a type of cell death. IL-1β in its mature state acts as a powerful inflammatory mediator (He et al., 2016). Numerous human disorders have been linked to the pathophysiology of NLRP3 inflammasome dysfunction. Unsaturated fatty acids, high glucose, ceramide, monosodium urate crystals (MSU), cholesterol crystals, amyloid-b aggregates, and other host-derived “danger signals” can all be sensed by the NLRP3 inflammasome to promote chronic inflammation and aid in the development of complex human diseases like gout, atherosclerosis, neurodegenerative diseases, type 2 diabetes, and NAFLD. Hence, NLPR3 has thus been considered a possible therapeutic target for treating inflammatory disorders (Huang et al., 2018). NLRP3 is activated through the activation of TLR4. Recently, it was discovered that

both in murine models and in human NASH patients, the expression of NLRP3 had dramatically increased. Additionally, pharmacological suppression of the NLRP3 gene or the use of gene-knockout mice reduced hepatic steatosis, hepatocyte inflammation, and fibrosis (Csak et al., 2011; Matsuzaka et al., 2012; Wree et al., 2014). According to these evidences, the NLRP3 inflammasome acts as a key player in the emergence of NASH and can serve as a molecular therapeutic target (Wan et al., 2016). Also, plant-derived compounds have been reported for its ability to inhibit NLRP3 activation (Ding et al., 2020). Therefore, the protein expression of NLRP3 in pretreatment with extract was studied in the OA-induced NFLD model. Interestingly, NLPR3 expression was significantly reduced with plant extract pretreatment.

JNK is a member of the mitogen-activated protein kinase (MAPK) family. In response to intra and extracellular stresses, JNK is activated. Multifaceted kinase cascade that activates the JNK signaling pathway (Czaja, 2010). Several studies have reported that the over-activation of JNK is associated with insulin resistance in obese individuals and NAFLD patients. Later research looking at JNK function in the hepatic symptoms of the metabolic syndrome reveals that JNK has a crucial mechanistic role in developing NAFLD (Schattenberg et al., 2006; Wan et al., 2016). Overactivation of JNK in adipose tissue causes peripheral insulin resistance. That leads to increased adipocyte lipolysis and the release of free fatty acids into the serum, which are then absorbed by the liver and stored as triglycerides. As most of the lipid that builds up in NAFLD is made up of serum fatty acids, adipose tissue JNK activation may encourage steatosis through this method (Donnelly et al., 2005). Lipid buildup in liver tissue promotes JNK activation. Activated JNK, in turn, activates TNF-α. Hepatocyte death is caused by necrotic and apoptotic processes, which are mediated by sustained JNK activation in response to TNF-α. Studies in animal models found that JNK mediates hepatic steatosis in high-fat diet-fed mice (Tuncman et al., 2006). Therefore, the regulation of JNK with plant extract pretreatment was studied in OA-induced NAFLD conditions. Results showed that phosphorylated JNK or p-JNK, the activated form was significantly downregulated with DI-HET extract pretreatment. Hence inflammation due to the activation of JNK was also reduced.

Autophagy plays a significant role in maintaining cellular homeostasis by controlling intracellular lipid stores, removing damaged organelles and misfolded

proteins in obesity, and managing intracellular lipid reserves (Kim et al., 2013; Singh et al., 2009). According to scientific studies, autophagy has a significant role in liver physiology and pathogenesis (Rautou et al., 2010). It has been observed that autophagy failure occurs in lipid-overloaded human hepatocytes, murine models of NAFLD, and NAFLD patients (Christian et al., 2013; Li et al., 2014; Ma et al., 2013). These findings suggested that autophagy might have a preventive role in liver damage. However, contradictory data have also been reported regarding the role of autophagy in the development of NAFLD (Singh et al., 2009). Autophagy is essentially a pro-survival mechanism; however, in certain conditions, it acts as death-promoting ones (Levine & Yuan, 2005). Inflammation activates autophagy signaling. For example, TNF-α, IFN-γ, IL-1, IL-2, IL-6, and TGF-β, is reported to induce autophagy. Scientific studies have demonstrated that the stimulation of the JNK signaling pathway activates TNF-α, which further activates autophagy gene expression LC3 and Beclin 1(Jia et al., 2006). Also, studies have reported that NF-κB also activates autophagy signaling (Pavel et al., 2022). Hence there is a relationship between inflammatory signaling and upregulation of autophagy. Therefore, the protein expression of two major proteins involved in autophagy was studied. Interestingly the result showed that both proteins Beclin-1 and LC3 were upregulated in OA-induced NAFLD in HepG2 cell lines, and protein expression was reversed with the DI-HET extract pretreatment. The reversal of autophagy may be due to the downregulation of inflammatory proteins with DI-HET extract pretreatment.

The present study revealed the significant antioxidant potential of polyphenol-rich DI-HET extract. The extract effectively lowered inflammatory proteins like TLR-4, TNF-α, NF-κB, p-JNK, and NLRP3. Due to the anti-inflammatory property of the extract, upregulated autophagy in OA-induced NAFLD condition is reversed with the plant extract pretreatment. Plant extract's antioxidant, anti-inflammatory, and autophagy-reversal properties demonstrate its hepatoprotective function in an OA-induced NAFLD model.

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Chapter 5

Anti-adipogenic potential of DI-HET in 3T3-L1 via modulating lipid metabolism, inflammation, and autophagy signaling pathway.

5.1 Introduction

Metabolic syndrome (MetS) now appears as an epidemic health issue that affects the metabolic equilibrium of most tissues like the heart, liver, pancreas, and adipose tissue. The characteristics of MetS include abdominal obesity, atherogenic dyslipidemia (raised TG, higher apo B, and lowered HDL-C), elevated blood pressure, elevated glucose levels, and proinflammatory and pro-thrombotic conditions. Proinflammatory and pro-thrombotic states are two major risk factors linked to cardiovascular diseases (CVDs). Around 20-40% of the world population suffers from MetS. Recent studies indicate that excess adipose tissue significantly contributes to the development of MetS (Grundy, 2015).

Over the past few years, there has been notable advancement in comprehending the multiple functions of the adipocyte, serving as both a secretory cell and a site for regulating energy storage. White adipose tissue's (WAT) traditional function has been described as energy storage, with fatty acids released when fuel is needed. However, WAT plays a complex role in metabolism. Adipose tissue is now considered metabolically active tissue with endocrine functions producing numerous metabolically significant adipokines. The tissue, for instance, is necessary for healthy glucose homeostasis and has been suggested to play a part in inflammatory processes (Grundy, 2015). Several external factors, like the autonomic nervous system, body energy reserve, blood flow rate, hormones, and other complex substances, regulate the multiple functions of adipose tissue. To reduce fat accumulation in adipose tissue, various metabolic activities are regulated, including the control of adipocyte differentiation and the formation of blood vessels. Nevertheless, adipocytes signal other tissues to adjust their energy metabolism to the body's nutritional condition. Finally, adipocyte fat storage must eventually balance the body's total energy excess or shortage (Frayn et al., 2003). The most important mechanism by which adipose tissue increases

the storage capacity of fat in adipose tissue by hypertrophy (cell size increases) and hyperplasia (cell number increases) of adipocytes. These two mechanisms predominantly accelerate in obese conditions (Jo et al., 2009). The major reason for obesity is the excessive build-up of body fat that leads to an imbalance between cellular lipogenic and lipolytic activity. The worldwide prevalence of overweight and obesity is such that over one-third of the world's population suffers from it. Additionally, the rate of NAFLD morbidity is rising. Obesity leads to the development of NAFLD (Pei et al., 2020). According to a study, 80% of NAFLD patients have obesity (body mass index (BMI) > 30 kg/m^2). Those who are severely obese (BMI > 40 kg/m^2) have a lot of visceral adipose tissue (VAT), which increases the risk of developing NAFLD (Milić et al., 2014). The liver and adipose tissue are the primary metabolic organs for storing and using energy. Excess or positive energy status of the body leads to metabolic distress like defective adipose tissue, dyslipidemia, secretion of adipokines, and the release of proinflammatory cytokines from adipose tissue, further enhancing lipid flux to the liver (Azzu et al., 2020). Hence the overall regulation of whole-body homeostasis is associated with proper communication between the liver and adipose tissue.

Adipogenesis or adipocyte differentiation is a complex and coordinated signaling process involving several cell cycle proteins and transcription factors. Finally, preadipocyte is converted into mature adipocytes (Lefterova & Lazar, 2009). A pre-existing population of undifferentiated progenitor cells called preadipocytes continuously produces new fat cells. The production of new fat cells significantly influences the development of obesity. PPAR-γ and C/EBP-α are the two major transcription factors that regulate the process of adipogenesis, and PPAR-γ is considered the master regulator of adipocyte differentiation. C/EBP- α activation directly activates the genes and proteins involved in lipogenesis and adipogenesis (Symonds, 2017).

Obesity is associated with chronic low-grade inflammation. Lipid overload in adipose tissue causes releases of proinflammatory cytokines and free fatty acids. That leads to the infiltration of macrophages into adipose tissue. Further, lead to the release of inflammatory cytokines. The major inflammatory pathways involved in adipose tissue inflammation are JNK and NF-κB (Musi & Guardado-Mendoza, 2014). Studies have shown that activation of lipid overload and release of proinflammatory cytokines

cause uncontrolled autophagy induction during obesity. *In vivo*, studies have also demonstrated that the proinflammatory transcription factor NF-κB has been associated with autophagy induction (Menikdiwela et al., 2020).

Hence the present chapter deals with the anti-adipogenic potential of DI-HET extract in the 3T3-L1 cell lines. More attention is given to elucidating the molecular mechanism behind the beneficial effect of extract against adipogenesis, lipogenesis, inflammation, and autophagy.

5.2 Materials and methods

5.2.1 Chemicals

Dulbecco's modified Eagle's medium (DMEM) was purchased from HiMedia, Mumbai, India. Fetal bovine serum (FBS), penicillin-streptomycin antibiotics, trypsin - ethylenediaminetetraacetate (EDTA), Hanks balanced saline solution (HBSS), and phosphate buffer saline (PBS) was obtained from Gibco, USA supplied. 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), skimmed milk powder, insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone and Oil Red O stain were purchased from Sigma-Aldrich Co. USA. The triglyceride assay kit was from Cayman, USA. BCA protein assay kit was from Bio-Rad Laboratories Pvt Ltd, and chemiluminescence detection Western Blot Hyper HRP Substrate kit from Takara Bio Inc, Japan. PPAR-γ, p-AMPK, AMPK, p-ACC, ACC, FAS, CD36, C/EBP-α, SREBP-1C, TLR4, NF-κB, p-JNK, JNK, and Resistin were from Santa Cruz Biotechnology, USA. I purchased FABP4, ATGL, NLRP-3, Lipin, p-ACLY, ACLY, and AceCS1 from Cell signalling technology, USA. AMBRA1, Beclin1, ATG7, ATG16L1, and LC3 were from Immunotag, Geno Technology Inc., USA. All secondary antibodies used in western blotting were from Santa Cruz Biotechnology, USA. All other chemicals and solvents used were of analytical grade.

5.2.2 Cell Culture and Treatments

The 3T3-L1 preadipocytes were acquired from the National Center for Cell Sciences (NCCS, Pune, India) and cultured until reaching confluence at 37 °C in a humidified 5% CO2 atmosphere. The culture medium used was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum and 1%

penicillin-streptomycin. On day 0, two days after reaching confluence, the 3T3-L1 preadipocytes were exposed to differentiation medium (DM) consisting of 10% fetal bovine serum, 10 µg/ mL insulin, 0.5 mM IBMX, and 1 µM dexamethasone. Two days after stimulation with differentiation media, including 0.5 mM IBMX, 1 µM dexamethasone, and 10 µg/ mL insulin (day 2), the medium was changed to a 10% FBS/DMEM medium containing 10 µg/ mL insulin. Subsequently, on day 4, the medium was switched to 10% FBS/DMEM containing 10 µg/ mL insulin. From thereon, the cells were cultured in 10% FBS/DMEM, with medium changes every two days. By day 8, full differentiation of the cells was achieved (Drissya et al., 2022). During the differentiation process, the DI-HET extract was utilized at concentrations of 5 µg/ mL and 10 µg/ mL to investigate its inhibitory effect on adipocyte differentiation in the 3T3-L1 cell culture, specifically between days 0 and 2 (48 h). In the control group (C1), the cells were cultured solely in 10% FBS/DMEM medium, with medium changes occurring every two days. The experimental group consists of:

(a) C1 group: 3T3-L1 preadipocyte cells without differentiation, and any treatment is designated as C1. (Without differentiation medium (DM))

(b) C2 group: Differentiated 3T3-L1 cells are indicated as C2. (With DM)

(c) DI 5 group: 3T3-L1 cells (C2) treated with 5 µg/ mL DI-HET extract.

(d) DI 10 group: 3T3-L1 cells treated (C2) with 10 µg/ mL DI-HET extract.

5.2.3 Evaluation of cell viability assay

For cell viability, 3T3-L1 cells (C1) were seeded in 96-well plates at a density of 5×10^3 per well, and MTT assay was performed after treating the cells with different concentrations of DI-HET for 24 h and 48 h (5, 10, 20, 50, 100, 200 and 400 µg/mL).

5.2.4 Intracellular lipid accumulation

5.2.4.1 Oil red O staining

After the respective treatments, the cells were subjected to two washes with PBS and were subsequently fixed with 4% paraformaldehyde for a duration of 1 hour. After the cells were fixed in 4% paraformaldehyde for 1 hour, they were washed with PBS and permeabilized with 0.1% Triton X 100. Subsequently, Oil Red O staining was conducted at room temperature for 20 minutes. The cells were then observed and examined under a light microscope. Lipid accumulation was assessed by measuring the absorbance at 490 nm using a spectrophotometer (Mohan et al., 2023).

5.2.4.2 Quantification of triglyceride content (TG)

The triglyceride concentration was measured using a colorimetric assay kit (Cayman, USA). This assay was based on lipase's enzymatic hydrolysis of triglycerides, and the absorbance was measured at 530–550 nm (Shyni et al., 2021).

5.2.5 Immunofluorescence

Cells were seeded in 96 well plates and subjected to various treatments. Cells were then washed three times with PBS and followed by fixation with 100µl of 4% paraformaldehyde in PBS for 20 min. After fixation of cells, with gentle agitation, the cells were permeabilized at room temperature for 15 minutes using 0.25% Triton X-100 in PBS. 10% natural goat serum was used for blocking of cells for 1 hour before primary antibodies (4 °C, overnight) and secondary antibodies were added (1 h, at room temperature). Using fluorescently labeled anti-rabbit and anti-mouse Alexa flour secondary antibodies (cell signalling) were used to detect the primary antibodies. Nuclei were counterstained with DAPI (1 mg/mL in PBS) and visualized with a fluorescence microscope (Olympus IX 83).

5.2.6 Western Blot analysis

After respective treatments, cells were lysed in RIPA buffer with the protease inhibitor cocktail (Sigma Aldrich, USA). Followed by incubation, cell suspensions were centrifuged at 12,000 RPM for 20 min at 4 ◦C. The supernatant was collected and used for further immunoblot analysis. The protein concentration was measured by using

the bicinchoninic acid kit. An equal amount of proteins were separated by 8 or 10% SDS-PAGE and transferred to PVDF. Afterward, the membranes were blocked in TBS-Tween 0.1% containing 5% nonfat skimmed milk (Bio-Rad, Hercules, CA, USA) and incubated with primary antibodies overnight at 4°C. The alterations of various proteins such as PPAR-γ, p-AMPK, AMPK, p-ACC, ACC, FAS, CD36, C/EBP-α, SREBP-1C, TLR4, NF-κB, p-JNK, JNK, Resistin, FABP4, ATGL, NLRP-3, Lipin, p-ACLY, ACLY, AceCS1, AMBRA1, Beclin1, ATG7, ATG16L1 and LC3 were analyzed. Following the washing step with TBS-Tween 0.1%, the membranes were exposed to HRP-conjugated corresponding secondary antibodies for 2-4 hours at room temperature. Subsequently, the membranes were washed three times with TBS-Tween 0.1%, then the membranes were developed using Western Blot Hyper HRP Substrate (Takara Bio Inc. USA), and the protein bands were analyzed (Bio-Rad ChemiDoc MP imaging systems, USA) (Mahmood & Yang, 2012).

5.2.8 Statistical analysis

Statistical analysis was done using the SPSS statistical program. The results were represented as mean ± SEM. The significant differences among the treatments were evaluated using one-way analysis of variance (ANOVA). It is followed by Duncan's multiple range to determine which mean values were significantly different at $p \leq 0.05$. Statistical significance was indicated as * for $p \leq 0.05$ Vs C1 and # for $p \leq 0.05$ Vs C2.

5.3 Results

5.3.1 Cytoprotective effect of DI-HET on 3T3-L1 cells

DI-HET was not toxic up to 100 µg/mL in 3T3-L1 cells (C1) in 24 h and 48 h study (Figures. 5.1a, b). Based on viability data, 5 and 10 µg/mL of DI-HET extract was selected for further investigation against adipogenesis in 3T3-L1 cell lines.

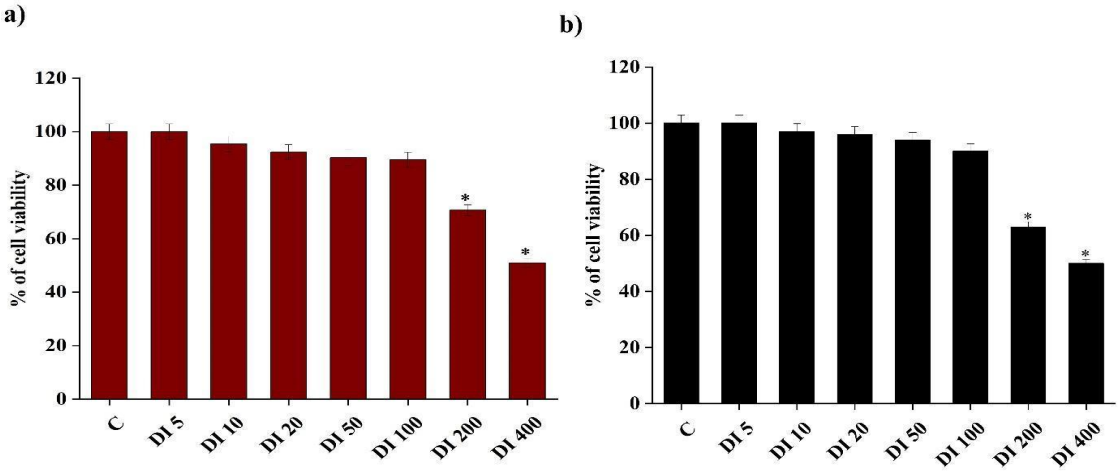


Figure. 5.1 Effect of DI-HET on viability of 3T3-L1 cells at 24 h and 48 h (a) 3T3-L1 cells were treated with DI-HET (5, 10, 20, 50, 100, 200, 400 µg/mL) for 24 h and cell viability was determined by MTT assay. (b) 3T3-L1 cells were treated with DI-HET (5, 10, 20,30, 40, 50, 100, 200 µg/mL) for 48 h, and cell viability was determined by MTT assay. Data are expressed as mean ± SEM where n = 6. * denotes significant difference from the control group ($p \leq 0.05$).

5.3.2 DI-HET inhibits intracellular lipid accumulation in 3T3-L1 cells

C2 group showed a significant increase in lipid accumulation by 38.10% ($p \leq 0.05$) compared to C1 Group. Extracts caused a significant reduction of lipid content in a dose-dependent manner (24.59% with DI 5 and 30.49% with DI 10; $p \leq 0.05$; Figures. 5.2a and b) when compared to the C2 group. For validating the result obtained from Oil Red O staining triglyceride accumulation was quantified in 3T3-L1 cells. The results showed that TG content was significantly increased in C2 group by 39.28% $p \leq (0.05)$

when compared to C1 group. TG content was significantly decreased in a dose-dependent manner with both extracts (16.71% with DI 5 and 30.92% with DI 10; $p \leq 0.05$; Figure. 5.3) when compared to the C2 group (39.28%).

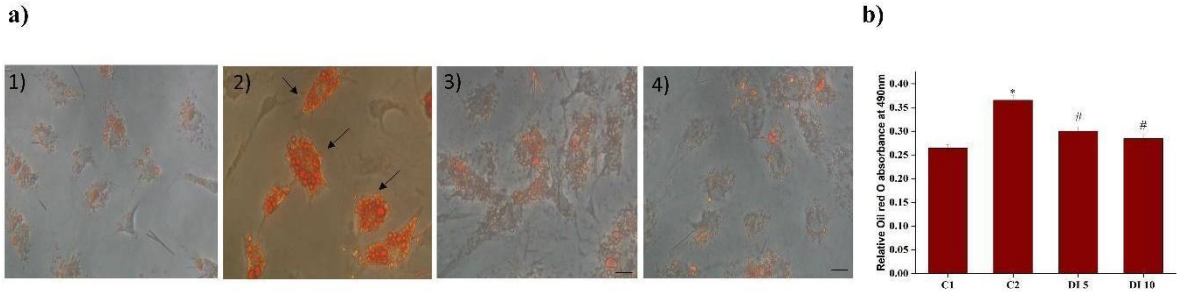


Figure. 5.2. Effect of DI-HET on lipid accumulation in 3T3-L1 cells: (a) The lipid accumulation was assessed by Oil Red O staining. Representative phase-contrast microscopic images of Oil Red O stained 3T3-L1 cells were presented, scale bar 50 μm. (1) Preadipocyte (C1) (2) Differentiated 3T3-L1 cells (C2) (3) Differentiated 3T3-L1 cells + DI 5 (4) Differentiated 3T3-L1 cells + DI 10 (b) Absorbance was read at 490 nm after Oil-Red-O staining. Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the C1 group ($p \leq 0.05$) and # denotes significant difference from the C2 group ($p \leq 0.05$).

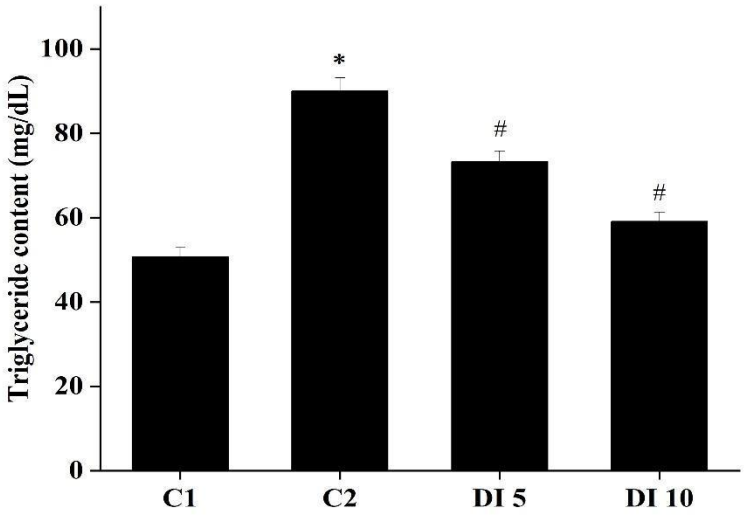


Figure. 5.3. Effect of DI-HET on triglyceride concentration in 3T3-L1 cells: Measurement of intracellular triglyceride content in 3T3-L1 cells (1) Pre-adipocyte(C1) (2) Differentiated 3T3-L1 cells (C2) (3) Differentiated 3T3-L1 cells + DI 5 (DI 5) (4)

Differentiated 3T3-L1 cells + DI 10 (DI 10). Data are expressed as mean ± SEM; where n=6. * denotes significant difference from the C1 group ($p \leq 0.05$) and # denotes significant difference from the C2 group ($p \leq 0.05$).

5.3.3 Effect of DI-HET on anti-adipogenic effect

The C2 group displayed increased PPAR-γ expression as compared to the C1 group (16.06 %; $p \leq 0.05$). Compared to the C2 group, the DI-HET extract-treated group inhibited the elevation of PPAR-γ levels (10.81 % with DI 5 and 28.14 % with DI 10; $p \leq 0.05$; Figures. 5.4 a, b). Two major transcription factors like C/EBP-α and SREBP-1C were studied. C/EBP-α helps in the differentiation of preadipocyte to mature adipocyte while SREBP-1C involved in fatty acid synthesis. Compared to the C1 group, C2 group showed enhanced C/EBP-α expression (15.78 %; $p \leq 0.05$). DI HET extract treated group prevented the upregulation of C/EBP-α levels when compared to the C2 group (14.43 % with DI 5 and 28.42 % with DI 10; $p \leq 0.05$; Figures. 5.4 a, b). Extract causes a significant downregulation of SREBP-1C (42.25 % with DI 5 and 32.18 % with DI 10; $p \leq 0.05$; Figures. 5.4 a, b) while its expression was upregulated in C2 group when compared to C1 group (8.86 %; $p \leq 0.05$).

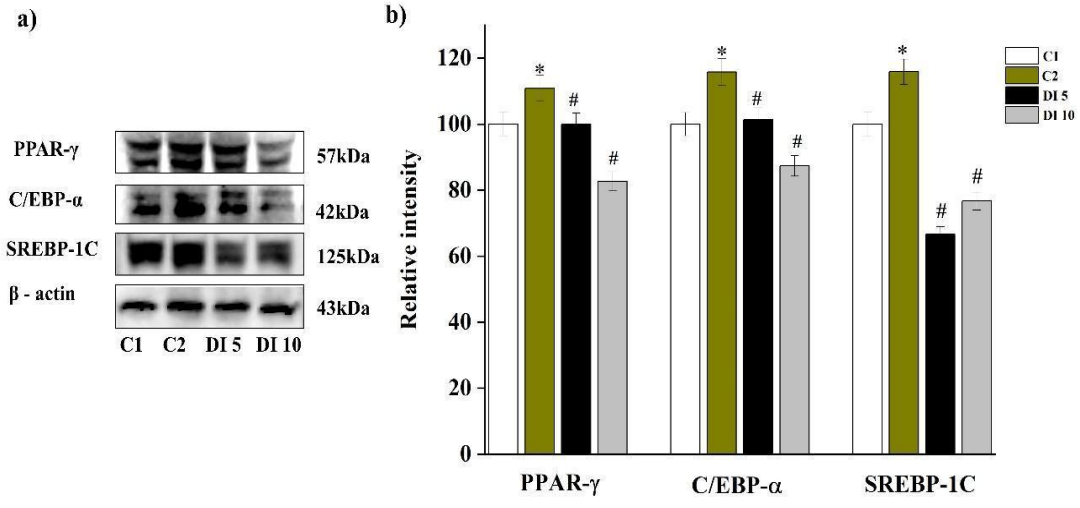


Figure. 5.4. Effect of DI-HET on PPAR-γ, C/EBP-α, and SREBP-1C (a) The protein expression in different experimental groups. b) The relative intensity of each band was quantified with β-actin. Pre-adipocyte (C1); Differentiated 3T3-L1 cells (C2); Differentiated 3T3-L1 cells + DI 5 (DI 5); Differentiated 3T3-L1 cells + DI 10 (DI 10). Data are expressed as

mean ± SEM; where n = 6. * denotes significant difference from the C1 group ($p \leq 0.05$) and # denotes significant difference from the C2 group ($p \leq 0.05$).

5.3.4 DI-HET on the activation of AMPK and associated proteins

The activation state of AMPK was assessed in different groups by calculating the ratio of phosphorylated AMPK to AMPK. Western blot showed phosphorylated AMPK was inhibited significantly in the C2 group (18.53%, $p \leq 0.05$) when compared to C1 group, and phosphorylated AMPK was significantly increased by DI-HET treatment (31.60% with DI 5 and 71.07% with DI 10; $p \leq 0.05$; Figures. 5.5a, b) compared to the C2 group.

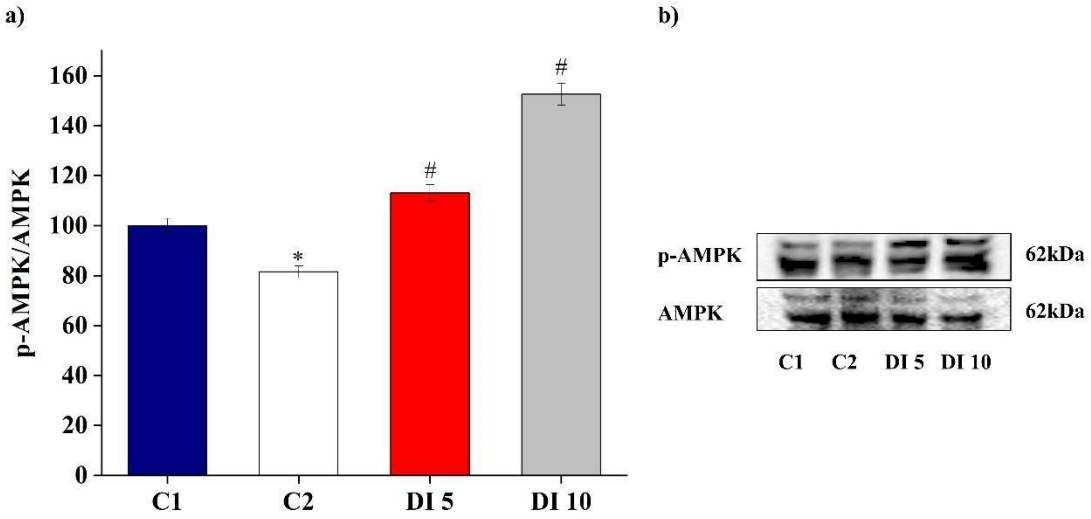


Figure. 5.5. Effect of DI-HET on AMPK a) The relative intensity of p-AMPK and AMPK. (b) The protein expression in different experimental groups. Pre-adipocyte (C1); Differentiated 3T3-L1 cells (C2); Differentiated 3T3-L1 cells + DI 5 (DI 5); Differentiated 3T3-L1 cells + DI 10 (DI 10). Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the C1 group ($p \leq 0.05$) and # denotes significant difference from the C2 group ($p \leq 0.05$).

5.3.4.1 Activation of AMPK inhibits ACC

The inhibition state of ACC was assessed in different groups by calculating the ratio of phosphorylated ACC to ACC. Western blot showed phosphorylated ACC was significantly decreased in the C2 group (28.17%; $p \leq 0.05$) when compared to C1 group. The expression of the phosphorylated form of ACC was significantly increased with DI-HET treatment (14.74% with DI 5 and 23.74% with DI 10; $p \leq 0.05$; Figures.

5.6 a,b) when compared to C2 group. Immunostaining study also showed the reduced expression of p-ACC with treatment (Figure. 5.6 c).

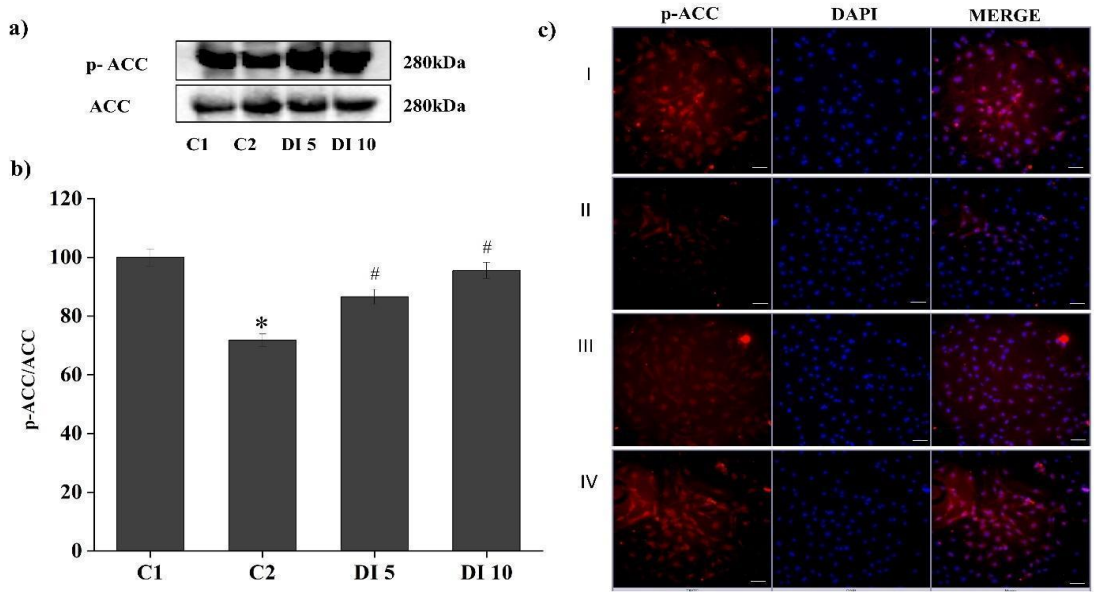


Figure. 5.6. Effect of DI-HET on ACC inhibition: (a) p-ACC/ACC protein expression in different experimental groups. b) Densitometric analysis of protein expression of p-ACC with respect to ACC. Pre-adipocyte (C1); Differentiated 3T3-L1 cells (C2); Differentiated 3T3-L1 cells + DI 5; Differentiated 3T3-L1 cells + DI 10. Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the C1 group ($p \leq 0.05$) and # denotes significant difference from the C2 group ($p \leq 0.05$). (C) Immunofluorescence staining showing p-ACC expression in different experimental groups. The cells were stained with DAPI (blue) and anti-p-ACC antibody (red). (i) Pre-adipocyte (C1); (ii) Differentiated 3T3-L1 cells (C2); (iii) Differentiated 3T3-L1 cells + DI 5; (iv) Differentiated 3T3-L1 cells + DI 10. Original magnification 20X.

5.3.4.2 Activation of AMPK inhibits FAS

FAS expression was significantly increased in the C2 group (29.84%, $p \leq 0.05$) compared to C1 group. Western blot showed that consistent with the activation of AMPK and suppression of SREBP-1C, FAS expression was inhibited significantly with DI-HET treatment (28.44% with DI 5 and 64.14% with DI 10; $p \leq 0.05$; Figures. 5.7b, c). Immunostaining study also showed the reduced expression of FAS with treatment (Figure. 5.7a).

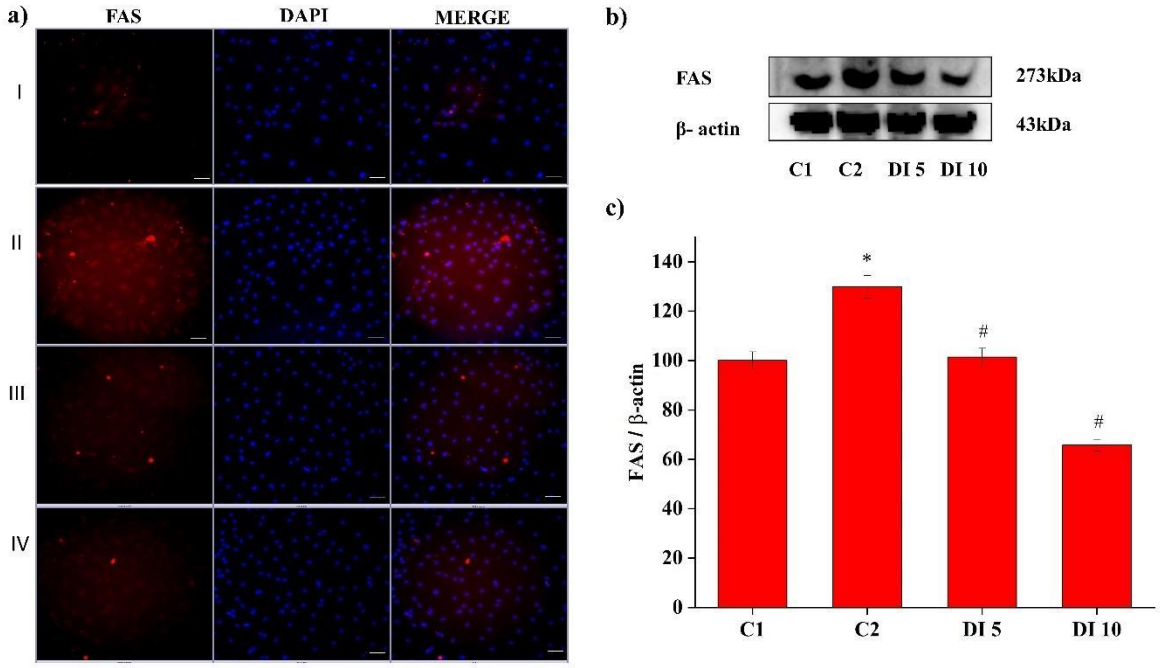


Figure. 5.7. Effect of DI-HET on FAS inhibition: (a)Immunofluorescence staining showing FAS expression in different experimental groups. The cells were stained by DAPI (blue) and anti- FAS antibody (red). (i) Pre-adipocyte (C1); (ii) Differentiated 3T3-L1 cells (C2); (iii) Differentiated 3T3-L1 cells + DI 5; (iv) Differentiated 3T3-L1 cells + DI 10. Original magnification 20X. b) FAS protein expression in different experimental groups. c) The relative intensity of each band was quantified with β-actin. Pre-adipocyte (C1); Differentiated 3T3-L1 cells (C2); Differentiated 3T3-L1 cells + DI 5; Differentiated 3T3-L1 cells + DI 10. Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the C1 group ($p \leq 0.05$) and # denotes significant difference from the C2 group ($p \leq 0.05$).

5.3.5 Effect of DI-HET on lipin expression

Lipin expression was also analysed. Western blot analysis showed that C2 group increased the expression of lipin when compared to with C1 group (35.61 %; $p \leq 0.05$). However, treatment with DI-HET shows reduced expression of lipin (17.2 % with DI 5 and 36.99 % with DI 10; $p \leq 0.05$; Figures. 5.8a, b). Immunostaining also showed the same results (Figure. 5.8c).

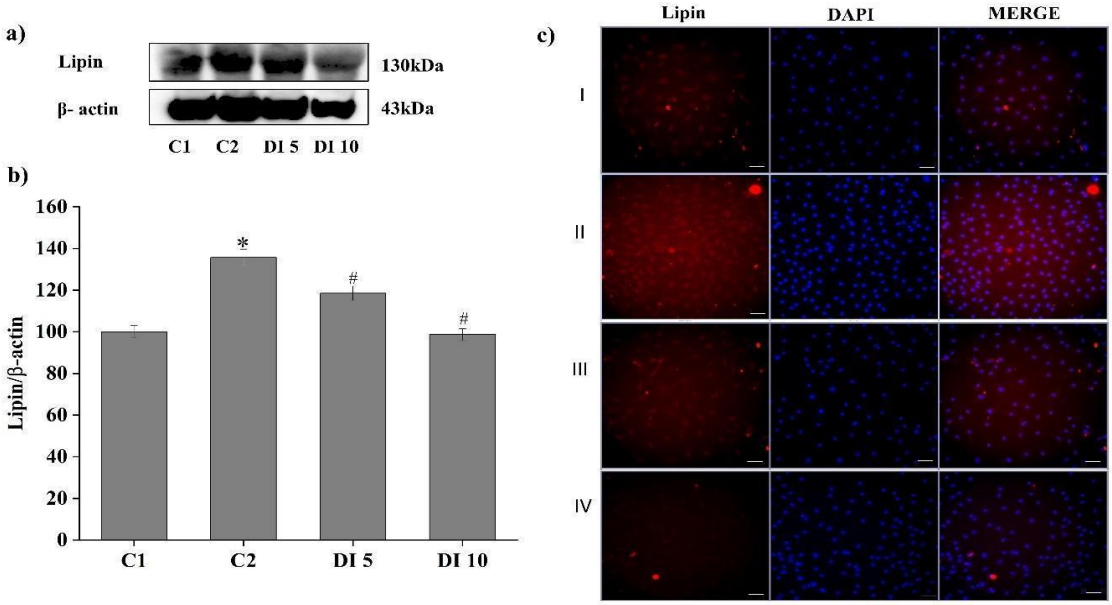


Figure. 5.8. Effect of DI-HET on lipin inhibition: (a) Lipin protein expression in different experimental groups. b) Densitometric analysis of protein expression of lipin with respect to β-actin. Pre-adipocyte (C1); Differentiated 3T3-L1 cells (C2); Differentiated 3T3-L1 cells + DI 5; Differentiated 3T3-L1 cells + DI 10. Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the C1 group ($p \leq 0.05$) and # denotes significant difference from the C2 group ($p \leq 0.05$). (C) Immunofluorescence staining showing lipin expression in different experimental groups. The cells were stained by DAPI (blue) and anti- lipin antibody (red). (i) Pre-adipocyte (C1); (ii) Differentiated 3T3-L1 cells (C2); (iii) Differentiated 3T3-L1 cells + DI 5; (iv) Differentiated 3T3-L1 cells + DI 10. Original magnification 20X.

5.3.6 Effect of DI-HET on p-ACLY/ACLY

The activation state of ACLY was assessed in different groups by calculating the ratio of phosphorylated ACLY to ACLY. Western blot showed phosphorylated ACLY was increased significantly in the C2 group (116.24%, $p \leq 0.05$) when compared to the C1 group. Phosphorylation of ACLY was inhibited significantly in DI-HET treated groups (20.10% with DI 5 and 37.47% with DI 10; $p \leq 0.05$; Figures. 5.9a, b), when compared to the C2 group.

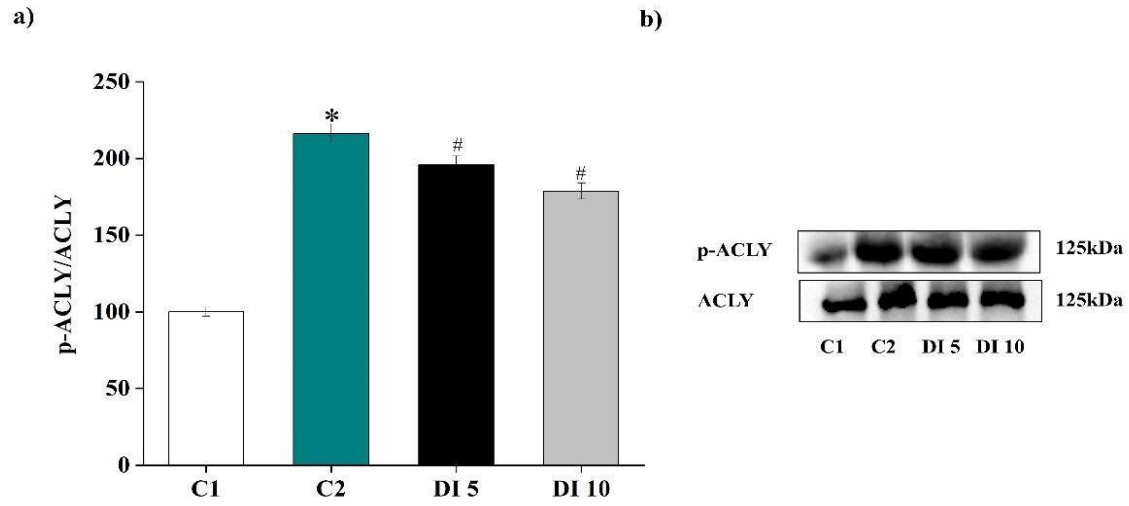


Figure. 5.9. Effect of DI-HET on p-ACLY/ACLY: a) The relative intensity of p-ACLY and ACLY. (b) The protein expression in different experimental groups. Pre-adipocyte (C1); Differentiated 3T3-L1 cells (C2); Differentiated 3T3-L1 cells + DI 5 (DI 5); Differentiated 3T3-L1 cells + DI 10 (DI 10). Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the C1 group ($p \leq 0.05$) and # denotes significant difference from the C2 group ($p \leq 0.05$).

5.3.7 Effect of DI-HET on AceCS1

The C2 group showed significant upregulation in the expression of AceCS1 by 28.68% ($p \leq 0.05$) compared to the C1 group. Treatment with DI-HET downregulated the expression of AceCS1 (16.18% with DI 5 and 19.48% with DI 10; $p \leq 0.05$; Figures. 5.10 a, b) compared to the C2 group. The C2 group showed significant upregulation in the expression of CD36 by 56.33% ($p \leq 0.05$) compared to the C1 group. Expression of CD36 was reduced with DI-HET treatment (78.61% with DI 5 and 70.13% with DI 10; $p \leq 0.05$; Figures. 5.10 a, b) when compared with C2 group.

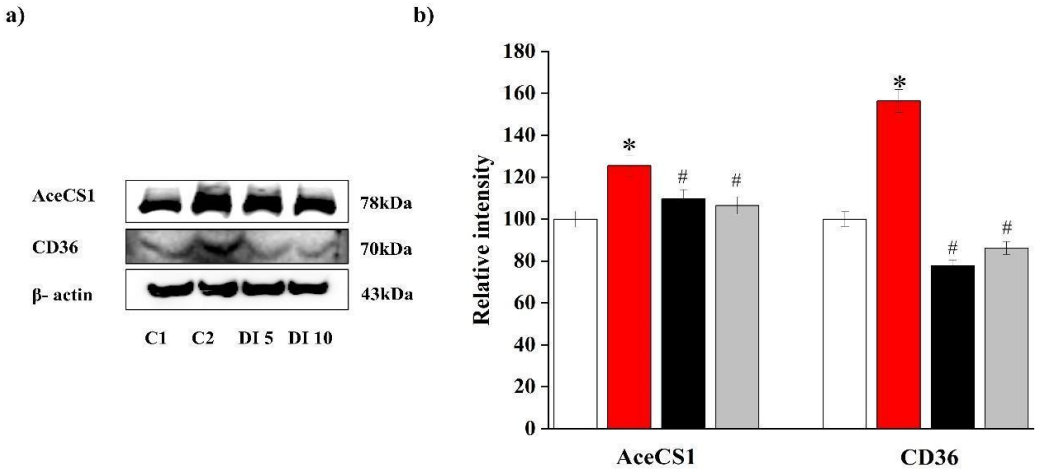


Figure. 5.10. Effect of DI-HET on AceCS1 (a) The protein expression in different experimental groups. b) The relative intensity of each band was quantified with β-actin. Pre-adipocyte (C1); Differentiated 3T3-L1 cells (C2); Differentiated 3T3-L1 cells + DI 5 (DI 5); Differentiated 3T3-L1 cells + DI 10 (DI 10). Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the C1 group ($p \leq 0.05$) and # denotes significant difference from the C2 group ($p \leq 0.05$).

5.3.8 Effect of DI-HET on FABP4 expression

The protein expression of FABP4 was significantly upregulated with the C2 group (32.25 %; $p \leq 0.05$) when compared to the C1 group. The expression of FABP4 was significantly reduced with DI-HET treatment (21.68 % with DI 5 and 46.06 % with DI 10; $p \leq 0.05$; Figures. 5.11a, b) when compared to the C2 group.

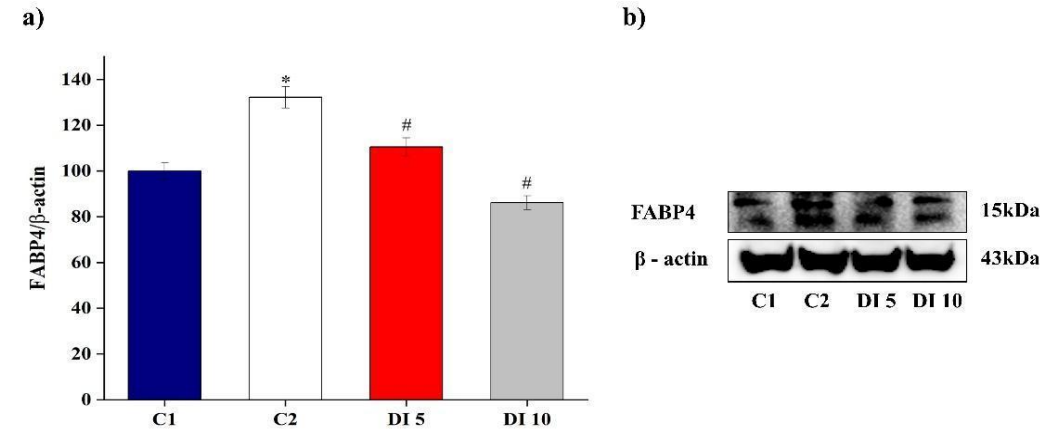


Figure. 5.11. Effect of DI-HET on FABP4: a) The relative intensity of FABP4 with β-actin. (b) The protein expression in different experimental groups. Pre-adipocyte (C1); Differentiated

3T3-L1 cells (C2); Differentiated 3T3-L1 cells + DI 5 (DI 5); Differentiated 3T3-L1 cells + DI 10 (DI 10). Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the C1 group ($p \leq 0.05$) and # denotes significant difference from the C2 group ($p \leq 0.05$).

5.3.9 Effect of DI-HET on ATGL expression

The protein expression of ATGL was significantly upregulated with the C2 group (17.06 %; $p \leq 0.05$) when compared to the C1 group. The expression of ATGL was significantly increased with DI-HET treatment (17.84 % with DI 5 and 25.55 % with DI 10; $p \leq 0.05$; Figures. 5.12 a, b) when compared to the C2 group.

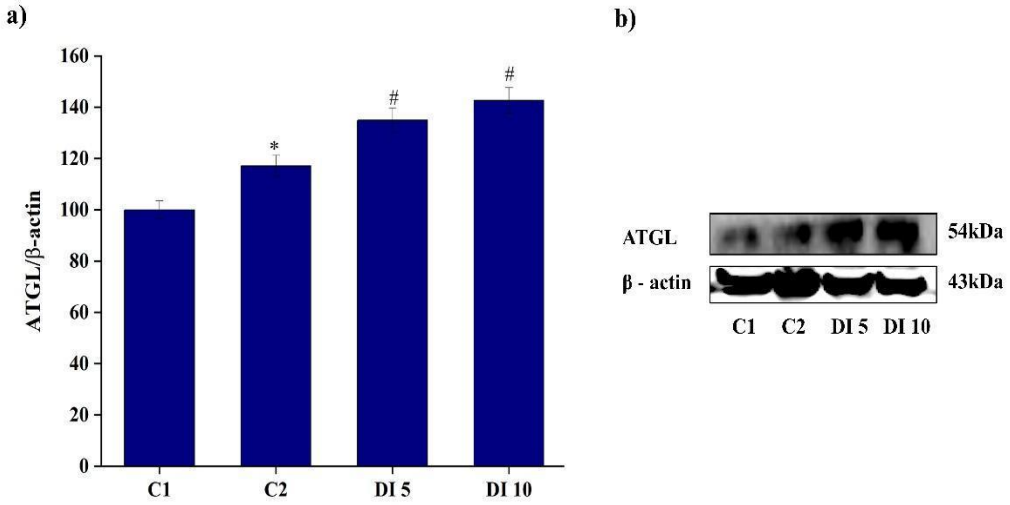


Figure. 5.12. Effect of DI-HET on ATGL: a) The relative intensity of ATGL with β-actin. (b) The protein expression in different experimental groups. Pre-adipocyte (C1); Differentiated 3T3-L1 cells (C2); Differentiated 3T3-L1 cells + DI 5 (DI 5); Differentiated 3T3-L1 cells + DI 10 (DI 10). Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the C1 group ($p \leq 0.05$) and # denotes significant difference from the C2 group ($p \leq 0.05$).

5.3.10 Effect of DI-HET on inflammation in 3T3-L1 cells

Major proteins involved in the inflammatory signaling pathway was evaluated to elucidate the mechanistic action of DI-HET on inflammation during preadipocyte differentiation. DI-HET extract treatment significantly reduced the expression of proinflammatory proteins, directly indicating the therapeutic potential of the extract.

The C2 group showed significant upregulation in the expression of TLR-4 by 20.15 % ($p \leq 0.05$) compared to the C1 group. Treatment with DI-HET downregulated the expression of TLR-4 (35.50 % with DI 5 and 79.36 % with DI 10; $p \leq 0.05$; Figures .5.13a, b) when compared to the C2 group.

Western blot showed significant upregulation in the expression of NF-κB in the C2 group by 50.27% ($p \leq 0.05$) compared to C1. NF-κB expression was reduced with DI-HET treatment (23.61% with DI 5 and 27.49% with DI 10; $p \leq 0.05$; Figures .5.13a, b) when compared to C2 group.

The activation state of JNK was assessed in different groups by calculating the ratio of phosphorylated JNK to JNK. Western blot showed JNK activation was significantly increased in the C2 group when compared to C1 (9.95%, $p \leq 0.05$). Phosphorylation of JNK was inhibited significantly in DI-HET treated groups (37.79 % with DI 5 and 43.36 % with DI 10; $p \leq 0.05$; Figures .5.13a, b) compared to the C2 group.

Western blot showed that NLRP3 expression was upregulated in C2 group by 10.23% ($p \leq 0.05$) compared to C1 group, whereas NLRP3 expression was reduced with DI-HET treatment (38.83 % with DI 5 and 60.16 % with DI 10; $p \leq 0.05$; Figures .5.13a, b) when compared with C2 group.

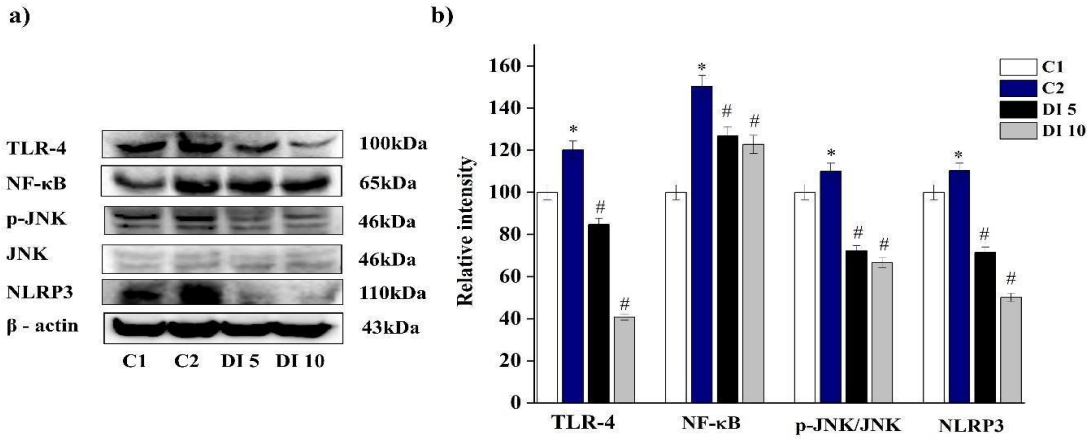


Figure. 5.13. Effect of DI-HET on TLR-4, NF-κB, p-JNK/JNK, NLRP3 (A) The protein expression in different experimental groups. B) The relative intensity of each band was quantified with β-actin. Pre-adipocyte (C1); Differentiated 3T3-L1 cells (C2); Differentiated

3T3-L1 cells + DI 5 (DI 5); Differentiated 3T3-L1 cells + DI 10 (DI 10). Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the C1 group ($p \leq 0.05$) and # denotes significant difference from the C2 group ($p \leq 0.05$).

5.3.10.1 Effect of DI-HET on Resistin

Compared with the C1 group, C2 showed enhanced expression of resistin by 33.71 % ($p \leq 0.05$). The expression of resistin was significantly reduced with DI-HET treatment (22.57 % with DI 5 and 49.7 % with DI 10; $p \leq 0.05$; Figures .5.14a, b) when compared to the C2 group.

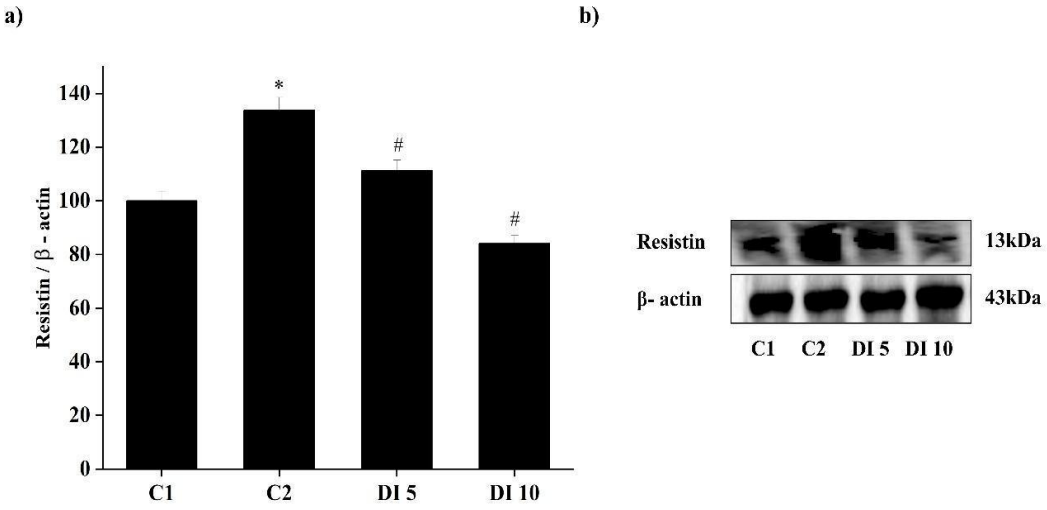


Figure. 5.14. Effect of DI-HET on Resistin: a) The relative intensity of Resistin with β-actin. (b) The protein expression in different experimental groups. Pre-adipocyte (C1); Differentiated 3T3-L1 cells (C2); Differentiated 3T3-L1 cells + DI 5 (DI 5); Differentiated 3T3-L1 cells + DI 10 (DI 10). Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the C1 group ($p \leq 0.05$) and # denotes significant difference from the C2 group ($p \leq 0.05$).

5.3.11 DI-HET regulates autophagy in 3T3-L1 cells

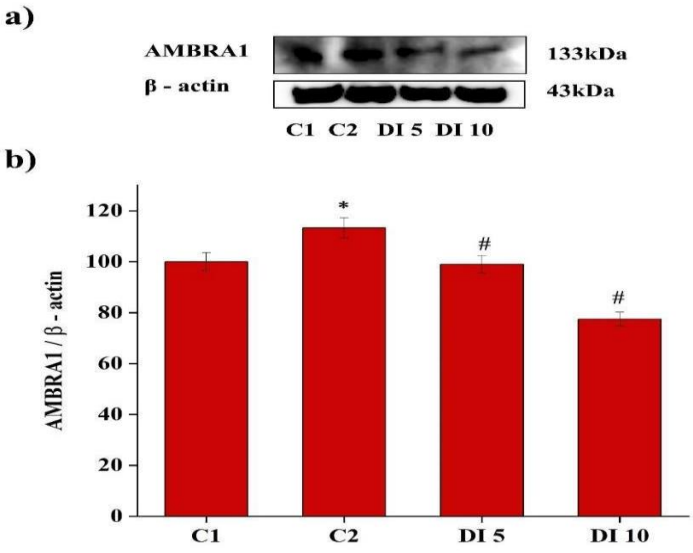
The C2 group showed significant upregulation in the expression of AMBRA1 by 13.32 % ($p \leq 0.05$) when compared to C1. Treatment with DI-HET downregulated the expression of AMBRA1 (14.33 % with DI 5 and 35.86 % with DI 10; $p \leq 0.05$; Figures .5.15a, b). Immunostaining also showed the same results (Figure .5.15c).

The protein expression of Beclin1 was significantly upregulated in the C2 group by 26.55 % ($p \leq 0.05$) compared to the C1 group, whereas expression of Beclin1 was reduced with DI-HET treatment (19.89 % with DI 5 and 26.01 % with DI 10; $p \leq 0.05$; Figures .5.16a, b) when compared with C2 group. Immunostaining also showed the same results (Figure .5.16c).

C2 group showed significant upregulation in the expression of ATG7 by 122.25 % ($p \leq 0.05$) when compared to C1, whereas expression of ATG7 was inhibited significantly in DI-HET treated groups (33.56 % with DI 5 and 54.41 % with DI 10; $p \leq 0.05$; Figures .5.17a, b) when compared to C2.

The protein expression of ATG16L1 was significantly upregulated in the C2 group by 29.50 % ($p \leq 0.05$) compared to the C1 group. Treatment with DI-HET significantly decreased the ATG16L1 expression (60.46 % with DI 5 and 74.61 % with DI 10; $p \leq 0.05$; Figures .5.17a, b) when compared with the C2 group.

LC3 expression was significantly upregulated in C2 group by 70.27 % ($p \leq 0.05$) compared to C2 group, whereas the expression of LC3 was significantly reduced with DI-HET treatment (104.89 % with DI 5 and 155.82 % with DI 10; $p \leq 0.05$; Figures .5.17a, b) when compared to the C2 group.



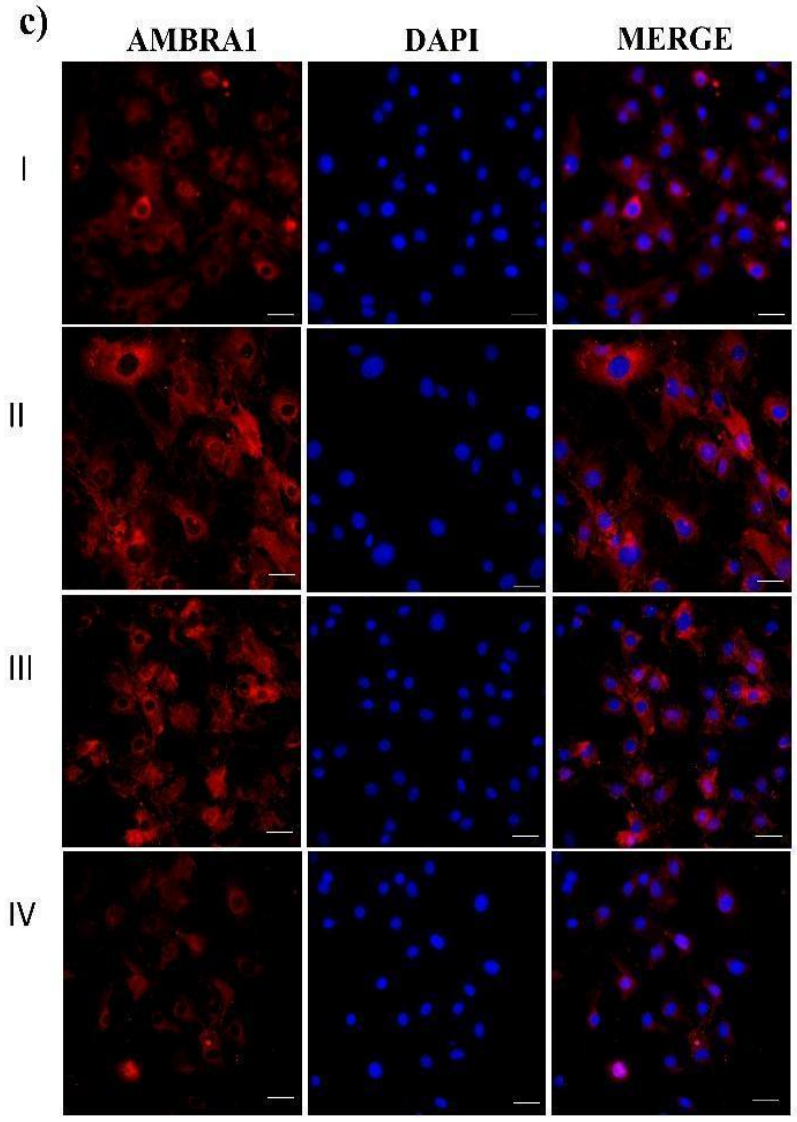


Figure. 5.15. Effect of DI-HET on AMBRA1 inhibition: (a) AMBRA1 protein expression in different experimental groups. b) Densitometric analysis of protein expression of AMBRA1 with respect to β-actin. Pre-adipocyte (C1); Differentiated 3T3-L1 cells (C2); Differentiated 3T3-L1 cells + DI 5; Differentiated 3T3-L1 cells + DI 10. Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the C1 group ($p \leq 0.05$) and # denotes significant difference from the C2 group ($p \leq 0.05$). (C) Immunofluorescence staining showing AMBRA1 expression in different experimental groups. The cells were stained by DAPI (blue) and anti- AMBRA1 antibody (red). (i) Pre-adipocyte (C1); (ii) Differentiated 3T3-L1 cells (C2); (iii) Differentiated 3T3-L1 cells + DI 5; (iv) Differentiated 3T3-L1 cells + DI 10. Original magnification 40X. Scale bar corresponds to 20 μm.

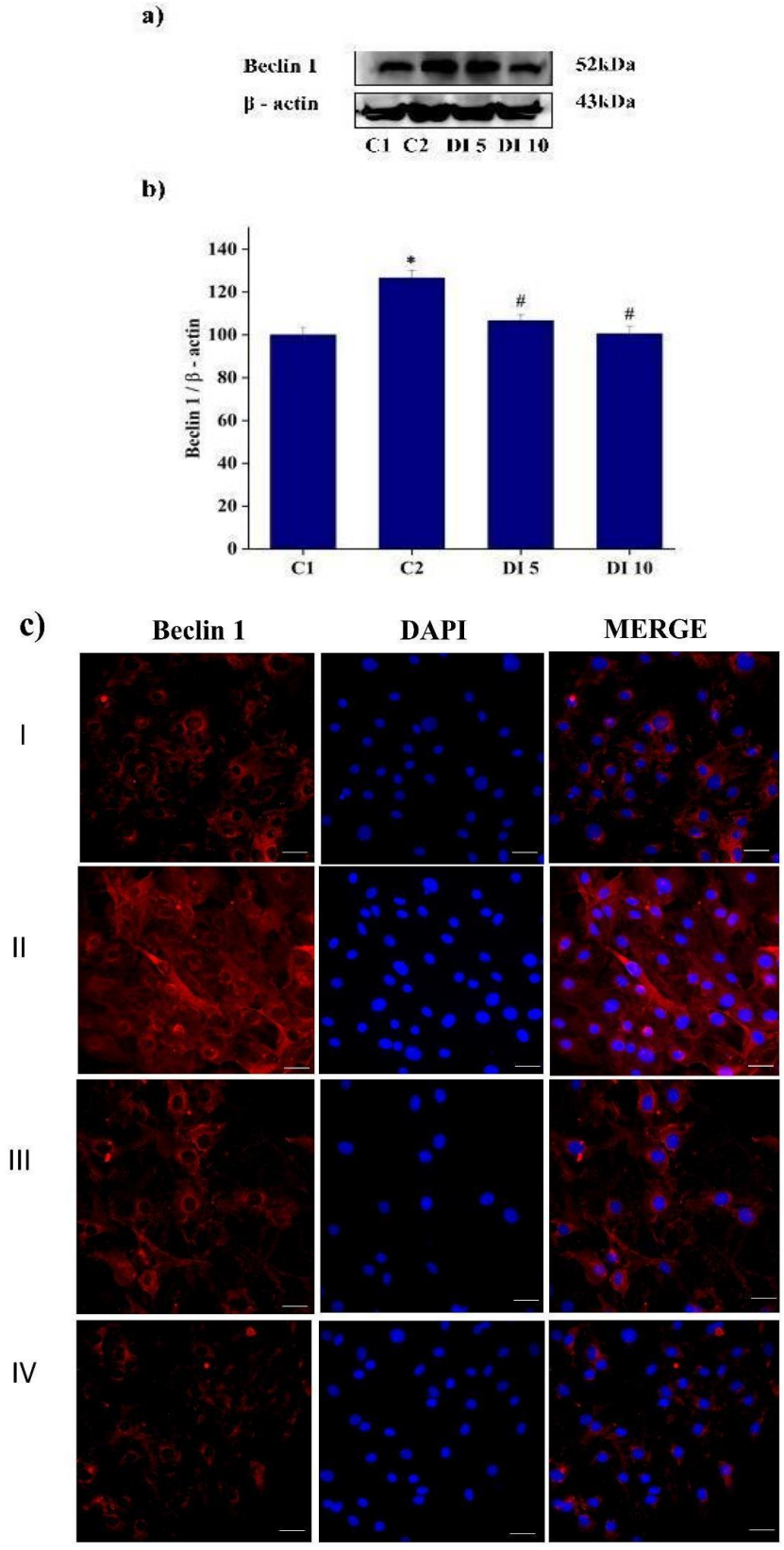


Figure. 5.16. Effect of DI-HET on Beclin1 inhibition: (a) Beclin1 protein expression in different experimental groups. b) Densitometric analysis of protein expression of Beclin1 with

respect to β-actin. Pre-adipocyte (C1); Differentiated 3T3-L1 cells (C2); Differentiated 3T3-L1 cells + DI 5; Differentiated 3T3-L1 cells + DI 10. Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the C1 group ($p \leq 0.05$) and # denotes significant difference from the C2 group ($p \leq 0.05$). (C) Immunofluorescence staining showing Beclin1 expression in different experimental groups. The cells were stained by DAPI (blue) and anti-Beclin1 antibody (red). (i) Pre-adipocyte (C1); (ii) Differentiated 3T3-L1 cells (C2); (iii) Differentiated 3T3-L1 cells + DI 5; (iv) Differentiated 3T3-L1 cells + DI 10. Original magnification 40X. Scale bar corresponds to 20 μm.

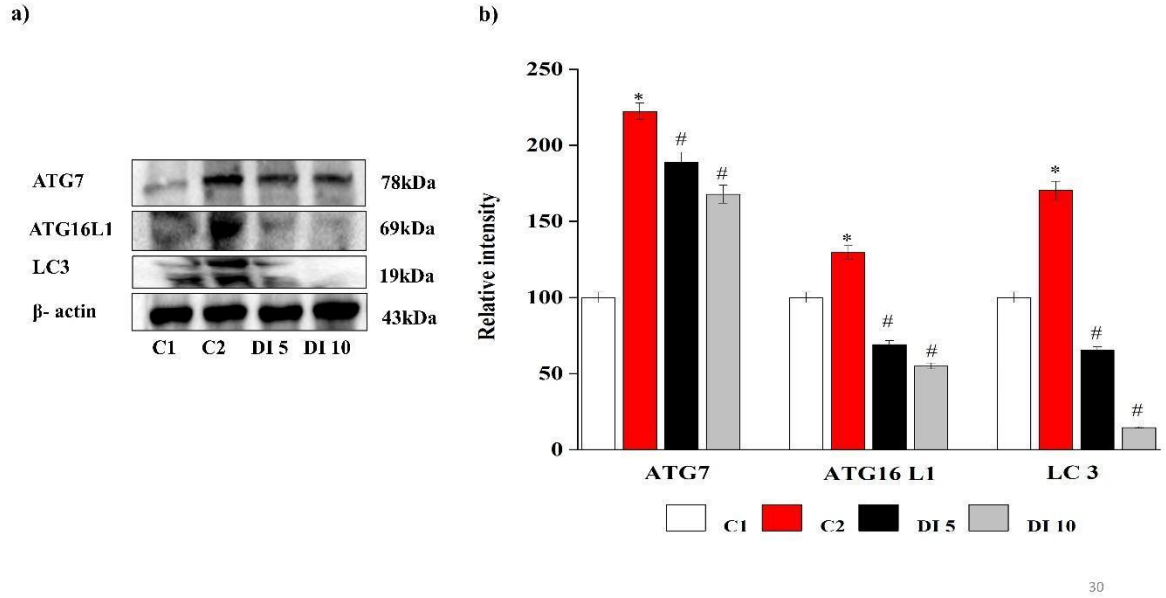


Figure. 5.17. Effect of DI-HET on ATG7, ATG16L1 and LC3 (A) The protein expression in different experimental groups. B) The relative intensity of each band was quantified with β-actin. Pre-adipocyte (C1); Differentiated 3T3-L1 cells (C2); Differentiated 3T3-L1 cells + DI 5 (DI 5); Differentiated 3T3-L1 cells + DI 10 (DI 10). Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the C1 group ($p \leq 0.05$) and # denotes significant difference from the C2 group ($p \leq 0.05$).

5.4 Discussion

Here, the present study investigated the antiadipogenic potential of DI-HET extract in 3T3-L1 cell lines. And also evaluated the protective role of the extract against inflammation and autophagy. Earlier studies suggest that several plant extracts have antiadipogenic potential. To address obesity and associated aberrant lipid metabolism, a thorough understanding of the molecular mechanisms driving adipose tissue

function is essential. Hence, this chapter deals with the regulatory effect of DI-HET on adipogenesis, lipogenesis, inflammation, and autophagy *in vitro* model.

Traditional medicine has utilized *D. indica* for the treatment of various diseases. However, its potential as an anti-adipogenic agent has not been reported thus far. In this regard, the anti-adipogenic potential of the DI-HET extract was studied. DI-HET extract effectively inhibits adipogenesis and lipid synthesis, while promoting lipolysis in adipocytes. Lipid accumulation and the expression of several proteins related to adipogenesis and lipolysis during the differentiation of 3T3-L1 adipocytes were studied.

Adipose cells' size varies considerably, primarily depending on how much triglyceride is kept in reserve. The majority of the extra energy is retained as triglycerides in adipose tissue when energy intake consistently exceeds energy expenditure. A growth in cell size, cell number, or both can result in an increase in adipose tissue mass. Mild obesity is primarily characterized by an enlargement in the size of adipose cells, known as hypertrophy. On the other hand, more severe obesity or obesity that manifests during childhood typically involves an increase in the number of fat cells, referred to as hyperplasia (Spiegelman & Flier, 1996). The molecular mechanisms that drive preadipose cell development, adipose differentiation, and lipogenesis in fat cells have received great attention because they play a significant role in a homeostatic system that regulates energy balance.

Adipogenesis is the maturation process of preadipocytes to differentiated adipocytes. Adipogenesis is a process that consists of two distinct phases: the determination phase and the terminal differentiation phase. In the determination phase, mesenchymal stem cells undergo a conversion into preadipocytes. This marks the initial commitment of the cells toward the adipocyte lineage. But the preadipocyte stage can't be distinguished morphologically from mesenchymal stem cells. However, preadipocyte lost their ability to differentiate into other cell types. Terminal differentiation has characterized the conversion of preadipocytes into mature adipocytes. At this stage, genes and proteins involved in adipogenesis, lipogenesis, and insulin action will be more prominent (Symonds, 2017). More than 2000 genes are involved in the regulation of adipogenesis, which is tightly controlled by numerous

other transcription factors (Farmer, 2006). Hence an intricate network of transcription factors are involved in the differentiation of preadipocytes into adipocytes. The two main adipogenic transcription factors, PPAR-γ and C/EBP-α, regulate the terminal differentiation process and control the synthesis of lipid metabolizing enzymes in mature adipocytes. PPAR-γ is considered the master regulator of adipocyte differentiation (Rosen et al., 2002). Our knowledge of the transcriptional basis of fat cell differentiation and adipose cell gene expression has greatly expanded in recent years, especially with the discovery of transcription factors.

Effect of DI-HET extract on lipid accumulation was studied. Morphological analysis, along with Oil Red O staining, revealed a significant reduction in lipid droplet accumulation in 3T3-L1 cells treated with DI-HET in comparison to the control group. Furthermore, quantitative measurements of cellular triglyceride levels also showed a significant decrease with DI-HET treatment. The inhibitory effect of DI-HET on adipogenesis was observed in a dose-dependent manner, as DI-HET efficiently reduced TG accumulation. This inhibitory action is attributed to DI-HET inducing lipolysis, leading to the hydrolysis of triglycerides and consequent reduction in intracellular lipid accumulation. The impact of DI-HET on adipogenesis was further confirmed by analyzing the expression of key regulator proteins involved in adipogenesis. Major transcription factors like PPAR-γ, C/EBP-α, and SREBP-1C were analyzed. Interestingly, DI-HET down-regulated the expression of PPAR-γ, C/EBP-α, and SREBP-1C in 3T3-L1 cells. During the initial stage of differentiation of preadipocytes, PPAR-γ and C/EBP- α expression are directly induced by both C/EBP-β and C/EBP-γ. After that, positive feedback is induced by PPAR-γ and C/EBP- α that activate a large number of downstream target genes and their own expression (Tang et al., 2005). The key lipogenic proteins, such as FAS and ACC, are expressed more when SREBP-1C is activated. That facilitates the activation of lipid production by insulin in adipocytes. Recent evidence suggests that the expression of SREBP-1C is activated by the activation of PPAR-γ and C/EBP-α. SREBP-1C expression occurs during the terminal differentiation of adipocytes (Tang & Lane, 2012). A key enzyme in the production of fatty acids is called ACC. Fatty acid production in cells is downregulated as a result of ACC phosphorylation. FAS expression was reduced with DI-HET treatment compared to differentiated adipocytes. Phosphorylated form of ACC was also increased with DI-

HET extract treatment, which is an inhibited form of ACC. Hence all these results indicate the anti-adipogenic potential of DI-HET.

Consistent with these results, PPAR-γ target gene FABP4 expression was also markedly downregulated in DI-HET treatment. It is known that activation of PPAR-γ induces preadipocyte differentiation. FABP4 is an intracellular protein that exerts a significant influence on lipid fluxes, metabolism, and intracellular signaling. In the context of obesity, FABP4 levels are elevated within adipocytes and actively secreted by these cells. Increased circulating levels of FABP4 are strongly correlated with obesity, metabolic syndrome, cardiac dysfunction, and hepatic glucose production stimulation. Given its prominent role in various metabolic diseases, targeting FABP4 presents a promising and innovative therapeutic approach. By modulating the activity or expression of FABP4, it may be possible to regulate lipid metabolism, enhance insulin sensitivity, and mitigate the adverse effects associated with obesity and metabolic disorders. Consequently, FABP4 represents an attractive target for the development of novel therapeutic interventions in the treatment of metabolic diseases (Garin-Shkolnik et al., 2014). ATGL, also known as patatin-like phospholipase domain 2. ATGL catalyses the first reaction of lipolysis, where triacylglycerols are hydrolysed to diacylglycerols. ATGL is highly expressed in the adipose tissue of mice and humans (Kim et al., 2006). Treatment with DI-HET upregulated the expression of ATGL.

During adipogenesis, *de novo* lipogenesis (DNL) and lipid uptake will increase. Differentiation of adipocytes promotes transcription of fat-specific marker genes like fatty acid translocase protein FAT or CD36, which helps in the uptake of long-chain fatty acids to cells for lipogenesis. CD36 expression is under the control of PPAR-γ (Cha et al., 2010). Treatment with DI-HET decreased the expression of the CD36 protein.

ACLY is another key enzyme that plays an important role in *de novo* lipogenesis that aids in the formation of acetyl CoA from citrate. In fact, it is the major enzyme that links carbohydrates to lipid metabolism. Dietary carbohydrates get through glycolysis and the tricarboxylic acid (TCA) cycle in the mitochondria to produce citrate, which is then transported to the cytosol and used by the enzyme ACLY to release acetyl-CoA. Then ACC converts the resultant acetyl-CoA into malonyl-CoA. Further,

FAS transforms malonyl-CoA into palmitate, the first fatty acid product in DNL. Ultimately, the complex fatty acids, such as stearic acid, palmitoleic acid, and oleic acid, are produced from palmitate by the elongation and desaturation events. Phosphorylation of ACLY (p-ACLY) at its Ser455 causes its activation. Transcription factor SREBP-1C regulates ACLY (Song et al., 2018). Overexpression of ACLY is associated with hyperlipidemia, cardiovascular diseases, and cancer. Studies have reported that finding efficient ACLY inhibitors will significantly advance our understanding of lipid-related diseases and cancer (Granchi, 2022). The expression of p-ACLY is reduced with DI-HET treatment. Consistent with the downregulation of SREBP-1C, ACLY is also downregulated. It inhibits the formation of acetyl CoA from citrate and reduces fatty acid synthesis.

As mentioned earlier, acetyl-CoA is an essential molecule that helps in synthesizing fatty acids and cholesterol and participates in the tricarboxylic acid cycle. AceCS1 catalyzes the ligation of acetate with CoA to create acetyl-CoA (Ikeda et al., 2001). AceCS1 is under the control of SREBP1C (Fujino et al., 2003). The protein expression of AceCS1 was reduced with DI-HET treatment. As a result, the formation of acetyl CoA is also reduced, which acts as the substrate for DNL.

AMPK has a crucial role to play in lipid metabolism in adipocytes by inactivating ACC. To investigate the effects of DI-HET on adipogenesis and lipid metabolism, a comprehensive analysis of various cellular and molecular pathways in 3T3-L1 adipocytes was studied. The primary focus was on DI-HET's role as an anti-adipogenic and anti-lipogenic agent, particularly its impact on PPARγ downregulation in *in vitro*. Treatment with DI-HET promotes the AMPK-dependent oxidation of fatty acids in 3T3-L1 cells. Activation of AMPK and subsequent inhibition of ACC activity were identified as critical signaling pathways in fatty acid oxidation, influencing the transport of fatty acids into mitochondria (Viollet et al., 2007). AMPK plays a pivotal role in regulating glucose and lipid metabolism, integrating cellular responses to nutritional signals (Long & Zierath, 2006). AMPK activation downregulates anabolic pathways and upregulates catabolic pathways, including the expression of lipolytic proteins. Protein expression analysis demonstrated that DI-HET activates AMPK, leading to the phosphorylation-mediated reduction of ACC activity and suppression of SREBP 1c protein expression. ACC is responsible for synthesizing malonyl-CoA from

acetyl-CoA, a crucial step in de novo lipogenesis (DNL). Therefore, DI-HET effectively inhibited lipid droplet accumulation in 3T3-L1 adipocytes by downregulating ACC expression.

The effect of the extract on inflammation was further studied. It is now well understood that adipose tissue is a major source of proinflammatory cytokines. Research in animal models is evident that the development of metabolic disease is significantly contributed by adipose tissue inflammation. Probably many variables play a role in the onset of inflammation in adipose tissue. Putative processes promoting inflammation in adipose tissue include ER stress, infiltration of macrophages to adipose tissue, hypoxia, adipocyte hypertrophy, FFA, mitochondrial dysfunction, and fibrosis. Research studies have reported that ER stress and inflammation interact strongly because the UPR (Unfolded protein response) stimulates NF-κB and JNK signaling and proinflammatory cytokines, which in turn activates UPR (Burhans et al., 2018). Activation of NF-κB and JNK was significantly reduced with DI-HET treatment. In addition to that, expression of NLRP3 and TLR-4 was evaluated. TLR4 is a crucial cell surface receptor that is activated by FFA. NLRP3 inflammasome acts as a lipid-responsive protein complex, which means excess lipid or FFA stimulates its activation (Esser et al., 2013). Studies have demonstrated that FFA activates the NLRP3 inflammasome via the TLR4 receptor (Reynolds et al., 2012). Expression of both proteins was found to be decreased with DI-HET treatment supporting its application against inflammation.

Resistin expression with DI-HET treatment was further studied. Resistin is a hormone secreted from adipose tissue. Resistin impacts various cell types and tissues and functions in an autocrine, paracrine, and endocrine manner (Qi et al., 2008; Shetty et al., 2004; Won et al., 2009). Both genetically and high-fat diet obese mice exhibit elevated serum levels of resistin. Treatment with the diabetes medication rosiglitazone lowers the serum resistin level. Elevated levels of resistin action resist insulin action and impair glucose homeostasis. This, in turn, leads to the development of T2D (Steppan et al., 2001). Its secretion induces the activation of NF-κB (Silswal et al., 2005). Resistin appears as a connecting link between visceral obesity and type 2 diabetes. Resistin is associated with conditions other than T2D or obesity are cancer,

atherosclerosis, arthritis, hypertension, and cardiovascular illnesses (CVD). Treatment with DI-HET extract significantly reduced the resistin expression.

Regulation of autophagy with DI-HET extract treatment during adipogenesis was analyzed. According to the most recent research, autophagy controls lipid storage in adipose tissue and liver tissues to maintain lipid metabolic balance. Also plays a crucial role in regulating lipid metabolism in fat cells and sustaining the activities of fat cells by increasing or decreasing the breakdown of lipid droplets in response to variations in the body's nutritional condition. Based on research findings, the knockout of the Atg7 gene has been shown to hinder fat-specific autophagy and reduce overall fat accumulation in adipose tissue (Frudd et al., 2018). This observation leads to the hypothesis that the absence of autophagy in adipose tissue prevents the formation of lipid droplets, consequently impeding fat buildup. However, it is postulated that maintaining a low basal level of autophagy is necessary for fat accumulation and adipose tissue differentiation (Tao & Xu, 2020). Studies in animal models also show that autophagy can be triggered by proinflammatory transcription factors like NF-κB. This is an example of the multiple regulation of autophagy. During obesity, upregulation of autophagy results in fewer mitochondria in adipocytes, resulting in reduced lipid oxidation and promoting fat storage instead of oxidation. Although autophagy has a protective function in the body, in obesity and MetS, it can play a seriously detrimental effect. Hence the advantage of autophagy activation or inhibition depends on the disease condition (Menikdiwela et al., 2020). Further research is needed to determine whether autophagy activation and downregulation are detrimental or beneficial in various tissues and disease conditions. Major five proteins involved in autophagy were studied. Downregulated expression of AMBRA1, Beclin1, LC3, ATG7, and ATG16L1 was observed with DI-HET treatment.

Hence the overall study demonstrated the beneficial effect of DI-HET against adipogenesis through the inhibition of major transcription factors like PPAR-γ, C/EBP-α, and SREBP-1C. And also, the activation of AMPK aids in lipolysis through the increased expression of lipolytic proteins and further inhibition of lipogenic proteins. The salient finding of this study also revealed the anti-inflammatory effect and the ability of the extract to regulate autophagy during adipogenesis. The protective effect of DI-HET extract is definitively through the synergetic effect of polyphenols

present in it. Therefore, these results show the importance of *D.indica*-based drug development in the future.

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Chapter 6

Summary and Conclusion

Nonalcoholic fatty liver disease (NAFLD) is a hepatic manifestation of metabolic syndrome. It is a spectrum of liver disorders caused by the buildup of fat in the liver. The prevalence rate of the disease has increased globally. This leads to a huge number of liver transplants in the world population. The lack of FDA-approved treatment for this condition urges the development of new therapeutic strategies. The complexity of NAFLD pathogenesis is the major hassle for making targeted therapeutic interventions for the disease. This is due to the factors like complex interaction between the genetic, metabolic, and environmental factors involved in disease progression. Our incomplete knowledge about the underlying mechanism for NAFLD makes it more difficult to design new medications. Researchers from all over the world are actively investigating possible drug candidates and therapeutic drug targets. But this process is time-consuming because it needs clinical studies, safety evaluation, and regulatory approval. Therefore, overcoming these obstacles needs more and continuous scientific research for a thorough understanding of disease pathogenesis to develop safe and effective medication for NAFLD.

Plant-based drugs play a major role in modern medicine because of their remarkable health benefits. Plants have long been used in traditional medicine to treat a variety of diseases including cancer. The beneficial effect of plant-based medications is because of the presence of bioactive compounds like alkaloids, flavonoids, terpenoids, and phenolic compounds. These bioactive compounds have different pharmacological impacts on target-specific activation and inhibition of metabolic pathways that make them valuable for drug discovery and development. In addition, plant-based drugs have a very good safety profile when compared to their synthetic counterparts. *Dillenia indica* L. is a well-known plant in Ayurveda and traditional medicine for its therapeutic benefits. The pharmacological potential of *Dillenia indica* L. is due to the presence of many bioactive compounds in the plant. Due to its robust medicinal property profile, *Dillenia indica* L. holds a promising resource for drug

development. This thesis deals with the evaluation of the beneficial effect of *Dillenia indica* L. against NAFLD employing *in vitro* models.

The objectives of the present study are the following

- Extraction and partial characterization of *Dillenia indica* L. leaf extract.
- Validation of the beneficial effect of extract against NAFLD *in vitro* models emphasizing pathological lipogenesis and adipogenesis.
- Detailed investigation on the effect extract on various proteins involved in lipid metabolism signaling pathway.
- Role of inflammation and autophagy signaling during lipid accumulation in cells and the effect of the extract.

Extraction and partial characterization of *Dillenia indica* L. extracts: Various *Dillenia indica* L. extracts were prepared through the sequential extraction method. Three extracts *Dillenia indica* L. n-hexane extract (DI-HX), *Dillenia indica* L. ethyl acetate extract (DI-EA), and *Dillenia indica* L. 70% hydroethanolic extract (DI-HET) were obtained. Next, the partial characterization of these extracts was done through HPLC and LCMS/MS analysis. Robust presence of polyphenols in all the extracts were found.

Effect of DI-HET in lipid metabolism in HepG2 cells: Further, the effect of extracts in the oleic acid (OA) induced NAFLD model in HepG2 cells was studied. OA is a monounsaturated fatty acid (18:1 n-9). Studies are reported that OA-induced steatosis in HepG2 cells occurs via the downregulation of nuclear receptor PPAR-α and its associated signaling pathway. That leads to excess lipid accumulation in HepG2 cells. Studies have also found that OA induction causes inflammation in HepG2 cells through the activation of proinflammatory cytokines. I found that OA treatment causes excess lipid accumulation and altered lipid metabolism in HepG2 cells. OA treatment also results in reduced protein expression of PPAR-α and elevated levels of proinflammatory cytokines.

ORO staining was conducted and observed that DI-HET extract reduced intracellular lipid accumulation in a better way in HepG2 cells when compared to the other two extracts, DI-HX and DI-EA. Hence, further evaluation was done with DI-HET extract on OA-induced NAFLD in HepG2 cells and anti-adipogenic potential in 3T3-L1 cells.

The effect of DI-HET extract on TG accumulation in HepG2 cells was also evaluated. The OA treated group showed a significant increase in TG accumulation in HepG2 cells. Pretreatment with DI-HET substantially reduced TG accumulation in HepG2 cells dose-dependently. Both these results showed the beneficial effect of DI-HET against NAFLD.

Further, the protective role of DI-HET extract on the aberrant lipid metabolism signaling pathway was studied. Results showed that treatment with OA inhibited AMPK activation, indicating dysregulation of proteins associated with lipid metabolism. This research demonstrated that pretreatment with DI-HET extract significantly reduced lipid and TG accumulation in HepG2 cells through the activation of AMPK. This therapeutic property was attributed to the activation of the SIRT-1/p-LKB-1/AMPK pathway by the DI-HET extract. Specifically, the extract stimulated upstream kinases such as LKB-1, through the activation of SIRT-1. The activated AMPK, in turn, triggers catabolic pathways like fatty acid oxidation while inhibiting anabolic pathways like DNL.

Activation of AMPK resulted in the inhibition of transcriptional factors like SREBP-1c, consequently downregulated the expression of ACC and FAS. Inhibition of ACC and FAS proteins represents promising drug targets in lipid metabolism. By targeting these key enzymes involved in fatty acid synthesis, it is possible to regulate lipid accumulation and potentially mitigate disorders such as NAFLD and obesity. These findings suggest that DI-HET treatment promotes mitochondrial β-oxidation and suppresses DNL, indicating its potential as a therapeutic approach for addressing NAFLD.

Furthermore, this study revealed that DI-HET treatment had additional impacts on protein expression associated with NAFLD. Specifically, DI-HET

upregulated the expression of PGC-1α and PPAR-α, which were found to be downregulated with OA treatment. The reversal of protein expression observed suggests that DI-HET extract has the potential to counteract the dysregulation induced by OA. Particularly, PPAR-α plays a vital role in the transcriptional regulation of genes involved in fatty acid beta-oxidation, making it a target of interest for NAFLD therapy. The upregulation of PGC-1α and PPAR-α by DI-HET extract offers promising implications for the modulation of glucose and lipid metabolism in the context of NAFLD.

ACOX-1 has been reported to be present in high amounts in patients with NAFLD. DI-HET treatment downregulated ACOX-1 expression. Therefore, the extract treatment can help to attenuate ROS-mediated NAFLD progression.

Moreover, the uptake of fatty acids by investigating CD36 expression was analyzed. There was an increased CD36 expression with OA treatment. Increased fatty acid uptake with OA treatment was reduced with DI-HET treatment through the downregulation of CD36.

Another crucial factor for NAFLD pathogenesis is associated with disturbed cholesterol metabolism. NAFLD patients are reported to have high levels of cholesterol. Major proteins involved in cholesterol biosynthesis were studied. Here, it was found that key proteins involved in cholesterol biosynthesis, like SREBP-2 and HMGCR, were upregulated with OA treatment. Thus, cholesterol accumulation in cells was also increased. Treatment with DI-HET extract reduced cholesterol accumulation by downregulating the transcription factor SREBP-2 and its target protein HMGCR. Therefore, these results demonstrated the effect of DI-HET on cholesterol biosynthesis.

Protective role of DI-HET in ROS generation, inflammation & autophagy in HepG2 cells: Further, the antioxidant potential of DI-HET has been studied because oxidative stress causes NAFLD progression. The present study showed that treatment with DI-HET extract reduced cellular and mitochondrial ROS generation. Moreover, an improved oxygen consumption rate was observed with DI-HET treatment. This is regarded as an additional advantageous action of the extract that enhances its capacity

to combat NAFLD. It also showed potent free radical scavenging activity in DPPH, ABTS, superoxide radical scavenging activity, and TRP assays.

The hallmarks of NAFLD are inflammation, hepatocyte damage, and death. To validate the effect of DI-HET extract on inflammation during OA-induced NAFLD, major proteins involved in inflammation were also studied. The DI-HET extract effectively lowered inflammatory proteins like TLR-4, TNF-α, NF-κB, p-JNK, and NLRP3. Studies have reported that inflammation activates autophagy signaling. Interestingly, we found that major autophagy proteins such as Beclin-1 and LC3 were upregulated with OA treatment. Pretreatment with DI-HET extract downregulated the protein expression of Beclin-1 and LC3. Hence, autophagy was reduced with plant extract treatment.

Effect of DI-HET in adipogenesis, inflammation & autophagy in 3T3-L1 cells: Adipose tissue also plays a major role in regulating whole-body metabolism. Dysfunctional lipid metabolism in adipose tissue contributes to metabolic syndrome, IR, low-grade inflammation, and the progression of NAFLD. Hence, the effect of DI-HET extract on adipogenesis in 3T3-L1 cells was evaluated.

To analyze the antiadipogenic potential of DI-HET extract, first, studies were conducted on lipid accumulation in 3T3-L1 cells. Treatment with DI-HET extract significantly reduced lipid and TG accumulation in 3T3-L1 cells. Then, major proteins involved in the differentiation of adipocyte were studied. DI-HET reduced the expression of major transcription factors such as PPAR-γ, C/EBP-α, and SREBP-1c. AMPK activation with DI-HET extract was also studied. Treatment with extract upregulated p-AMPK expression. Reduced expression of PPAR-γ, C/EBP-α, and SREBP-1c, along with the activation of AMPK, leads to lipolysis, reduced lipogenesis, and inhibition of differentiation of 3T3-L1 cells with DI-HET extract treatment.

Further, the anti-inflammatory property of DI-HET extract in adipocytes was studied. The extract was found to effectively lower the expression of major proteins like TLR-4, NF-κB, p-JNK, and NLRP-3. Besides, changes in autophagy in 3T3-L1 cells were also studied. The major autophagy proteins like AMBRA1, Beclin1, LC3, ATG7, and ATG16L1 were upregulated with adipocyte differentiation. Treatment with

DI-HET reduced autophagy by downregulating the expression of proteins. The anti-inflammatory effect and the ability of the extract to regulate autophagy during adipogenesis is found to contribute its beneficial effects against NAFLD.

In conclusion, the overall results showed the beneficial effect of DI-HET against OA-induced NAFLD in HepG2 cell lines through the activation of the SIRT-1/p-LKB-1/AMPK pathway, inhibition of lipogenic proteins, reduced inflammation, and reversal of autophagy. The extract also showed antiadipogenic potential. The robust presence of pharmacologically active polyphenols in the plant extract are expected to play an important role in its above-mentioned properties Therefore, from the study, we can infer that *Dillenia indica* L. has significant therapeutic potential against NAFLD due to the synergistic effect of polyphenols in the plant extract.

ABSTRACT

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Title of the thesis: **An *in vitro* investigation on *Dillenia indica* L. for its beneficial effects against pathological lipogenesis and adipogenesis**

Surplus lipogenesis is a major factor in the genesis of NAFLD. In addition, altered adipogenesis is a risk factor for NAFLD due to its significant impact on liver function. Based on these, this thesis deals with studies on various biochemical pathways associated with NAFLD and effect of plant extract of *Dillenia indica*. NAFLD is a chronic liver disease, affecting a high proportion of the world's population. However, to date, no effective medical interventions exist that completely reverse NAFLD. The increased prevalence of NAFLD in the world population, the absence of targeted medicine for NAFLD, and the possibility of drug discovery based on Ayurvedic or traditional knowledge have driven this research. *Dillenia indica* L. is an edible plant, present in the forests of Asian countries. Different parts of this plant are used in traditional medicine for treating various diseases. The aim of this research was to evaluate the beneficial effect of *Dillenia indica* L. against NAFLD employing *in vitro* models. We have employed two *in vitro* models in this study such as HepG2 and 3T3-L1 cell lines based. HepG2 cells were incubated with 100 µM of oleic acid (OA) for 24 h. For evaluation of the effect of the extracts (5 or 10 µg/mL), extracts were pretreated to the OA group. Oleic acid (OA) induced NAFLD in HepG2 cells causes surplus lipid accumulation and aberrant lipid metabolism. Fenofibrate was used as the positive control. Adipogenesis was studied using 3T3L-1 cells. During the differentiation process, the DI-HET extract was utilized at concentrations of 5 µg/mL and 10 µg/mL to investigate its inhibitory effect on adipocyte differentiation in the 3T3-L1 cell culture, specifically between days 0 and 2 (48 h). Partial characterization of plant extract by HPLC and LCMS/MS showed the robust presence of polyphenols in *D. indica* L. leaf extracts. The initial study consists of three extracts DI-HX, DI-EA, and DI-HET. The most effective DI-HET extract was selected for further studies based on the ability of the extract to reduce lipid accumulation. Various parameters relevant to lipid metabolism, inflammation, and autophagy were studied in HepG2 cell lines. DI-HET was effective against NAFLD by activating the SIRT-1/p-LKB-1/AMPK signaling pathway. DI-HET extract treatment was effective in lowering inflammation and reversal of autophagy. DI-HET treatment also showed strong antioxidant properties. This study also observed the effect of DI-HET against surplus adipogenesis through the inhibition of major transcription factors like PPAR-γ, C/EBP-α, and SREBP-1C. The activation of AMPK aids in lipolysis through the increased expression of lipolytic proteins and further inhibition of lipogenic proteins. The salient finding of this study also revealed the anti-inflammatory effect and the ability of the extract to regulate autophagy during adipogenesis. Thus, *Dillenia indica* L. was found to be effective against NAFLD.

Publications

Emanating from thesis

1. **Poornima, M. S**., Sindhu, G., Billu, A., Sruthi, C. R., Nisha, P., Gogoi, P., Baishya, G., & G Raghu, K. (2022). Pretreatment of hydroethanolic extract of Dillenia indica L. attenuates oleic acid induced NAFLD in HepG2 cells via modulating SIRT-1/p-LKB-1/AMPK, HMGCR & PPAR-α signaling pathways. *Journal of Ethnopharmacology*, *292*, 115237. https://doi.org/10.1016/j.jep.2022.115237

Other than thesis

1. Drissya, T., Induja, D. K., **Poornima, M. S**., Jesmina, A. R. S., Prabha, B., Saumini, M., Suresh, C. H., Raghu, K. G., Kumar, B. S. D., & Lankalapalli, R. S. (2022). A novel aureothin diepoxide derivative from Streptomyces sp. NIIST-D31 strain. *The Journal of Antibiotics*, *75*(9), Article 9. https://doi.org/10.1038/s41429-022-00547-1
2. Mohan, S., Nair, A., **Poornima, M. S**., & Raghu, K. G. (2023). Vanillic acid mitigates hyperinsulinemia induced ER stress mediated altered calcium homeostasis, MAMs distortion and surplus lipogenesis in HepG2 cells. *Chemico-Biological Interactions*, *375*, 110365. https://doi.org/10.1016/j.cbi.2023.110365

Scientific conferences

- International Conference on Deliberation on Translation of Basic Scientific Insights into Affordable Health Care Products, On 25th-27th February 2019 at CSIR-national Institute for Interdisciplinary Science & Technology, Trivandrum.

- **Poornima M S & Raghu K G.** Exploring the anti-obesity potential of *Dillenia indica* L. International Conference of Indian Academy of Biomedical Sciences Held at Department of Biochemistry, D. Y. Patil Medical College, Kolhapur, Maharashtra from 27th To 29th February 2020. (**Best Poster Award**)

- **Poornima M S & Raghu K G.** Exploring the anti-obesity potential of *Dillenia indica* L. International Congress on Obesity and Metabolic Syndrome Hosted by Korean Society for The Study of Obesity Held at Conrad Hotel in Seoul from 2nd to 4th September 2021. (**Online Poster presentation**)

- **Poornima M S & Raghu K G.** *Dillenia indica* L. attenuates Non-alcoholic fatty liver disease in HepG2 cells via SIRT-1/p-LKB-1/AMPK, HMGCR, PPAR-α, and NF-κB signaling pathways. 10th Annual conference of Indian Academy of Biomedical Sciences, Department of Biochemistry, Kings George's Medical University, Lucknow and Department of Biotechnology Era University, Lucknow (**Poster presentation**)

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Pretreatment of hydroethanolic extract of *Dillenia indica* L. attenuates oleic acid induced NAFLD in HepG2 cells via modulating SIRT-1/p-LKB-1/AMPK, HMGCR & PPAR-α signaling pathways

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ABSTRACT

Ethnopharmacological relevance: Dillenia indica L. is an edible plant from the Dilleniaceae family present in the forest of India and other Asian countries. Different parts of this plant are being used in the traditional system of medicines for various diseases like diabetes, indigestion, asthma, jaundice, and rheumatic pain by various rural communities. This plant is very common among Khamptis traditional healers, the rural community of the Dhemaji district of Assam, ethnic communities of Dibru-Saikhowa Biosphere Reserve of Northeast, India for various medicinal uses. It is observed as a 'vat' suppressant and 'pitta' boosting medicine in Ayurveda.

Aim of the study: The aim of this research was to evaluate the effect of hydroethanolic extract of *Dillenia indica* leaf (DI-HET) against non-alcoholic fatty liver disease (NAFLD) as it is reported effective against jaundice in traditional medicine. We are also planning to see the various molecular mechanisms responsible for its effect if it is efficacious.

Study design/method: An *in vitro* model for NAFLD was employed in this study. For this HepG2 cells were incubated with 100 μM of oleic acid (OA) for 24 h. For evaluation of the effect of DI-HET, the extracts (5 or 10 μg/mL) were pretreated to the OA group. Fenofibrate was the positive control. Various parameters relevant to lipogenesis and β-oxidation of fatty acids like intracellular lipid accumulation, reactive oxygen species (ROS), mitochondrial stress, and key proteins were studied.

Results: DI-HET significantly reduced the intracellular lipid accumulation in OA treated cells. And also substantially decreased the expression of lipogenic proteins and increased β-oxidation in the OA group. OA induced ROS generation was found to reduce with DI-HET treatment. Western blot analysis showed that the expression of LXR-α, SREBP-1C, SREBP-2, HMGCR, FAS, CD-36, and ACOX-1 were downregulated while that of SIRT-1, p-LKB-, p-AMPK, p-ACC, CPT-1, and PPAR-α upregulated in DI-HET treatment. LCMS/MS analysis showed the presence of polyphenols like naringenin, catechin, epicatechin, shikimic acid, syringic acid, vanillic acid, and kaempferol.

Conclusion: These results suggest that DI-HET is effective against NAFLD by activation of the SIRT-1/p-LKB-1/AMPK signaling pathway via polyphenols present in the extract.

Abbreviations: ACC, Acetyl-CoA carboxylase; ACOX-1, Peroxisomal acyl-coenzyme A oxidase-1; AMPK, Adenosine monophosphate-activated protein kinase; ANOVA, One-way analysis of variance; CD36, Cluster of differentiation 36; ChREBP, Carbohydrate response element binding protein; CPT-1, Carnitine palmitoyltransferase; DI-HET, *Dillenia indica* L. hydroethanolic extract; ER, Endoplasmic reticulum; FAS, Fatty acid synthase; HMGCR, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; LXR-α, Liver X receptor-α; NAFLD, Non-alcoholic fatty liver disease; OA, Oleic acid; p-ACC, Phosphorylated ACC; p-AMPK, Phosphorylated AMP-activated kinase; p-LKB-1, Phosphorylated liver kinase B-1; PPAR-α, Peroxisome proliferator-activated receptor-α; SIRT-1, Sirtuin-1; SREBP-2, Sterol regulatory element-binding protein-2; SREBP-IC, Sterol regulatory element-binding protein-1C; TG, Triglyceride.

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1. Introduction

Current drug discovery is encountering serious challenges due to low success rate and rising cost. This scenario has compelled us to come out with a novel approach of consolidated drug discovery, where Ayurvedic/traditional knowledge can synergize with drug discovery. Starting steps in new drug discovery is identification of new chemical entities. This can be obtained from natural sources through activity based extraction and fractionation. The starting point for plant-based new drug discovery is identification of the right plants. This is done by utilizing Ayurvedic insight, traditional knowledge and elaborate literature search. The integration of traditional practice with drug discovery brings a fundamental change in the extraction methods. Bioassay-based extraction and fractionation of the selected plant may lead to standardized extract or druggable compound as the new drug. This integrated approach would save the cost and time, coupled with enhanced success rate in drug discovery. The present paper describes a traditional knowledge based exploratory research for the identification of effective extract against non-alcoholic fatty liver diseases (NAFLD). NAFLD is characterized by the surplus fat in the hepatocytes of people with little or no consumption of alcohol. This causes various pathologies ranging from hepatic steatosis to steatohepatitis, a serious stage of the disease (Papatheodoridi and Cholongitas, 2018). This is associated with various conditions like metabolic abnormalities, insulin resistance, steatosis, oxidative stress, inflammation, fibrosis, etc. (Cobbina and Akhlaghi, 2017). No effective treatment or approved drug therapy for NAFLD is available now. Recently, the effect of obeticholic acid, a farnesoid X nuclear receptor ligand, has been tested in adult patients and found that it eased the liver histology suggesting the potential efficacy in the treatment of NAFLD (Sumida et al., 2020). However, the details of the limited benefits and safety of obeticholic acid in the long-term application are still unknown. Lifestyle modifications like increase in physical activity, weight reduction, consumption of antioxidants, restriction of high energy food, etc. are recommended for control and management. These actions have only a limited impact on the incidence and severity of NAFLD at the population level and may not be effective for complete recovery. So, it is essential to have an effective therapy for NAFLD.

Although there are advances in conventional medicine, plant derived traditional medicines are easily accessible, affordable, and do not require stringent synthesis. Thus, indigenous medicines seem highly attractive for the effective management of diseases including NAFLD. Herbal medicines, described as entire plants and unpurified plant extracts, have been traditionally used in various countries for liver diseases (Okaiyeto et al., 2018). There are many reports about various extracts and natural compounds with liver function promoting properties (Mohamed Saleem et al., 2010). Plant based formulations for NAFLD were evaluated generally in various signaling pathways relevant to hepatic lipogenesis, beta-oxidation of fatty acids, antioxidant potential, anti-inflammatory potential, cellular lipid transport, etc. (Bagherniya et al., 2018). Here we are exploring the potential of *Dillenia indica* L., an edible plant against NAFLD in an *in vitro* model employing HepG2 cells. It is an evergreen tree, from the Dilleniaceae family with various medicinal properties like anticancer, antidiabetic, antioxidant, anti-inflammatory (Singh and Saha, 2019). It is also found to be good for fever, jaundice, diarrhoea, etc. (Singh and Saha, 2019). In India, different parts of *D. indica* are being used in traditional medicines. Native communities in Mizoram, rural communities in Dhemaji district of Assam, and Khamptis traditional healers in Arunachal Himalaya have used various parts of this plant for the treatment of diabetes, jaundice, dysentery, and other diseases (Saiful Yazan and Armania, 2014; Namsa et al., 2011; Tag et al., 2012). Taungya community in Terai Arc Landscape, India is using this plant for cough, fever, and constipation (Poonam and Singh, 2009). In Ayurveda, it is considered a 'vat' suppressant and 'pitta' supplementing (Rai and Sajwan, 2020). In addition, that D. *indica* extract has shown cardiac and hepatoprotective properties (Tene et al., 2021; Padhya et al., 2008; Himakar Reddy et al., 2010). Hence in this study, we have conducted detailed *in vitro* experiments to investigate its potential against NAFLD in an oleic acid induced model in Hepg2 cells. For this various signaling pathways relevant to lipogenesis, beta-oxidation of fatty acid, and trafficking of lipid have been studied. In addition, LCMS/MS characterization of the extract was performed to identify partially the chemical constituents responsible for its beneficial properties against NAFLD.

2. Materials and methods

2.1. Chemicals

Minimum essential medium eagle (MEM) was from HiMedia (Mumbai, India). Fetal bovine serum (FBS), penicillin-streptomycin antibiotics, trypsin - ethylenediaminetetraacetate (EDTA), Hanks balanced saline solution (HBSS), and phosphate buffer saline (PBS) were bought from Gibco, USA. 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), oleic acid, dimethyl sulfoxide (DMSO), 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA), skimmed milk powder, and Oil Red O stain were purchased from Sigma-Aldrich Co. USA. The triglyceride and oxygen consumption assay kits were from the Cayman chemical company in the USA. MitoSOX red was from Carlsbad, USA. BCA protein assay kit was from Bio-Rad Laboratories Pvt Ltd, and chemiluminescence detection kit from Advansta, USA. All primary and secondary antibodies used in western blotting were from Santa Cruz Biotechnology, USA. All other chemicals and solvents used were of analytical grade.

2.2. Preparation of D. indica hydroethanolic extract (DI-HET)

D. indica was collected from Malampuzha, Palakkad district of Kerala (Latitude: 10° 49' 29.87" N; Longitude 76° 41 0.89" E) during the month of January 2020 and identified with the help of a taxonomist and a voucher specimen of the same was kept in the herbarium at CSIR-NIIST (AC-3/2020) for future reference. Ethanolic extract of the leaf of *D. indica* was prepared according to previous reports (Prathapan et al., 2013). The plant leaves were dried, ground to powder, and extracted (240 g) with n-hexane (1 L) to remove low polarity contaminants, and filtered through the Whatman No.1 filter paper. Then the supernatant was concentrated using rotavapor at reduced pressure, to get *D. indica* n-hexane extract (DI-HX). After this, the powder was extracted further with ethyl acetate at ambient temperature (27 ± 1 °C) under stirring for 6 h, and the extraction process was repeated until the solvent became colourless. The supernatant was filtered through Whatman No.1 filter paper and concentrated *in vacuo* under reduced pressure in a rotavapor followed by lyophilization to get DI-EA extract. Again, the powder of DI-EA extract was extracted using hydroethanolic solvent (70% ethanol) and it was processed as above to get the DI-HET extract. The lyophilized DI-HET leaf extract was stored at 4 °C until use. Each extract (DI-HX, DI-EA, and DI-HET) was evaluated for its lipolytic activity by Oil Red O staining against oleic acid (OA) induced lipogenesis in HepG2 cells. DI-HET was found more effective in lipolytic activity compared to the rest of the extracts (data not shown). So, DI-HET was taken forward for detailed evaluation.

2.3. Polyphenol profiling of DI-HET using liquid chromatography and mass spectrometry (LCMS/MS)

On the basis of literature support (Singh and Saha, 2019) the compounds mentioned in table (1) were identified and quantified from leaves of *D. indica* hydroethanolic extract using LCMS/MS (Nexera with LCMS-8045, Shimadzu, Japan) (Abraham et al., 2020).

2.4. Cell culture and treatments

Hepatocellular carcinoma cells (HepG2) from National Centre for

Cell Sciences (NCCS, Pune, India) were cultured and maintained in modified eagle medium (MEM) supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin solution. The cells were cultured in a humidified atmosphere with 5% CO_2 and 37 °C. Cultures were used at 80% confluency. For subculturing, the cells were washed with pre-warmed PBS and detached by treating with 0.25% trypsin. The experimental group consists of.

(a) Control group: HepG2 cells without any treatment are designated as C.
(b) OA group: HepG2 cells were incubated with 100 μM of OA for 24 h and indicated as OA
(c) DI 5 group: HepG2 cells were pretreated with 5 μg/mL of extract for 2 h and followed by 100 μM of OA for 24 h designated as DI 5.
(d) DI 10 group: HepG2 cells were pretreated with 10 μg/mL of extract for 2 h and followed by 100 μM of OA for 24 h designated as DI 10.
(e) PC group: Cells were pretreated with fenofibrate (20 μM) for 2 h followed by OA (100 μM) for 24 h designated as PC.

Fenofibrate is a safe and effective medication approved by the FDA for the treatment of hypertriglyceridemia, primary cholesterolemia, and dyslipidemia (Sidhu and Tripp, 2020). Since there are no effective drugs for NAFLD, fenofibrate is commonly used as a positive control in most studies (Swapna Sasi et al., 2020).

2.5. Preparation of BSA- OA complex

OA stock solution (10 mM) was prepared by dissolving OA in 0.1 N of NaOH at room temperature. The stock solution was then mixed with fatty acid-free BSA (10% in MEM) to make a final concentration of 1 mM. The solution was then diluted in 1% culture media to give a final concentration of 100 μM (Cousin et al., 2001).

2.6. Cell viability assay

For cell viability HepG2 cells were seeded in 96-well plates at a density of 5×10^3 per well and MTT assay was performed after treating the cells with different concentrations of DI-HET for 24 h (5, 10, 20, 50, 100, 200 and 400 μg/mL). Cytotoxicity of fenofibrate was also studied with different concentrations (5, 10, 20, 30,40, 50,100, and 200 μM) (Mosmann, 1983).

2.7. Oil Red-O-staining

After respective treatment, the cells were washed twice with PBS and fixed in 4% paraformaldehyde for 1 h. After fixation, the cells were washed with PBS and permeabilized with 0.1% Triton X 100 followed by Oil Red O staining for 20 min at room temperature. The cells were then visualized under a light microscope. Absorbance was spectrophotometrically measured at 490 nm (Swapna Sasi et al., 2020).

2.8. Quantification of triglyceride content

The concentration of triglyceride was measured using a triglyceride colorimetric assay kit (Cayman, USA). This assay was based on the enzymatic hydrolysis of triglycerides by lipase and the absorbance was measured at 530–550 nm (Shyni et al., 2021).

2.9. Detection of intracellular ROS generation

ROS in treated HepG2 cells were assessed by employing DCFH-DA (Wang and Joseph, 1999), fluorescent images were captured with a fluorescent microscope (Olympus IX 83) and fluorescence was read at 488 nm excitation and 525 nm emission (Infinite® M200 PRO, Tecan Group Ltd, Switzerland).

2.10. Detection of mitochondrial superoxide generation

Changes in mitochondrial superoxide production were monitored using MitoSOX™ red and fluorescent images were captured with a fluorescent microscope (Olympus IX 83) at an excitation/emission range of 514/580 nm (Wojtala et al., 2014).

2.11. Oxygen consumption assay

The oxygen consumption rate was determined by using Cayman's cell-based assay kit, using antimycin A as a standard inhibitor. Phosphorescent oxygen probe was added to all wells except the blank wells. The wells were then covered with HS mineral oil and fluorescence was read at an excitation of 380 nm and emission of 650 nm for 120 min (Sruthi and Raghu, 2022).

2.12. Western blot analysis

After respective treatments, cells were lysed in RIPA buffer with the protease inhibitor cocktail (Sigma Aldrich, USA). Followed by incubation, cell suspensions were centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was collected and used for further immunoblot analysis. The protein concentration was measured by using the bicinchoninic acid kit (Pierce, Rockford, IL, USA). An equal amount of proteins were separated by 8 or 10% SDS-PAGE and transferred to PVDF. After blocking in TBS-Tween 0.1% containing 5% nonfat skimmed milk (Bio-Rad, Hercules, CA, USA), membranes were incubated with primary antibodies overnight at 4 °C. The alterations of various protein such as acetyl-CoA carboxylase (ACC), phosphorylated ACC (p-ACC), fatty acid synthase (FAS), adenosine monophosphate-activated protein kinase (AMPK), phosphorylated AMP-activated kinase (p-AMPK), 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR), peroxisome proliferator-activated receptor-α (PPAR-α), sirtuin-1 (SIRT-1), sterol regulatory element-binding protein-1C (SREBP-IC), sterol regulatory element-binding protein-2 (SREBP-2), cluster of differentiation (CD36), peroxisomal acyl-coenzyme A oxidase-1 (ACOX-1), liver X receptor-α (LXR-α), carnitine palmitoyltransferase (CPT-1) and phosphorylated liver kinase B-1(p-LKB-1) were analyzed. After washing with TBS-Tween 0.1%, the membranes were incubated with HRP -conjugated corresponding secondary antibodies for 2–4 h at room temperature. Again, washed three times with TBS-Tween 0.1%, then the membranes were developed using Western Blot Hyper HRP Substrate (Takara Bio Inc. USA), and the protein bands were analyzed (Bio -Rad ChemiDoc MP imaging systems, USA) (Mahmood and Yang, 2012).

2.13. Statistical analysis

Statistical analysis was done using the SPSS statistical program. The results were represented as mean ± SEM. The significant differences among the treatments were evaluated using one-way analysis of variance (ANOVA). It is followed by Duncan's multiple range to determine which mean values were significantly different at $p \leq 0.05$. Statistical significance was indicated as * for $p \leq 0.05$ Vs C and # for $p \leq 0.05$ Vs OA.

3. Results

3.1. Effect of DI-HET on the viability of HepG2 cells

DI-HET was not toxic up to 100 μg/mL in HepG2 cells (Fig. 1A). Based on viability data, we selected 5 and 10 μg/mL of DI-HET extract for investigation against NAFLD.

3.2. Effect of fenofibrate on the viability of HepG2 cells

There is no significant cell death up to 100 μM (Fig. 1B).

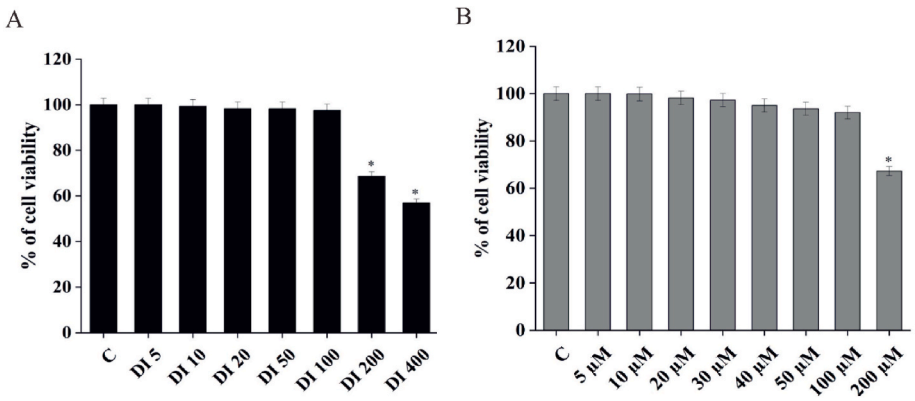

Fig. 1. Effect of DI-HET and fenofibrate on viability of HepG2 cells. (A) HepG2 cells were treated with DI-HET (5, 10, 20, 50, 100, 200, 400 μg/mL) for 24 h and cell viability was determined by MTT assay. (B) HepG2 cells were treated with fenofibrate (5, 10, 20,30, 40, 50, 100, 200 μM) for 24 h, and cell viability was determined by MTT assay. Data are expressed as mean ± SEM where n = 6. * denotes significant difference from the control group ($p \leq 0.05$).

3.3. DI-HET inhibits lipid accumulation in HepG2 cells

Both extracts caused a significant reduction of lipid content in a dose-dependent manner (22.40% with DI 5 and 33.80% with DI 10; $p \leq 0.05$; Fig. 2A and B) when compared to the OA treated group which showed a significant increase (36.34%). Treatment with fenofibrate significantly reduced the lipid accumulation by 37.59%. For validating the result obtained from Oil Red O staining we further quantified triglyceride accumulation in HepG2 cells. Here also the TG content was significantly decreased in a dose-dependent manner with both extracts (30.38% with DI 5 and 41.18% with DI 10; $p \leq 0.05$; Fig. 2C) when compared to the increase observed in the OA group (45.86%). Fenofibrate treated cells also showed a substantial reduction in TG levels by 42.71%.

3.4. Effect of DI-HET on intracellular ROS generation

OA caused surplus (24.14%; $p \leq 0.05$; Fig. 3A and B) generation of ROS when compared to the control group. Pretreatment with DI-HET (15.49% with DI 5 and 16.95% with DI 10; $p \leq 0.05$; Fig. 3A and B) and fenofibrate (19%) appreciably reduced the same as evident as in the (Fig. 3A and B).

3.5. Effect of DI-HET on mitochondrial superoxide generation

Mitochondrial superoxide generation was significantly increased in the OA group (57.39%; $p \leq 0.05$: Fig. 4A and B) when compared to the control group. With both extracts of DI-HET, superoxide generation was found to be inhibited (23.25% with DI 5 and 56.72% with DI 10; Fig. 4A and B). A significant reduction (54.69%) in superoxide generation was also observed with fenofibrate (Fig. 4A and B).

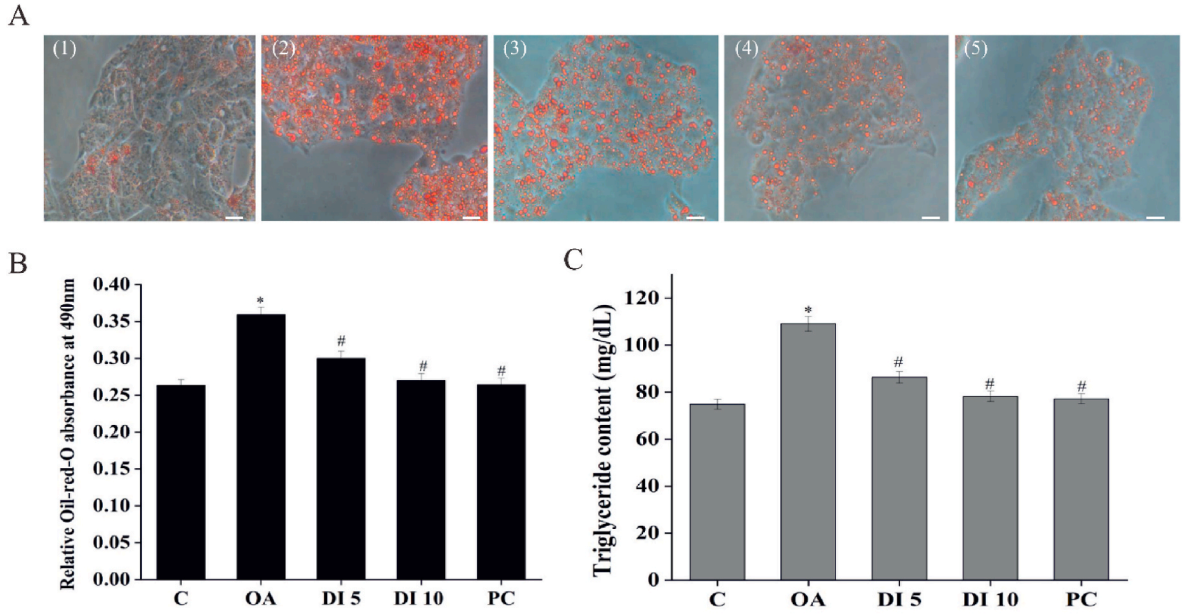

Fig. 2. Effect of DI-HET on oleic acid induced lipid accumulation in Hep G2 cells: (A)The lipid accumulation was assessed by Oil Red O staining. Representative phase-contrast microscopic images of Oil Red O stained HepG2 were presented, scale bar 50 μm. (1) Control (C) (2) Oleic acid 100 μM (OA) (3) Oleic acid + 5 μg/mL extract (DI 5) (4) Oleic acid +10 μg/mL extract (DI 10) (5) Oleic acid 100 μM + fenofibrate 20 μM (PC). (B) Absorbance was read at 490 nm after Oil-Red-O staining (C) Measurement of intracellular triglyceride content in HepG2 cells. Absorbance was spectrophotometrically measured at 530 nm. Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the control group ($p \leq 0.05$) and # denotes significant difference from the OA treated group ($p \leq 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

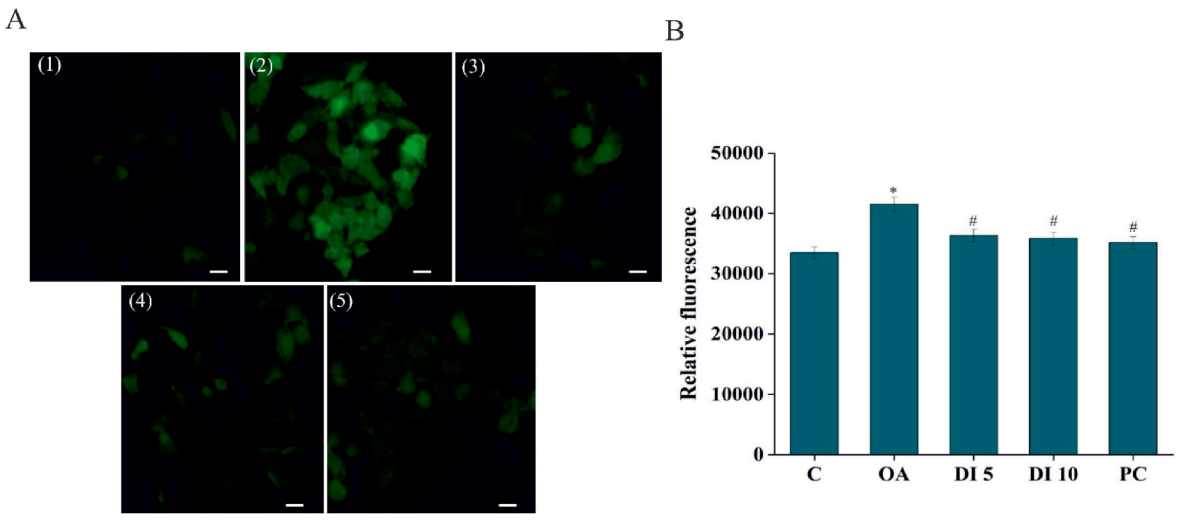

Fig: 3. Effect of DI-HET on oleic acid induced ROS generation in HepG2 cells. A) ROS generation in various groups (1) Control (C) (2) Oleic acid 100 μM (OA) (3) Oleic acid + 5 μg/mL extract (DI 5) (4) Oleic acid + 10 μg/mL extract (DI 10) (5) Oleic acid + fenofibrate 20 μM (PC). B) Relative fluorescence intensity of each group. Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the control group ($p \leq 0.05$) and # denotes significant difference from the OA treated group ($p \leq 0.05$).

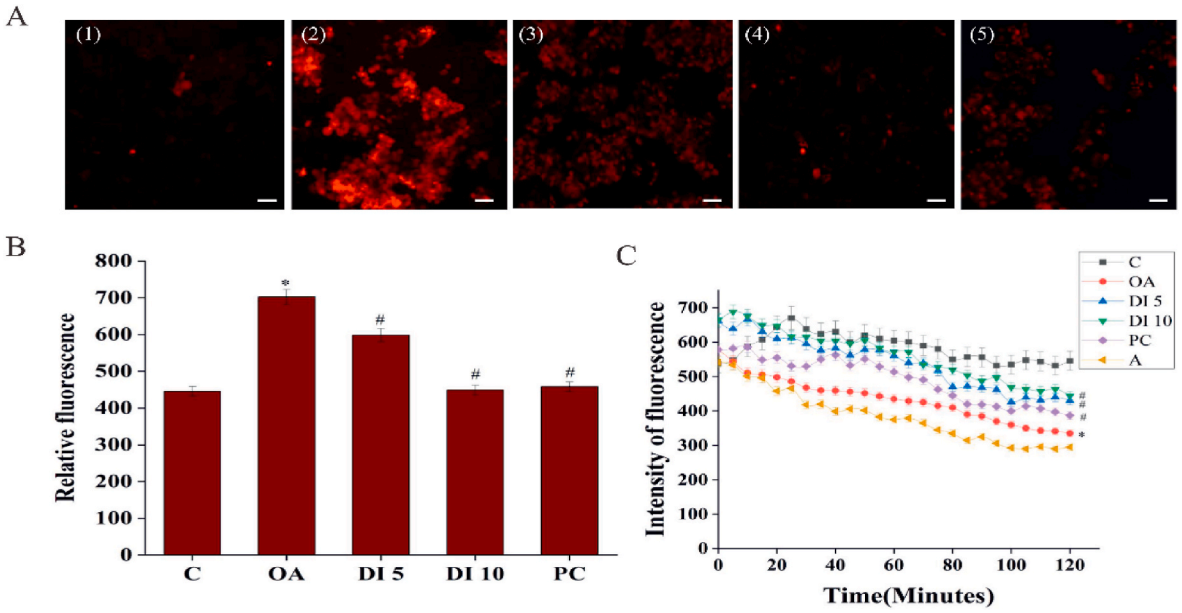

Fig. 4. Effect of DI-HET on oleic acid induced mitochondrial superoxide generation and oxygen consumption rate in HepG2: A) The fluorescent microscopic images of cells stained with MitoSOX^{TM} Red indicator, scale bar 20 μm. (1) Control (C) (2) Oleic acid 100 μM (OA) (3) Oleic acid + 5 μg/mL extract (DI 5) (4) Oleic acid + 10 μg/mL extract (DI 10) (5) Oleic acid 100 μM + fenofibrate 20 μM (PC). (B) Fluorescence intensity emitted by MitoSOX^{TM} in control and treated cells. C) Effect of DI-HET on oxygen consumption rate. Control (C), Oleic acid 100 μM (OA), Oleic acid + 5 μg/mL extract (DI 5), Oleic acid + 10 μg/mL extract (DI 10), Oleic acid + fenofibrate 20 μM (PC) and Antimycin A (A). Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the control group ($p \leq 0.05$) and # denotes significant difference from the OA treated group ($p \leq 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.6. Increased oxygen consumption rate by DI-HET treatment in HepG2 cells

The oxygen consumption rate was found decreased (38.64% $p \leq 0.05$; Fig. 4C) in the OA group compared to control. However, extracts improved oxygen consumption rate significantly (17.39% with DI 5 and 19.96% with DI 10; Fig. 4C) compared to the OA group. Fenofibrate also caused an improvement in oxygen consumption rate by 9.52% (Fig. 4C).

3.7. Effect of DI-HET on SIRT-1 and p-LKB-1 activation

Pretreatment with DI-HET significantly increased the expression of SIRT-1(32.51% with DI 5 and 54.16% with DI 10; $p \leq 0.05$; Fig. 5A and B) when compared to the OA group (38.85%; $p \leq 0.05$). The expression of SIRT-1 was found to be enhanced by 35.94% (Fig. 5A and B) with fenofibrate treatment. And the expression of the phosphorylated form of LKB-1 was enhanced with DI-HET (29.73% with DI 5 and 51.86% with DI 10; Fig. 5A, C) when compared to the OA group (15.38%; Fig. 5A, C) and with fenofibrate treatment p-LKB-1 expression was increased significantly by 52.15% (Fig. 5A, C).

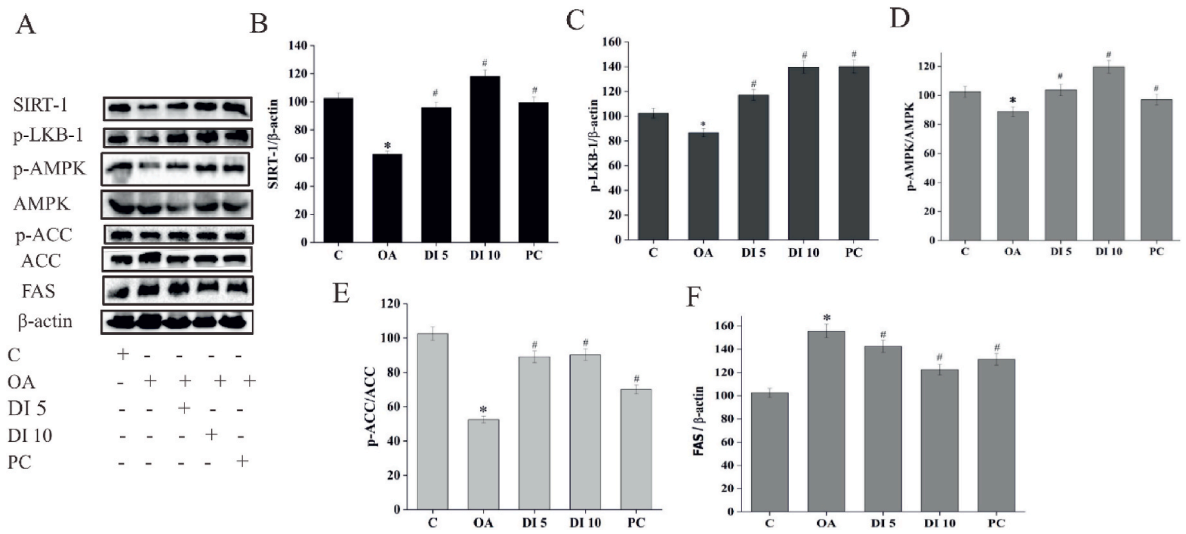

Fig. 5. Effect of DI-HET on SIRT-1, p-LKB-1, p-AMPK/AMPK, p-ACC/ACC, and FAS: (A) The protein expression in different experimental groups. (B–F) The relative intensity of each band was quantified with β-actin. Control (C); Oleic acid 100 μM (OA); Oleic acid + 5 μg/mL extract (DI 5); Oleic acid + 10 μg/mL extract (DI 10); Oleic acid 100 μM + fenofibrate 20 μM (PC). Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the control group ($p \leq 0.05$) and # denotes significant difference from the OA treated group ($p \leq 0.05$).

3.8. Effect of DI-HET on AMPK activation

Western blot showed phosphorylation of AMPK was inhibited significantly in the OA group (13.33%, $p \leq 0.05$), and OA-induced AMPK inactivation was significantly increased by DI-HET treatment (14.63% with DI 5 and 30.15% with DI 10; $p \leq 0.05$; Fig. 5A, D). Fenofibrate treatment also significantly increased the expression of p-AMPK (8.10%; $p \leq 0.05$) compared to the OA.

3.9. Effect of DI-HET on inhibition of lipogenic enzymes ACC, p-ACC and FAS

The expression of the phosphorylated form of ACC was significantly increased with DI-HET treatment (35.63% with DI 5 and 36.74% with DI 10; $p \leq 0.05$; Fig. 5A, E) when compared to OA treatment (48.73%; $p \leq 0.05$). Fenofibrate treatment also increased the expression of phosphorylated forms of ACC by 17.10% ($p \leq 0.05$). Consistent with the suppression of SREBP1-C the expression of FAS, target protein was also downregulated with DI-HET pretreatment (12.84% with DI 5 and 32.47% with DI 10; $p \leq 0.05$ Fig. 5A, F) when compared to the OA group (51.95%; $p \leq 0.05$). The expression of FAS was also significantly reduced

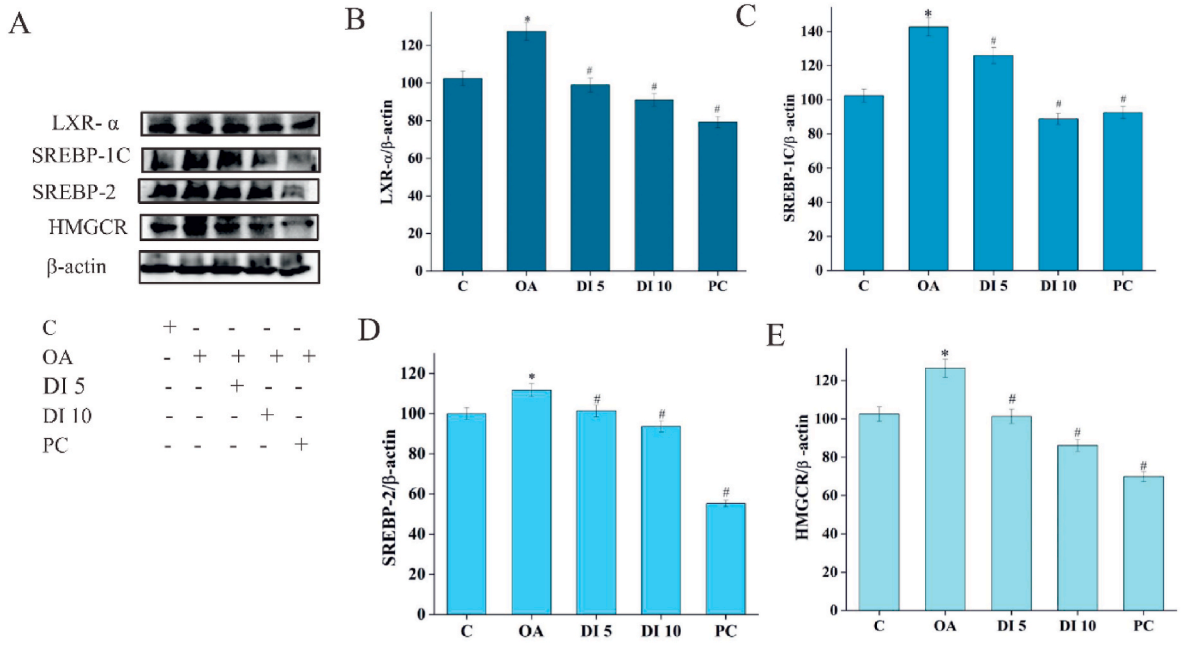

Fig. 6. Effect of DI-HET on LXR-α, SREBP-1C, SREBP-2, and HMGCR. (A) The protein expression in different experimental groups. (B–E) The relative intensity of each band was quantified with β-actin. Control (C); Oleic acid 100 μM (OA); Oleic acid + 5 μg/mL extract (DI 5); Oleic acid +10 μg/mL extract (DI 10); Oleic acid 100 μM + fenofibrate 20 μM (PC). Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the control group ($p \leq 0.05$) and # denotes significant difference from the OA treated group ($p \leq 0.05$).

(23.84%; $p \leq 0.05$) with fenofibrate treatment when compared to the OA (51.95%; $p \leq 0.05$).

3.10. Inhibition of LXR-α and SREBP-1C by pretreatment with DI-HET

Upregulated LXR-α (24.4%; $p \leq 0.05$; Fig. 6A and B) and SREBP-1C (39.31%; $p \leq 0.05$; Fig. 6A, C) expression in the OA group was reversed with pretreatment with DI-HET. The expression of both LXR-α (27.82% with DI 5 and 35.65% with DI 10; $p \leq 0.05$; Fig. 6A and B) and SREBP-1C were down-regulated with DI-HET (16.4% with DI 5 and 52.59% with DI 10%; $p \leq 0.05$; Fig. 6A, C). Fenofibrate treatment also decreased the expression of LXR-α and SREBP-1C (47.03% and 48.89%; $p \leq 0.05$).

3.11. Effect of DI-HET on SREBP-2 and HMGCR

Treatment with OA upregulated the expression of SREBP-2 by 11.70% ($p \leq 0.05$). Pretreatment with DI-HET caused a significant drop in the expression of SREBP-2 (10.42% with DI 5 and 18.13% with DI 10; $p \leq 0.05$; Fig. 6A and B) and with fenofibrate treatment, SREBP-2 expression was reduced by 56.43%. Consistent with the reduction in SREBP-2 the expression of HMGCR was also reduced with DI-HET treatment (24.43% with DI 5 and 39.28% with DI 10; $p \leq 0.05$; Fig. 6A, E) when compared with an increase seen with OA (23.23%). Treatment with fenofibrate also caused a significant reduction in HMGCR expression by 55.18%.

3.12. Effect of DI-HET on PPAR-α expression

The expression of PPAR-α was decreased in the OA group (40%; $p \leq 0.05$) when compared to control (Fig. 7A and B). There was an increased expression of PPAR-α with extract (33.31% with DI 5 and 34.37% DI 10). Fenofibrate treatment also increased the expression of PPAR-α (24.46%).

3.13. Effect of DI-HET on CD36 expression

The expression of CD36 was significantly reduced with DI-HET pretreatment (31.54% with DI 5 and 59.59% with DI 10; $p \leq 0.05$; Fig. 7A, C) when compared to OA-induced diseased conditions (26.84%; $p \leq 0.05$). With fenofibrate treatment, CD36 expression has been reduced by 64.85%.

3.14. Effect of DI-HET on CPT-1 expression

Pretreatment of DI-HET with HepG2 cells significantly increased the expression of CPT-1(28.35% with DI 5 and 27.71% with DI 10; $p \leq 0.05$; Fig. 7A, D) when compared to the reduction caused by OA (25.63%; $p \leq 0.05$ Fig. 7A, D). Fenofibrate treatment also upregulated the expression of CPT-1 significantly (29.16%).

3.15. Inhibition of ACOX-1 with DI-HET treatment

The expression of ACOX-1 was significantly reduced with DI-HET pretreatment (7.58% with DI 5 and 36.04% with DI 10; $p \leq 0.05$; Fig. 7A, E) when compared to the OA group (41%; $p \leq 0.05$). ACOX expression was also reduced with fenofibrate treatment by 25.88%.

3.16. LCMS/MS polyphenol profiling

The LCMS/MS data revealed the presence of polyphenols (for details see chromatograms Fig. 8A and B) which are depicted in Table 1 and structure of major polyphenols in Fig. 9.

4. Discussion

NAFLD is one of the most prevalent liver diseases in the world. It is a complex pathogenesis condition, which involves body tissues such as hepatocytes, adipose tissue, gut, and muscle. Since there is no effective approved treatment for NAFLD so far there is high demand for novel drugs. So, there is very hectic research for drug development for the same in different laboratories in the world. In this regard medicinal plants have been explored for drug discovery for the treatment of NAFLD. Some studies have shown that plant based formulations are effective against hepatocellular carcinoma (Nenni et al., 2021). Here in, various biochemical pathways involved in the OA induced lipogenesis in HepG2 cells were studied to evaluate the potential of hydroethanolic

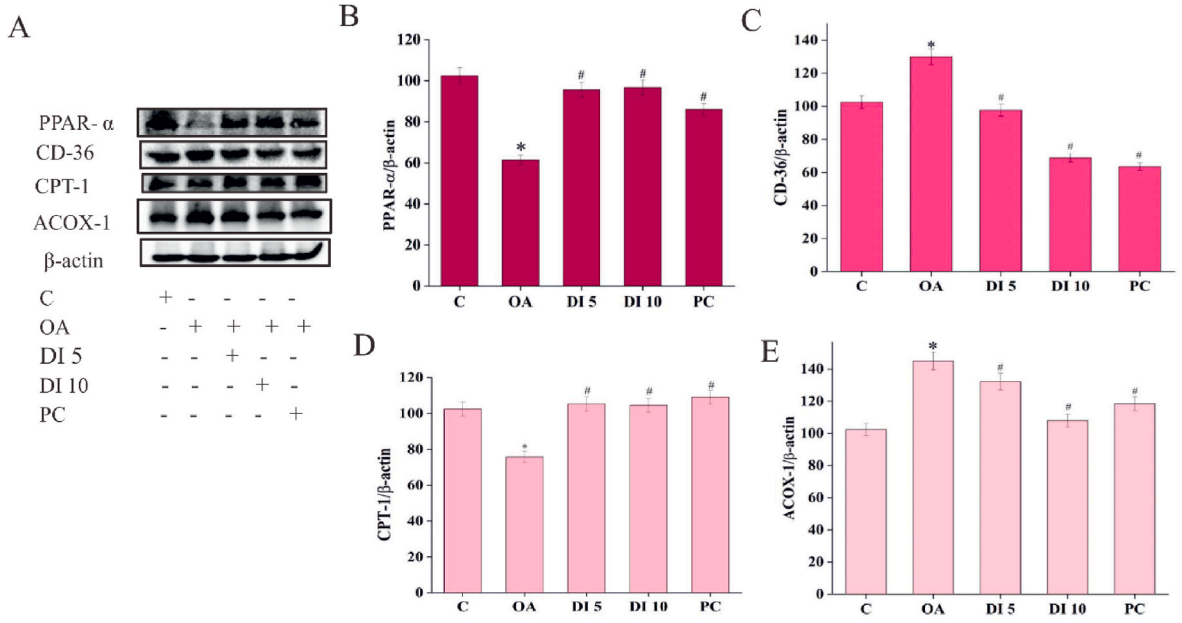

Fig. 7. Effect of DI-HET on PPAR-α, CD36, CPT-1, and ACOX-1. (A) The protein expression in different experimental groups. (B–E) The relative intensity of each band was quantified with β-actin. Control (C); Oleic acid 100 μM (OA); Oleic acid + 5 μg/mL extract (DI 5); Oleic acid + 10 μg/mL extract (DI 10); Oleic acid 100 μM + fenofibrate 20 μM (PC). Data are expressed as mean ± SEM; where n = 6. * denotes significant difference from the control group ($p \leq 0.05$) and # denotes significant difference from the OA treated group ($p \leq 0.05$).

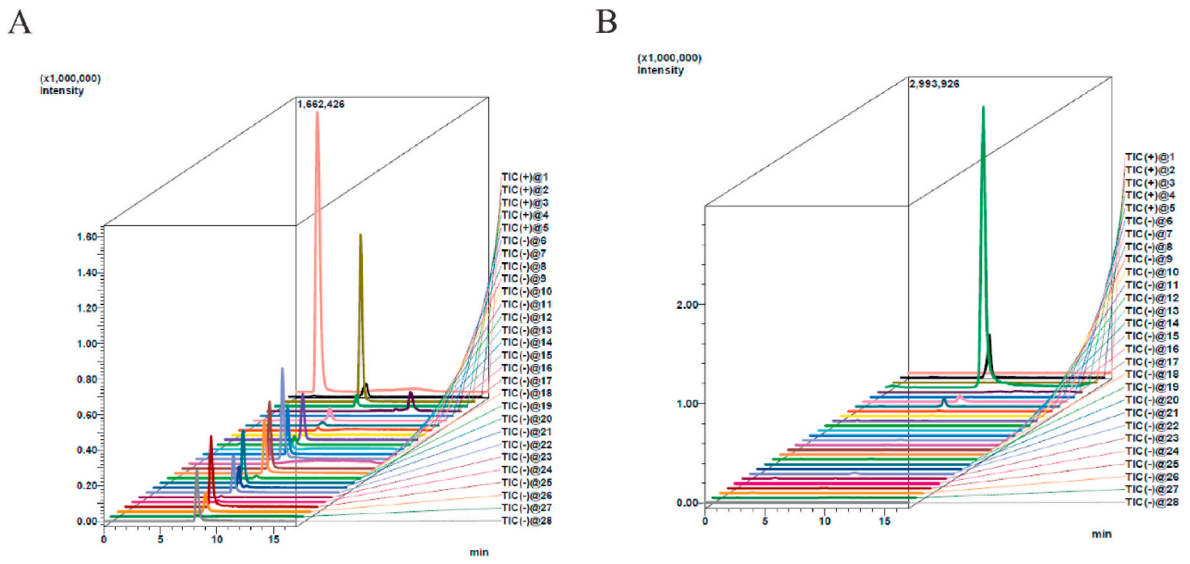

Fig. 8. Phytochemical analysis of DI-HET by LCMS/MS method a) LCMS/MS Chromatogram of standard compounds with retention time in minutes 1) Catechol (1.87 min), 2), Catechin(6.75 min), 3) Quinine(6.88 min), 4) Naringenin(7.28 min), 5) Tocopherol (12.87 min) 6) Gallic acid (1.91 min) 7) Chlorogenic acid (6.81 min) 8) Epicatechin (6.77 min), 9) Syringic acid (7.20 min) 10) Vanillic acid (6.79 min) 11) Caffeic acid (6.91 min) 12) Epigallocatechin(2.01 min) 13) Ferulic acid (7.36 min) 14) Myricetin (7.65 min), 15) Quercetin (7.92 min), 16) p-Coumaric acid (7.34 min), 17) Luteolin (7.92 min), 18) Apigenin (8.18 min), 19) Kaempferol (7.83 min) 20) Rutin (7.34 min), 21) Diadzein (7.91 min), 22) Hesperetin (7.86 min) 23) Shikimic acid (1.76 min) 24) Ellagic acid (7.56 min), 25) Morin (7.74 min), 26) Genstein(7.81 min), 27) Cinnamic acid (7.93 min), 28) Chrysin (8.39 min) b) LCMS/MS Chromatogram of DI-HET showing corresponding peaks of polyphenols with retention time.

Table 1
Shows the details of polyphenols with retention time and their corresponding concentration (μg/g) identified in DI-HET.

SL No	Name of polyphenol, peak position in chromatogram & retention time	Concentration (μg/g)
1	Naringenin-4 (7.28 min)	6768.581
2	Catechin- 2 (6.75 min)	687.073
3	Epicatechin- 8 (6.77 min)	539.969
4	Shikimic acid-23 (1.76 min)	126.535
5	Syringic acid- 9 (7.20 min)	75.687
6	Vanillic acid-10 (6.79 min)	67.82
7	Kaempferol-19 (7.83 min)	60.159
8	Tocopherol-5 (12.87 min)	7.011
9	Ferulic acid-13 (7.36 min)	3.382
10	Cinnamic acid-27 (7.93 min)	3.094
11	Gallic acid- 6 (1.91 min)	2.681
12	Luteolin-17 (7.92 min)	1.751
13	p-Coumaric acid-16 (7.34 min)	1.646
14	Myricetin-14 (7.65 min)	1.161
15	Quercetin-15 (7.92 min)	1.119
16	Hesperetin-22 (7.86 min)	1.017
17	Apigenin-18 (8.18 min)	1.002
18	Caffeic acid- 11 (6.91 min)	0.964
19	Chrysin- 28 (8.39 min)	0.884
20	Epigallocatechin-12 (2.01 min)	0.703
21	Genistein- 26 (7.81 min)	0.579
22	Quinine- 3 (6.88 min)	0.413

extract of *D. indica* against NAFLD. OA incubation caused the build-up of triglyceride in HepG2 cells via surplus lipogenesis. It is through down-regulation of p-AMPK and associated downstream pathways such as p-ACC, CPT -1, and PPAR-α. On the other hand, it also upregulated lipogenesis, promoting enzymes and transporters like CD36, ACOX-1, FAS, HMGCR, SREBP-1C, SREBP-2, and LXR-α. AMPK is a vital energy detector of intracellular energy metabolism (Grahame Hardie, 2016). It regulates cellular triglyceride and cholesterol production. The phosphorylation of AMPK abates free fatty acid-mediated *de novo* lipogenesis genes and hepatic lipid build-up. Based on this, AMPK phosphorylation has been studied as an important biochemical pathway customarily in hepatic lipid metabolism to assess the potential of many herbal medicines (Viollet et al., 2006; Leng et al., 2018). Our extract has increased phosphorylation of AMPK, revealing its potential to reduce lipid accumulation via promoting fatty acid oxidation in HepG2 cells. AMPK activation also occurs by upstream kinase such as LKB-1 and is also found increased with the extract. LKB-1 is a primary upstream kinase of AMPK, a necessary element in cell metabolism that is required for maintaining energy homeostasis. LKB-1 regulates lipid metabolism in its uptake, *de novo* synthesis, and fatty acid β-oxidation (Kwon et al., 2018). SIRT-1 is a regulator of LKB-1 that is also found to increase with extract, which again confirms AMPK phosphorylation.

The role of SREBP-1c for the regulation of lipogenesis in the liver has been well established by several lines of evidence (Hao et al., 2016). It is known that activated AMPK also inhibits SREBP-1C. This, in turn, will downregulate the expression of ACC and FAS (Shimano, 2001; Horton et al., 2002). This will result in increased mitochondrial β-oxidation and decreased fatty acid synthesis. The extract was found to decrease SREBP-1C. ACC is an enzyme involved in fatty acid biosynthesis. Phosphorylation of ACC causes the downregulation of fatty acid biosynthesis in cells. (Randy et al., 2016). The same has been found enhanced with extract preventing biosynthesis of fat. In the liver, FAS has long been considered as a housekeeping protein, producing fat for the storage of energy when nutrients are present in abundance (Jensen-Urstad and Semenkovich, 2012). Based on this it is an attractive therapeutic target for treating fatty liver and dyslipidemia. Interestingly FAS was found to decrease with extract supporting its benefits against NAFLD. From these results, there is a clue for its liver function promoting properties via phosphorylation of AMPK during acute lipogenesis.

Hereafter cholesterol synthesis pathway was studied with the extract. For this alteration of SREBP-2 and HMGCR with extract were seen at the protein level. SREBP-2 regulates genes involved in cholesterol biosynthesis and homeostasis. SREBP-2 activates the transcription of mevalonate pathway genes and HMGCR enzymes (Brown and Goldstein, 1997). Both SREBP-2 and HMGCR were found to have decreased with extract revealing its potential to prevent hypercholesterolemia. Hypercholesterolemia is a serious risk factor for NAFLD. Hereafter the fatty acid translocase protein, CD36 which facilitates the transport of long-chain fatty acids and is regulated by peroxisome

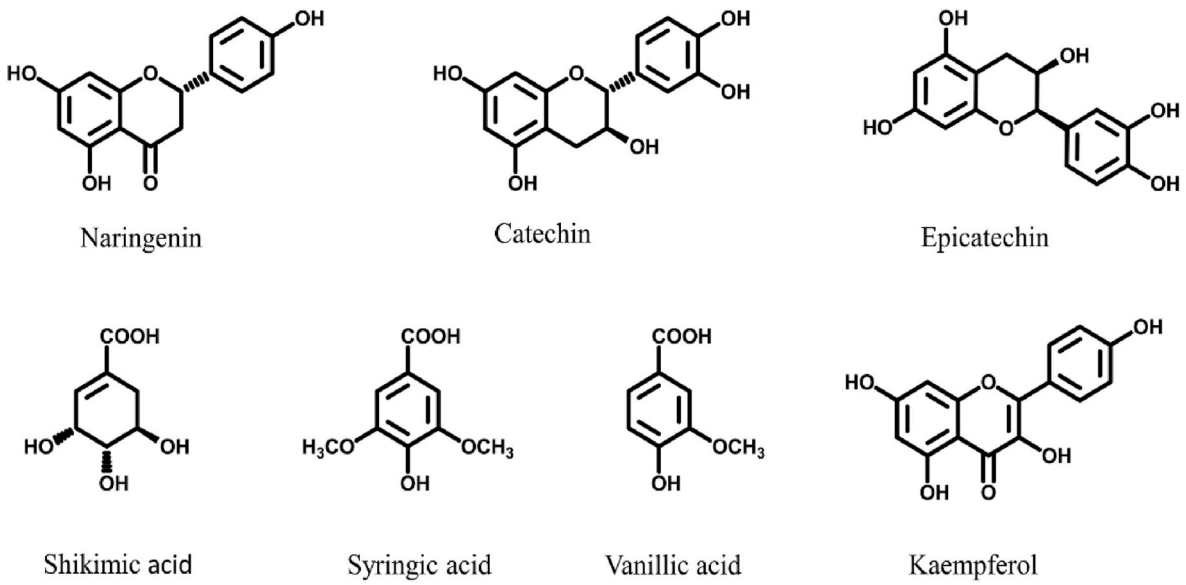

Fig. 9. Structure of major compounds present in the DI-HET using LCMS/MS analysis.

proliferator-activated receptor (PPAR) γ, pregnane X receptor, and liver X receptor (Rada et al., 2020) was studied. In adolescents and adults of both genders, diagnosed with hepatic steatosis, CD36 gene and protein expression were increased compared to healthy controls (Ipsen et al., 2018). Interestingly enough, the extract was found to decrease its activity supporting its application for NAFLD.

LXRs play a critical role in cholesterol homeostasis and bile acid metabolism (Zhu et al., 2012). This establishes LXR-α as a master lipogenic transcription factor, as it directly regulates both SREBP-1c and ChREBP to enhance hepatic fatty acid synthesis. LXR-α is found to decrease with extract paving way for inhibition of fatty acid synthesis.

ACOX-1, a rate-limiting enzyme in peroxisomal fatty acid β-oxidation, regulates metabolism, spontaneous hepatic steatosis, and hepatocellular damage over time (Chen et al., 2018). ACOX-1 is reported in a very high amount in patients with NAFLD compared to controls (Ipsen et al., 2018). However, the peroxisomes generate ROS as they oxidize fatty acids, and likewise, the peroxisomes may induce oxidative stress and promote disease progression (Islam et al., 2020).

Mitochondria play a major role in energy supply to cells as well as critical players in cell injury and cell death. And also, mitochondria are the greatest source of ROS generation (Chen et al., 2020). Oxygen consumption rate is an important indicator of normal cellular physiological function. Impaired oxygen consumption is associated with unhealthy mitochondria or diseased conditions of cells. Mitochondrial dysfunction is an important feature of NAFLD resulting in increased generation of ROS and utilization of cytochrome and peroxisome-mediated oxidation (Ipsen et al., 2018). This further promotes oxidative stress, in turn inducing damage to the mitochondrial membranes, compromising cellular respiration and metabolism, and impairing liver function by direct and indirect cellular damage (Muriel et al., 2017). Lipogenesis was found to induce oxidative stress, alteration in the mitochondrial innate antioxidant defense system (Chen et al., 2020). The extract was found to reduce oxidative stress in mitochondria in Hepg2 cells treated with OA. This is considered an additional beneficial activity of the extract in strengthening its potential against NAFLD. This has caused the enhancement of the activity of CPT-1 with the extract accelerating fatty acid beta-oxidation.

PPAR-α is a ligand-activated transcriptional factor that belongs to the family of nuclear receptors. PPAR-α regulates the expression of genes involved in fatty acid beta-oxidation and energy homeostasis (Wang et al., 2020). Because of their key role in the transcriptional regulation of glucose and lipid metabolism, PPAR ligands have been investigated as possible therapeutic agents for NAFLD. The extract was found to upregulate its expression against OA induced inhibition. It is worth mentioning that DI 10 was found more effective than DI 5 though both extracts were beneficial. Similarly, fenofibrate was also found effective in this *in vitro* study.

LCMS/MS characterization showed the robust presence of pharmacologically active phytochemicals, especially liver function promoting ones. These include naringenin, catechin, epicatechin, shikimic acid, syringic acid, vanillic acid, kaempferol with traces of other compounds (table no. 1 & Fig. 9). Naringenin acts as an antidyslipidemic through the regulation of hepatocyte TG accumulation by activating PPAR-α, SIRT-1, and LKB-1 (Goldwasser et al., 2010; Hua et al., 2021). Catechin is found to have robust antioxidant activity and antidyslipidemic activity through the inhibition of cholesterol and triglyceride synthesis by the activation of LKB-1/AMPK (Hui et al., 2020; Murase et al., 2009). Shikimic acid is found to attenuate mRNA expression of *de novo* lipogenesis related genes such as FAS, SREBP-1C, and LXR- α in the HepG2 and 3T3L-1(Kim et al., 2019). In addition, shikimic acid was found to activate AMPK and p-ACC (Kim et al., 2019). Syringic acid is a potent antioxidant and antidyslipidemic in hepatocytes (Vo et al., 2020). It also decreases hepatic lipogenic enzymes and elevates fatty acid oxidation enzymes (Ham et al., 2016). Vanillic acid is found to activate AMPK to mediate its therapeutic activity against NAFLD (Shekari et al., 2021). Kaempferol decreases the expression of LXR-α and also has antioxidant properties (Xiang et al., 2021). It also reduces TG and cholesterol and activates PPAR-α in hepatocytes (Chang et al., 2011). In addition to the above-mentioned phytochemicals, we also found the presence of pharmacologically important bioactives with comparatively lesser concentrations. These bioactives were also expected to contribute to the anti-NAFLD properties of DI-HET.

5. Conclusion

From the overall data we conclude that DI-HET is effective against OA induced NAFLD in HepG2 cells. This is accomplished via activation of the SIRT-1/p-LKB-1/AMPK pathway, promotion of mitochondrial function, and inhibition of lipogenesis. In addition, antioxidant, anti-inflammatory and ER protection properties of *D. indica* also add to its beneficial properties. Various bioactives compounds such as naringenin, catechin, epicatechin, shikimic acid, syringic acid, vanillic acid, kaempferol, and traces of other compounds found in the extract are expected to contribute to its beneficial effects. Based on this *in vitro*

study, detailed studies are recommended for the development of *D. indica* based phytopharmaceutical for NAFLD.

Declaration of competing interest

The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

M.S. Poornima: Data curation, Formal analysis, Writing – original draft, conducted the entire experiments, collected, analyzed, interpreted the data, and wrote the original manuscript. **G. Sindhu:** Writing – review & editing, assisted in conducting the in vitro experiments and manuscript editing. **Abraham Billu:** did the polyphenol characterization of the plant. **C.R. Sruthi:** Writing – review & editing, assisted in conducting the in vitro experiments and manuscript editing. **P. Nisha:** did the polyphenol characterization of the plant. **Pinku Gogoi:** Plant extraction was done by. **Gakul Baishya:** Plant extraction was done by. **K. G Raghu:** designed the work plan, concept, interpreted the data, contributed intellectual content.

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