Doctor of Philosophy

Protective Effect of Filbertone against Liver Diseases via Regulating Ferroptosis

The Graduate School of the

University of Ulsan

Department of Food and Nutrition

Sonam Chandrawanshi

Protective Effect of Filbertone against Liver Diseases via Regulating Ferroptosis

Supervisor: Prof. Dr. Yu Rina

A Dissertation

Submitted to

The Graduate School of the University of Ulsan

In the partial Fulfillment of the Requirements

for the degree of

DOCTOR OF PHILOSOPHY

BY

CHANDRAWANSHI SONAM

Department of Food and Nutrition

University of Ulsan, Korea

December 2023

Protective Effect of Filbertone against Liver Diseases via Regulating Ferroptosis

This certifies that the dissertation of
Chandrawanshi Sonam is approved by

Committee Chair	Dr. Ahn Byung Yong
Committee Member	Dr. Yu Ri Na
Committee Member	Dr. Jang Yu Mi
Committee Member	Dr. Park Jeong Woo
Committee Member	Dr. Sung Mi Kyung

Department of Food and Nutrition

University of Ulsan, Korea

December 2023

Acknowledgment

I would like to acknowledge and give my warmest thanks to my parents and brothers for their whole continuous support and understanding. You’re blessing for me was what sustained me this far and pass me through all the difficulties.

I would like to thank my supervisor Prof. Rina Yu who made this work possible. Her guidance and advice carried me through all the stages of writing my project. I would also like to thank my committee members for letting my defense be an enjoyable moment and for your brilliant comments and suggestions, thanks to you.

I would also like to thank Prof. Chung Hun Taeg and Dr. Yeonsoo Jeo for always encouraging and guiding me. I am very grateful to all the lab members Dr. Jeongmin Park, Dr. Jeongheon Gong, who gave so much of their time, and energy to support me.

Finally, I would like to thank my friends for their support. I have experienced your guidance day by day. You all are the one who let me finish my degree. I will keep on trusting you for my future.

Abstract

Background: Obesity is an ongoing issue associated with abnormal fat accumulation in the liver that can damage the organ and lead to serious complications. The accumulation of excessive fat in the liver, resulting in liver steatosis, can progress to liver fibrosis, leading to irreversible harm and frequently culminating in the development of cirrhosis and liver cancer. Ferroptosis is an iron-dependent form of oxidative programmed cell death characterized by the inactivation of glutathione peroxidase 4 (GPX4) and lipid peroxidation. Filbertone (C8H14O) is a natural compound found in hazelnuts and has a molecular weight of 126.2 g/mol. It has beneficial properties for anti-obesity and regulating lipid homeostasis. However, the effect of filbertone on liver diseases and its related molecular mechanisms remain unknown.

Aim: In this study, we investigated the potential effects of filbertone on liver disease via modulating ferroptosis.

Chapter I: We hypothesized that filbertone can protect against a fatty liver-induced hepatic injury by inhibiting ferroptosis. Filbertone treatment significantly reduced liver damage in HFD/MCD-fed mice by inhibiting hepatic ferroptosis. To examine the mechanism of filbertone on the ferroptosis-regulated pathway, we analyzed the expression levels of ferroptosis-related proteins, such as Nrf2, GPX4, HO-1, and PTGS2 in in vivo and in vitro experiments. Furthermore, we showed that filbertone inhibited ferroptosis by activating the PERK-Nrf2 pathway.

Chapter II: We aimed to investigate the role of filbertone in cancer cells and its mechanisms. We have shown that filbertone decreases the cell viability of B16F10 and HepG2 cells, as determined by the WST-8 assay. Our mechanistic studies indicated that filbertone inhibits YAP activity in cancer cells and promotes ferroptotic cell death. This study demonstrated that filbertone modulates YAP activity by generating mitochondrial ROS. Furthermore, the inhibition of YAP by mitochondrial ROS resulted in the decrease of SLC7A11 expression. Therefore, filbertone inhibits YAP by generating mitochondrial ROS, which can induce ferroptosis by downregulating SLC7A11 expression.

Conclusion: Thus, we suggest that filbertone has an emerging potential effect on fatty liver-induced hepatic damage by inhibiting lipid peroxidation and decreasing ferroptosis. This study also indicates that filbertone has strong potential as an inducer of ferroptotic cell death in cancer cells. Therefore, filbertone may be a valid food component against obesity-induced liver diseases.

Chapter I. An overview of ferroptosis in liver diseases.

CONTENTS

Chapter II. Protective effect of Filbertone against fatty liver-related oxidative damage by inhibiting hepatic ferroptosis

Chapter III. Protective effect of Filbertone against hepatocellular carcinoma by stimulating ferroptotic cell death.

CHAPTER I

An overview of ferroptosis in liver diseases

1. Introduction

1) Obesity and liver diseases

Obesity is characterized by the atypical or excessive accumulation of fat in the body. The worldwide incidence of obesity is increasing irrespective of age, ethnic background, gender, or socioeconomic status. It is a complex combination of genetic, environmental, and mental factors causing an independent risk factor of cancer-related, insulin resistance, type 2 diabetes high blood pressure, hyperlipidemia, and cardiovascular diseases outcomes. Fatty liver disease is a prevalent health condition characterized by the accumulation of excess fat within the liver, commonly associated with obesity (Figure 1). Obesity is correlated with the development of non-alcoholic fatty liver disease (NAFLD)-associated steatosis and enhances the growth of various other hepatic disorders (1). Numerous studies validated the data suggesting an evident association between obesity and the development and progression of hepatic disorders (2). The liver is a highly active organ that plays a crucial role in maintaining metabolic balance throughout the body by performing a wide range of biochemical activities. For a healthy liver, a rich blood supply is essential for delivering and exporting substrates, hormones, and nutrients. When the liver is insufficiently functional to carry out its physiological processes- the accumulation of triglycerides within the liver, known as intrahepatic triglyceride (IHTG, associated with disruptions in glucose, fatty acid (FA), and lipoprotein metabolism) or steatosis, leads to cause hepatic injury. The modification of liver lipid metabolism plays a significant role in various pathological states, including obesity, non-alcoholic fatty liver disease, diabetes mellitus, atherosclerosis, and cardiovascular disease.

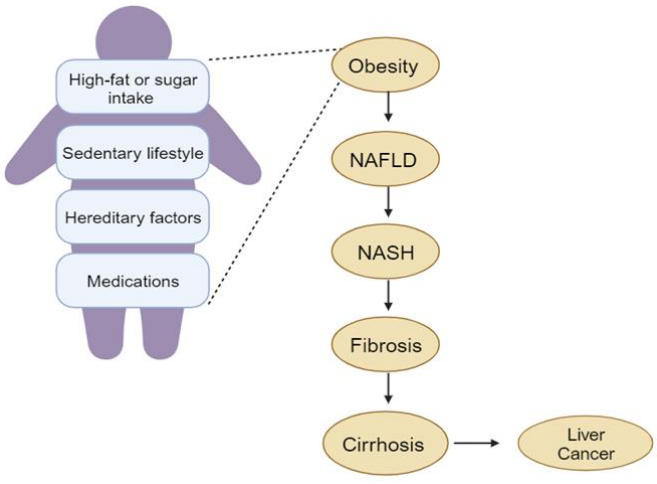

Figure 1. Obesity and liver diseases

2) Non-alcoholic fatty liver disease (NAFLD)

Non-alcoholic fatty liver disease (NAFLD) poses a significant health concern, affecting overall population. This prevalence is even higher, ranging from 70% to 80%, among those with diabetes and obesity, indicating a disordered hepatic lipid metabolism. The growing incidence of non-alcoholic fatty liver disease (NAFLD) is a major threat to public health and is currently the most prominent chronic liver disease in Western societies. Non-alcoholic fatty liver disease (NAFLD) extends to an array of ailments ranging from the reversible condition to the more severe non-alcoholic steatohepatitis (NASH), characterized by the presence of hepatic steatosis accompanied by lobular inflammation. In contrast, non-alcoholic steatohepatitis (NASH) is defined by the presence of hepatic steatosis accompanied by lobular inflammation and cell death, leading to the potential progression of fibrosis and cirrhosis (Figure 2) (3).

3) Liver fibrosis and cirrhosis

NASH, a major cause of liver fibrosis, ultimately develops liver cirrhosis and eventually leads to liver cancer, is the consequence of multiple biological processes. Liver fibrosis refers to the pathological condition characterized by the abnormal and excessive buildup of extracellular matrix proteins, particularly collagen, which is observed in most chronic liver disorders (3). Liver cirrhosis is a complex condition with multiple contributing factors, including the degeneration and necrosis of hepatocytes, the replacement of liver parenchyma by fibrotic tissues, regenerative nodules, and the subsequent loss of liver function (3). On the other hand, chronic liver illness facilitates the impairment of hepatocytes by inducing hepatotoxicity, which encompasses factors such as hepatitis viruses, alcohol metabolites, and bile acids. Hepatocytes that have undergone damage release reactive oxygen species (ROS) and fibrogenic mediators, which facilitate cell death and ultimately result in liver injury (4).

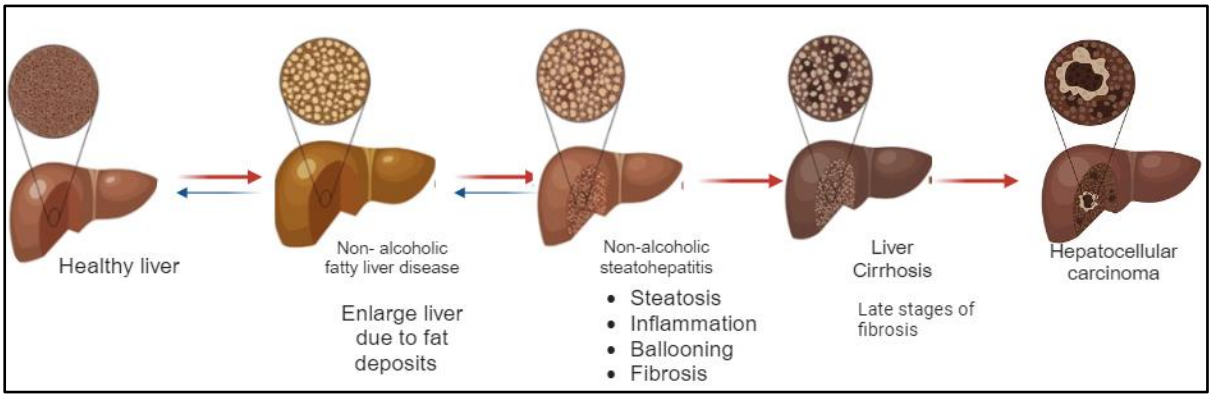

Figure 2. The stages of liver disease

4) Liver cancer

Primary liver cancer, hepatocellular carcinoma, commonly referred to as hepatoma, is a cancer that arises in the liver. It is globally recognized as the sixth most prevalent type of cancer and ranks as the fourth leading cause of cancer-related deaths. In recent years, liver metastases have become more common, which refers to the development of secondary cancer in the liver that begins from other places in the body. The gastrointestinal tract is a common anatomical area for the development of diverse forms of malignancies, such as pancreatic cancer, stomach cancer, colon cancer, and carcinoid tumors predominantly located in the appendix. This is due to the proximity of the liver to these metabolically active organs, which are rich in blood vessels and lymph nodes. Additionally, cancers originating from breast, ovarian, lung, renal, and prostate tissues can also spread to the liver.

2. Cell death and liver diseases

Cell death is considered the most fundamental pathological phenomenon in liver disease. Cell death can be classified into two main categories, namely accidental cell death (ACD) and regulated cell death (RCD), which are distinguished based on their functional characteristics. Regulated cell death involves intricate signaling cascades, which are governed through effector molecules and result in distinct biochemical, functional, and immunological outcomes. Regulated cell death is considered the most fundamental pathological phenomenon in liver disease. The initiation of hepatocyte cell death has a pivotal role in the growth of liver disease, as it triggers an inflammatory response that ultimately leads to the development of fibrosis. RCD is classified based on its molecular characteristics into various subroutines, including necroptosis and pyroptosis, which can occur during development or in the presence of viral infections. Conversely, other subroutines, including ferroptosis (an iron-lipid dependent

cell death), entotic cell death, netotic cell death, parthanatos, lysosome-dependent cell death, autophagy-dependent cell death, alkaliptosis, and oxeiptosis, have received less extensive research and may primarily pertain to cellular responses triggered by specific toxins, rather than reflecting normal physiological conditions (5, 6). Multiple forms of cell death can be induced by oxidative stress. The dysregulation of various forms of cell death, including both individual and combined types, plays a significant role in the development and progression of human disorders, whereas the death of specific kinds of liver cells could potentially lead to hepatic damage in various disease types (7, 8, 9). Multiple studies have reported the signaling cascades involved in each cellular death pathway and their implications in hepatic disorders, with particular focus on research models related to non-alcoholic fatty liver disease and steatohepatitis (NASH/NAFLD), acetaminophen (APAP)-induced hepatotoxicity, autoimmune hepatitis, alcoholic liver disease, cholestatic liver disease, and viral hepatitis, however, more research is needed.

3. Biochemical features of ferroptosis

Ferroptosis is a discovered in 2012 by Dixon et. al. is an iron- lipotoxicity dependent form of regulated cell death (81). Ferroptosis exhibits unique morphological, biochemical, and genetic features that distinguish it from other well-defined forms of controlled cell death. Ferroptosis is associated with two main biochemical characteristics - iron accumulation and lipid peroxidation (10). An excess intracellular free ferrous iron promotes reactive oxygen species (ROS) through the Fenton reaction, which in turn initiates lipid peroxidation and triggers the process of ferroptosis. The predominant form of iron found in food is Fe3+. Upon entering the bloodstream, Fe3+ binds with transferrin (TF) in the serum. Subsequently, the Fe3+-TF complex is recognized by the transferrin receptor 1 (TFR1) located on the cell membrane. Upon being internalized by the transferrin receptor 1 (TFR1), ferric ions (Fe3+) undergo reduction to ferrous ions (Fe2+) facilitated by the STEAP3 metalloreductase within the endosome. Subsequently, the ferrous ions are released from the endosome into the cytosol through solute carrier family 11 member 2 (SLC11A2/DMT1). The protein ferritin, which is involved in iron storage, exhibits an anti-ferroptosis function. This includes the ferritin light chain and FTH1. Additionally, lysosomes can breakdown ferritin, hence increasing the amounts of free iron. The process of ferritin breakdown is enhanced by NCOA4-mediated ferritinophagy, resulting in a reduction in iron storage and the promotion of ferroptosis. The iron-efflux protein FPN is responsible for the transportation of iron out of cells. The inhibition of the iron release route in cellular membranes leads to heightened susceptibility to ferroptosis

(11).

4. Molecular mechanism of ferroptosis

Ferroptosis is primarily characterized by the occurrence of lipid peroxidation, which is triggered by diverse oxidative stress factors. The process of lipid peroxidation is closely linked with antioxidant systems (12). Multiple antioxidant systems have been involved in the regulation of ferroptosis, including the System Xc-GSH-GPX4 pathway, the transsulfuration pathway, the mevalonate pathway, the FSP1-CoQ10 pathway, the DHODH-CoQH2 pathway, and the GCH1-BH4 pathway.

The System **Xc-GSH-GPX4** pathway defines it to be the most essential antioxidant system to prevent ferroptosis (13). System Xc – a cell membrane cystine-glutamate antiporter, composed of two heterodimer the light chain subunit SLC7A11 and the heavy chain subunit SLC3A2 and, is responsible for the transport of intracellular glutamate to the extracellular space, as well as the transport of extracellular cystine into the intracellular space. Intracellular cystine converts to cysteine, subsequently combined with glutamic acid and glycine to form glutathione (GSH) through the processes of glutamate cysteine ligase (GCL) and glutathione synthase (GSS) (14). The enzyme GPX4 catalyzes the conversion of lipid hydrogen peroxide into lipid alcohol, a non-toxic compound. This enzymatic reaction is facilitated by the presence of glutathione (GSH) as a cofactor. The conversion of lipid hydrogen peroxide into lipid alcohol by GPX4 serves as a protective mechanism for cells, effectively mitigating the harmful effects of lipid peroxides (15). Therefore, the inhibition of System Xc- leads to a reduction in glutathione (GSH) synthesis and the inactivation of glutathione peroxidase 4 (GPX4), resulting in the induction of ferroptosis. Consequently, the targeting of this pathway plays a crucial role in the modulation of ferroptosis, serving as a significant antioxidant system.

Reactive oxidative stress (ROS), mostly produced within mitochondria from usual metabolism and energy generation, are crucial for cell signaling and tissue homeostasis, but excess ROS damage cells by depleting biomolecules (16). While, reactive oxygen species (ROS) are effectively neutralized through the action of many antioxidants, including glutathione peroxidase (GPX), vitamin E (Vit-E), and coenzyme Q10 (CoQ10) (17). The dysfunction within the generation and elimination of ROS causes several kinds of diseases, linked to oxidative stress (18).

Lipid peroxidation refers to the mechanism via which several free radical species, including oxygen radicals, peroxyl radicals, and hydroxyl radicals, initiate an attack on the diallylidene moiety of

polyunsaturated fatty acids (PUFA). This process ultimately leads to the buildup of lipid peroxidation free radicals and hydroperoxide (19). The presence of polyunsaturated fatty acids (PUFAs) in cellular and organelle membranes is substantial, thereby providing them susceptible to lipid peroxidation, which subsequently results in compromised cellular integrity and function (20). Notably, various investigations have proven the essential role of lipid peroxidation in ferroptosis.

5. Ferroptosis involved in various diseases

In recent years, the dysregulation of ferroptosis has drawn considerable interest due to its involvement in a variety of diseases (Figure 3), including cancer, kidney injury, and neurodegenerative disorders (11). Cancer cells exhibit a higher susceptibility for iron accumulation in comparison to their normal cells. Multiple studies have provided evidence supporting the dysregulation of iron homeostasis in several cancer forms, such as breast cancer, ovarian cancer, renal cancer, and lung cancer (21).

Cancer cells requires high greater metabolic rate to sustain their raised proliferation, elevated by increased ROS production (22). Consequently, tumors possess higher ROS levels, therefore cancer cells trigger their antioxidant defense mechanisms to mitigate the enhanced oxidative stress, could be a potent strategy to treat them against oxidative stress- induced cell deaths (24). Ferroptosis inducing clinical drugs in cancer cells, including sorafenib, sulfasalazine, artemisinin, and ibuprofen, can promote the chemotherapy effects in the treatment of tumors (18, 24, 25, 20).

The presence of iron dyshomeostasis and lipid peroxidation, are characteristic features of ferroptosis, have been extensively observed in the pathology of Alzheimer's disease (AD) and Parkinson's disease (PD) (26). Ageing is widely recognized as the main risk factor for the development of neurodegenerative diseases, defined as the progressive degeneration of neurons (26). Additionally, ageing can be associated with the accumulation of iron in the brain. Various neurological disorders have been established to exhibit iron formation in the affected brain regions. Therefore, iron has been proposed as a vital component that contributes to the neurodegenerative processes. According to other reports, loss of ceruloplasmin (Cp), a protein responsible for iron export, is found to be associated with iron-dependent parkinsonism in mice (27), and in the cerebrospinal fluid (CSF) of PD patients (28). The vulnerability of brain tissues to oxidative stress is due to their high metabolic activity. Several neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease, are marked by an excess in oxidative stress.

Inflammation is a defensive reaction of the body to tissue injury, contributing as physiological mechanism, associated with observable symptoms such as redness, swelling, heat, discomfort, and impaired functionality. Many studies have demonstrated the significance of polyunsaturated fatty acids (PUFAs) and their associated metabolic enzymes as key regulators in modulating the body's inflammatory response (29). Inflammation is linked to ferroptosis, as GPX4 inhibits AA and NF-κB pathways and reduces ROS from lipid peroxidation, indicating that inflammation is related to ferroptosis (30).

Kidney, responsible for metabolizing, reabsorb and retain water in the body. A series of complications causing sharp decline in renal function, promoting acute kidney injury (AKI), has been found to have great association with ferroptosis (31). Friedmann's research has revealed a significant involvement of the GSH/Gpx4 axis in the Gpx4 deletion mice model of acute renal failure. Furthermore, it has been established that GPX4 functions as a negative regulator of AKI (30). Tonnus, showed the loss of FSP1 or GPX4 enhance the sensitivity to renal tubular ferroptosis (30). Additionally, Muller also represented the increased expression of ACSL4, a fatty acid metabolism enzyme that can regulate ferroptosis, promoting AKI (32).

Skeletal muscle is an essential part of the motor system, facilitating a diverse range of bodily movements through the process of muscular contraction. Acute compartment syndrome (ACS) has the potential to exacerbate skeletal muscle injury, which involves an increase in the content of the fascial compartment, a decrease in the volume of the fascial compartment, and an elevation in internal pressure. Wang elucidated that the downregulation of cystathionine γ-lyase/hydrogen sulfide (CSE/H2S) signaling triggers ferroptosis and increases acetylation of related proteins in skeletal muscle of mice, associated with Skeletal muscle injury and aging via MuRF-1-dependent pathways (33). Rhabdomyolysis (RM), a common syndrome caused by severe skeletal muscle damage, also reported induced by ferroptosis. It has been shown that increasing the expression level of GPX4 in muscle cells by inhibiting ACSL4 reduces lipid peroxidation and improves RM (34).

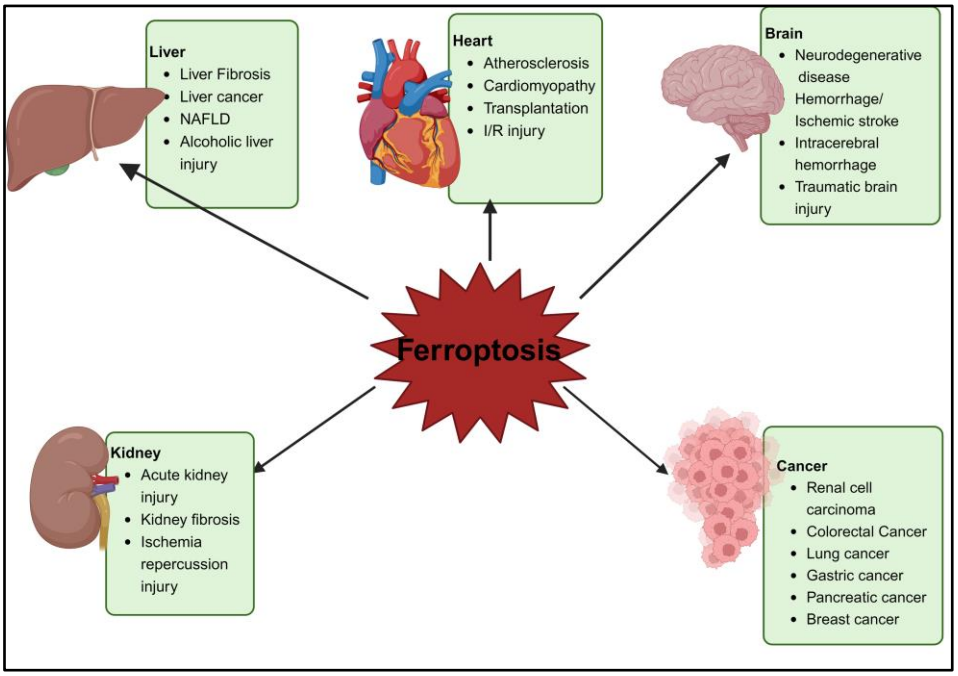

Figure 3. Ferroptosis is involved in several diseases

6. Ferroptosis and liver diseases

As one of the main organs responsible for iron processing, the liver is the first to suffer once iron homeostasis is disrupted. Studies on the mechanism of ferroptosis in the pathogenesis of liver diseases gradually emerged, and ferroptosis-based diagnosis and treatment strategies for liver diseases are now being developed (Figure 6). Even though ferroptosis threatens the survival of all cells, including hepatocytes, stellate cells, Kupffer cells, and cancer cells, its clinical relevance has been questioned due to the diverse properties and roles of different cells in liver diseases. Researchers found that targeted inhibition of ferroptosis had an important role in preventing lipid peroxidation-induced liver injuries, inflammatory infiltration, and immune disorders, while inducing ferroptosis reversed resistance to targeted therapy in liver cancer. There is still a need to investigate how ferroptosis contributes to the progression from hepatitis to cirrhosis or HCC in basic and clinical studies (35). Accommodating evidence indicates that ferroptosis acquires a variety of functions in a vast array of liver diseases. One possible approach to mitigating the pathophysiological progression of several liver diseases, including alcoholic liver injury, nonalcoholic steatosis hepatitis, and fibrosis, involves the inhibition of ferroptosis. In the context of hepatocellular carcinoma (HCC) treatment, the induction of ferroptosis has the potential to limit the development of secondary resistance to existing pharmaceuticals, such as sorafenib (36, 37). The researchers have reported that ACSL4, a key regulator of ferroptosis, was increased in rat model of non-alcoholic steatohepatitis (NASH) induced by arsenic. Subsequent investigations have

revealed that the suppression of the Mfn2/IRE1α-ACSL4 pathway might be a significant strategy for preventing the development and progression of non-alcoholic steatohepatitis (NASH). According to Qi et al. (2020), found that the progression of NASH can be slowed down by administering a GPX4 activator, iron chelator, or ferroptosis inhibitor. These therapies enhance GPX4 activity and influence iron metabolism. Furthermore, Li et al. (2020) conducted a study demonstrating the inhibition of ferroptosis could decrease liver fibrosis in a NASH mice model induced by MCD diet. Wang demonstrated that ferrostatin-1, a ferroptosis inhibitor prevent the anti-fibrotic action of artemether (ART) on the liver by blocking ferroptosis, also the upstream molecule P53 inducing ferroptosis in hepatic stellate cells (HSC) (38).

Hepatic ischemia-reperfusion injury (IRI) is a major obstacle in the context of liver transplantation. Recent evidence suggesting that ferroptosis is involved in the development of ischemia-reperfusion injury (IRI), therefore, inhibition of ferroptosis could be a therapeutic strategy (39, 40). Previous studies demonstrated the inactivation of GPX4 contributes to hepatic IRI via liver injury, lipid peroxidation, and iron overload by upregulating PTGS2 (41). The development of ferroptosis cell death in the liver was first observed through hemochromatosis (HH) diet-induced and transgenic mice. In HH, SLC7A11 serves as biomarker for ferroptosis, and it has been observed that elevated levels of SLC7A11 may be associated with the activation of the ROS-Nrf2 antioxidant response element, which is understood to be the mechanism responsible for the inhibition of ferroptosis (42). On the other hand, several studies have shown ferroptosis sensitivity could inhibit the progression of liver cancer while suppressing GPX4 expression (43). Wang, Jin, et al, demonstrated the role of YAP1 in septic liver injury via, disrupting the link among NCOA4 and FTH1 and inhibit the degradation of ferritin to Fe2+, which results in a subsequent reduction of ROS generation and lead to suppression of especially ferritinophagy-mediated ferroptosis (44). Another mediator of oxidative homeostasis, activating transcription factor 4 (ATF4) has also been elucidated to inhibit sorafenib, RSL3, erastin- induced ferroptosis via SLC7A11 activation (45, 46). Gao et al., demonstrated that ATF4 inhibition can promote sorafenib- induced ferroptosis via increasing lipid peroxidation, and reducing GSH level, which could further be prevented by forced expression of SLC7A11 and ferrostatin-1 in HCC. Importantly, the activation of YAP/TAZ resulted in the translocation of ATF4 to nucleus, triggering its transcriptional expression, showing YAP/TAZ association with ATF4 (47).

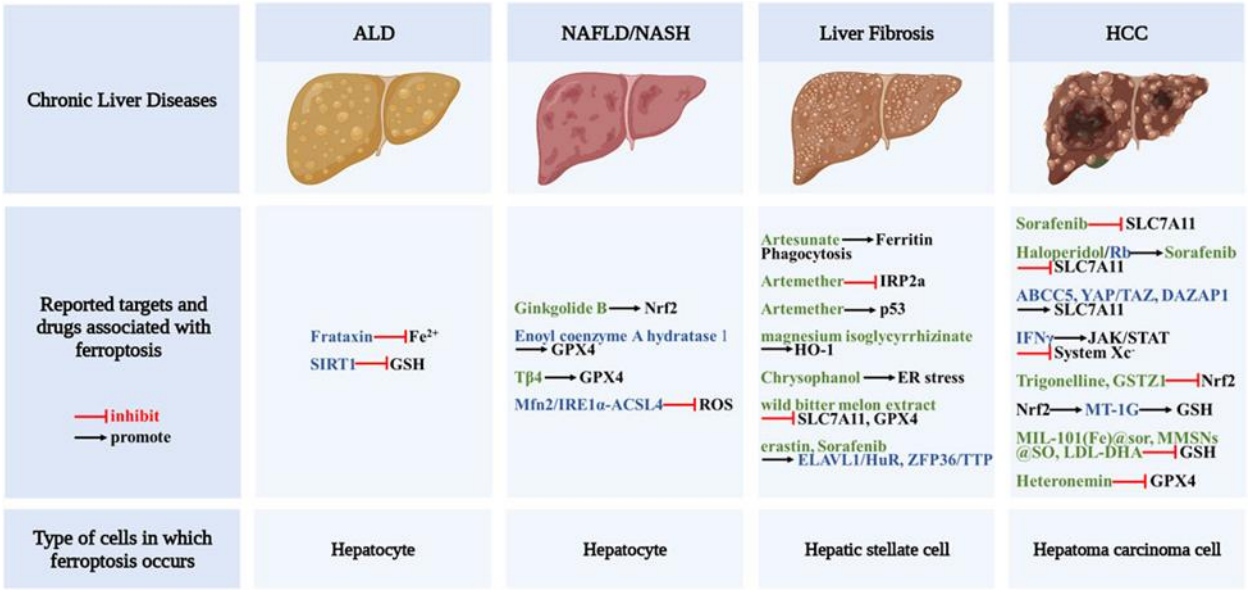

Figure 4. Chronic liver diseases and ferroptosis (48)

7. Phytochemicals involved in prevention of liver diseases

Natural polyphenols contain a variety of phytochemicals that possess a similar phenolic structure, categorized into two main classes: flavonoids and non-flavonoids. Polyphenols are a class of bioactive chemicals synthesized by plants, which exhibit a range of diverse functions including defense mechanisms against microbial pathogens, attraction of pollinators, and promotion of seed dissemination through animal carriers. Dietary flavonoids and non-flavonoids have garnered considerable attention due to their presumed health-promoting properties found in various fruits and vegetables. Several bioactive compounds are widely recognized for their well-known hepatoprotective properties (Figure 5) (49, 50, 51, 52). Over a prolonged time, plant-based diet with a high fiber content and low-fat intake as a viable strategy for the management of liver-related conditions has been recommended. Currently, lifestyle modifications influencing dietary and exercise strategies are widely regarded as the primary approach for managing non-alcoholic fatty liver disease liver-related conditions. However, there is emerging evidence suggesting that certain herbal medicines play a significant role in mitigating oxidative stress by reducing insulin resistance and hepatic lipid accumulation, as well as interacting with lipid metabolism in the liver (50). According to Jiao et al, it was discovered that curcumin possesses the ability to inhibit the production of hepcidin, a peptide hormone mostly synthesized in the liver. This inhibition of hepcidin synthesis by curcumin subsequently influences the body iron metabolism and mitigates inflammation induced by lipopolysaccharide (LPS) (53).

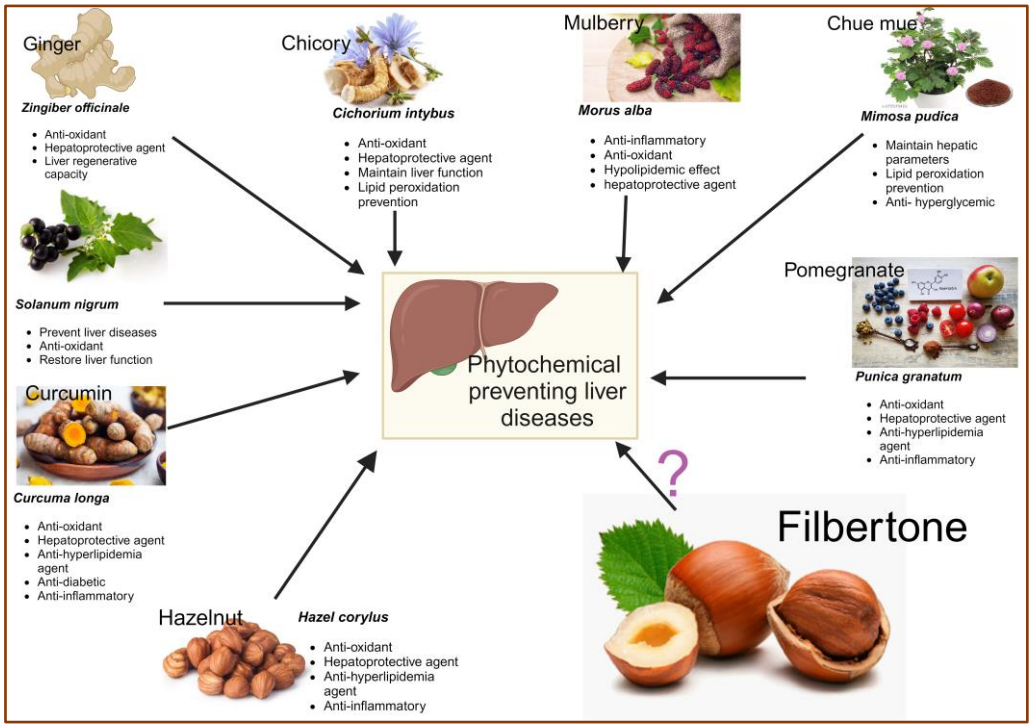

Figure 5. Phytochemicals are involved in liver diseases (54, 55).

Table I. Ferroptosis regulating phytochemicals.

Compounds	Mechanisms	Functions	Ref.
Chrysin	promoted selective autophagy and FTH1 degradation	inducer	56
Luteolin	promoted heme degradation by up-regulating HO-1 expression	inducer	57
Baicalein	regulated the NRF2/SLC7A11/GPX4 pathway	inhibitor	58
Apigenin	associated with the increased GPX4 and FTH1 levels and the decreased ACSL4 and TFR levels	inhibitor	59
Quercetin	regulated NCOA4, iron storage protein FTH1	inhibitor	60
Kaempferol	activated the NRF2/SLC7A11/GPX4 signaling pathway	inhibitor	43
Fisetin	promoted the expression of FTH1 and HO-1 by activating SIRT1/NRF2 pathway	inhibitor	61
Rutin	regulated the NRF2/HO-1 pathway	inhibitor	62
Silibinin	suppressed the enzymatic activity of ACSL4 by directly binding to it	inhibitor	63
Wogonoside	regulated the SOCS1/P53/SLC7A11 pathway	inducer	64
Acacetin	inhibited endoplasmic reticulum stress	inhibitor	65
Nobiletin	regulated the GSK3β-mediated Keap1/NRF2/HO-1 pathway	inducer	66, 67
Icariin	inhibited ferroptosis by activating the system xc−/GPX4 axis	inhibitor	68, 69
Dihydromyricetin	inhibited the SPHK1/mTOR signaling pathway	inhibitor	70

8. Filbertone

Filbertone is a primary flavor compound discovered in hazelnut oil (71). Hazelnuts are recognized for their composition of diverse phytochemicals, which are associated with anti-inflammatory, antioxidant, and lipid-lowering attributes (72). Filbertone has emerged as a viable marker for the purpose of analyzing hazelnut constituents in diverse product formulations. The methods used to determine filbertone can be categorized based on the specific objectives associated with these analyses, include: (a) identifying the addition of hazelnut oil in the presence of olive oil and other fatty acids with similar properties, and (b) detecting allergens and assessing the authenticity of hazelnut products. Based on a report, it has been demonstrated that racemic filbertone displays a flavor-perception threshold of around 5 parts per trillion (ppt) and a recognition threshold of roughly 30 ppt when dissolved in a 3% sucrose solution. Filbertone is utilized in this context as a 5% sugar solution, administered at a dosage of 0.1

parts per million (ppm) (73, 74). According to the Flavor and Extract Manufacturers Association (FEMA), filbertone is recognized as a chemically safe compound. It is commonly employed in fragrances to provide a natural flavor reminiscent of tropical fruits and citrus (73). Recent research has provided evidence indicating that filbertone possesses beneficial in vitro and in vivo features, in relation to its anti-inflammatory, anti-hyperglycemic, and anti-obesity activities (75, 72, 16).

In a previous study, we demonstrated the potential neurodegenerative disease mitigating effects of filbertone (16). In this study, we provide findings indicating that the activation of PERK by filbertone is mediated by the production of reactive oxygen species in the mitochondria. The administration of filbertone therapy was found to decrease the accumulation of α-synuclein generated by a high-fat diet, potentially via increasing the activity of the autophagy-lysosomal pathway (16). Recently, filbertone has been proposed as a potential therapeutic agent for obesity and inflammation. Filbertone alleviated high-fat diet induced obesity, hyperglycemia, and hyperinsulinemia by activating AMPK signaling (75). Furthermore, filbertone has been discovered to inhibit the MAPK and NF-κB signalling pathways, thereby attenuating the production of inflammatory mediators from lipopolysaccharide (LPS)-activated microglia. Furthermore, the treatment of filbertone led to a reduction in the expression levels of inflammatory cytokines and the activation marker of microglia in the hypothalamus (72). A recent study examined the potential impact of filbertone on the modulation of myocyte lipid metabolism and thermogenesis in muscle, specifically under conditions of a high-fat diet (HFD). They also revealed a significant impact of filbertone in muscle lipid accumulation. Filbertone demonstrated a notable reduction in the formation of muscle triacylglycerol (TAG) in the presence of chronic food excess, led to a suppression of lipid accumulation in C2C12 myotubes treated with oleic acid (76).

CHAPTER II

Protective effect of Filbertone against fatty liver-related oxidative damage by inhibiting hepatic ferroptosis

1. Introduction

Nonalcoholic fatty liver disease (NAFLD), a kind of chronic liver disease, characterized by simple steatosis to steatohepatitis, is the most endemic liver disease in the world. Nonalcoholic steatohepatitis (NASH) is an acute form of NAFLD, manifested by hepatic cell death, lipid droplet accumulation in hepatocytes (77). The incidence rate of NAFLD has reached 25%, making it the leading cause of chronic liver disease worldwide due to the prevalence of obesity (78). Notably, it is more concerning because obese now account for 80% of the incidence rate. Moreover, increased ROS generation in NAFLD mediates inflammation and oxidative stress (5), and lipid peroxidation (79) is a sign of mitochondrial malfunction caused by oxidative stress. As it is well known that ROS promote lipid peroxidation by targeting the double bonds of polyunsaturated fatty acids, resulting in the formation of lipid peroxides (LPO), specifically 4-hydroxy-2-nonenal (4-HNE) and MDA, which cause intracellular damage (6, 30, 10).

Ferroptosis, an iron- dependent form of oxidative programmed cell death defined by inactivation of glutathione peroxidase 4 (GPX4) and higher lipid hydroperoxide level. It has been reported the association between ferroptosis and NAFLD-induced liver injury. During the formation of NAFLD, lipid peroxidation is initiated by ROS, and directly inhibits GPX4 activity, which acts as the main source to enhance ferroptosis (80, 81). Recent investigations have shown that ferroptosis is closely associated with the pathogenesis of several liver disorders (3). ER stress plays an important role in ferroptosis via stimulating ROS (82, 3), activating PERK pathway, a protein kinase located in ER-membrane. It has been reported that ferroptosis inducer sorafenib promotes ER stress, GSH depletion and lipid ROS accumulation in liver disease (83). PERK phosphorylation subsequently phosphorylates Nrf2, and activate the antioxidant genes i.e., GPX4, HO-1 and SLC7A11 to inhibit lipid ROS level and hepatic ferroptosis (84) in liver disease.

Multiple studies have provided evidence demonstrating that several other phytochemicals, like curcumin and quercetin have the potential to regulate antioxidant genes to prevent fatty liver diseases, via suppression or activation of ferroptosis (Table 1). Filbertone, a major component of hazelnut oil displays anti-obesity, anti-inflammatory and lipid reducing properties (75, 72). For example, it has been shown that filbertone inhibits HFD-induced adiposity, lipid accumulation and reduces plasma levels of inflammatory cytokines via activating cAMP pathway (75). Recent study has also shown the protective role of filbertone against obesity- induced hypothalamic inflammation in HFD-fed mice (72). Filbertone

has an antioxidant potential to prevent neurodegenerative disease in HFD-treated mice by activation of mtROS-PERK pathway (16). Recently, several studies have reported that ferroptosis is also closely linked to inflammation- related signaling pathways (29, 85). Based on antioxidant properties of filbertone, we aimed to investigate whether filbertone ameliorates fatty liver- associated hepatic injury by reducing lipid peroxidation and oxidative stress -induced ferroptosis its mechanism.

2. Materials and method

2.1. Reagents and chemicals

Filbertone, Ferrostatin-1, and Palmitic acid (PA) were purchased from Sigma- Aldrich (St Louis, MO, USA). The ferroptosis inducer RSL3 from selleckchem (Houston, TX, USA).

2.2. Cell culture

AML12 (ATCC; CRL-2254) mouse hepatocyte cells were grown in Dulbecco's modified Eagle's medium (DMEM/F12; GIBCO, Grand Island, NY) and HepG2 (ATCC; HB-8065) human hepatocyte carcinoma cell lines were cultured in Minimum Essential Medium (MEM, GIBCO, Grand Island, USA) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin (P/S) (Gibco, Grand Island NY, USA). Cells were grown in humidified incubators, at 37°C with 5% CO2.

2.3. Animal experiment and organ collection

All animal care and procedures were conducted according to the protocols and guidelines approved by the University of Ulsan Animal Care and Use Committee (LYN-15-010). Nine-week-old male C57BL/6 mice were purchased from Koatech (Pyeongtaek South korea). Mice were maintained under specific pathogen-free conditions at 22 °C and given access to food and water ad libitum.

2.4. HFD- diet induced- liver injury and filbertone treatment.

To examine the effects of filbertone on ferroptosis-induced NAFLD in mice were adapted for 1 week, then randomly divided into three dietary groups (n = 9 per group) and fed for 16 weeks on (1) a low-fat diet (LFD; 10% calories from fat; Research Diet Inc., New Brunswick, NJ, USA); (2) a high-fat diet (HFD; 60% of calories from fat; Research Diets Inc.); and (3) the HFD supplemented with 0.2% filbertone. Body weight was measured weekly. After 16 weeks of feeding the mice were scarified and collected liver tissues, as well as serum were collected.

2.5. MCD- diet induced- liver injury and filbertone treatment.

For NASH mouse model, C57BL/6 mice were randomly divided into seven groups (n-5, in each group): Normal diet (NCD), methionine and choline-deficient (MCD) diet, MCD+ RSL3, MCD+RSL3+Filbertone, MCD+RSL3+Filbertone+GSK, MCD+RSL3+Ferostatin 1 and RSL3-treated groups. After 10 days of feeding the mice were scarified and collected liver tissues, as well as serum were collected. The

filbertone groups received 0.2% filbertone orally once daily via oral gavage. The RSL3 (10 mg/kg), Ferostatin1 (1mg/kg), and GSK (5 mg/kg) were injected daily intraperitoneal (i.p.).

2.6. WST-8 assay.

To investigate cell viability, Cell counting kit 8 (WST-8/ CCk8) assay was used. After reaching 70% confluence of AML12 and HepG2 cell in 96-well plates, the cells PA in concentration manner (100, 250, 500, 1000 μM) for 24 hrs. Also, pre-treated with filbertone (20μM) for 4hrs, ferrostatin 1 (1 μM) for 1hr followed by RSL3 (1 μM) and PA (250 μM) for 24hrs. And then, the medium was replaced with medium containing WST-8 (Biomax, Seoul, Korea) for 1h. The optical density of samples was measured at 450nm using spectrophotometer.

2.7. Western blot

Liver tissues and cells were lysed in RIPA buffer (Thermo Scientific, Waltham, MA, USA) containing phosphatase and protease inhibitors (Sigma-Aldrich), and the total protein concentration was determined by using BCA protein assay reagents (Pierce Biotechnology, Rockford, IL, USA). Prepared lysate sample were boiled in sample buffer containing β-mercaptoethanol for 5 min. An equivalent volume of proteins for all samples was subjected to electrophoresis and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Burlington, MA, USA). The membranes were blocked with 5% skim milk (BD bioscience, San Jose, CA, USA) in PBS containing 0.1% Tween 20 (PBS-T) for 30 min and incubated at 4 °C overnight with primary antibodies, as follows: anti–phospho-PERK (1:1000, Cell Signaling Technology), anti-PERK (1:1000, Cell Signaling Technology), anti–phospho-eIF2α (1:1000, Cell Signaling Technology), anti-eIF2α (1:1000, Cell Signaling Technology), anti-ATF4 (1:1000, Santa Cruz Biotechnology, Santa Cruz), HO-1 (1:1000, Enzo Life Sciences) or β-actin (1:2500, Cell Signaling Technology), GPX4 (1:2000, abcam), PTGS2 (1:1000, abcam), p-Nrf2 (1:5000, abcam), Nrf2 (1:1000, Invitrogen), 4-HNE (1:1000, abcam), MDA (1:1000, abcam), SLC7A11 (1:1000, Invitrogen). Membranes were washed with 1X PBS-T thrice for 10 mins and incubated secondary antibodies conjugated with horseradish peroxidase. Antibody binding was visualized by ECL chemiluminescence (Pierce Biotechnology) using Azure Biosystems C300 analyzer (Azure Biosystems, Dublin, CA, USA).

2.8. RNA isolation and RT-PCR

Total RNA was isolated from cells and liver tissues using TRIzol reagent (Invitrogen, CA, USA)

according to manufacturer's instructions. 2μg of total RNA was used to synthesize cDNA by using M-MLV reverse transcriptase (Promega Corporation, WI, USA). The following primers were-

Table II. RT-PCR primers-

Genes	Primer sequence
h GAPDH	F-ccacccatggcaaattccatggca, R-tctagacggcaggtcaggtccacc
h HO-1	F-cttcgcccctgtctacttcc, R-gtccttggtgtcatgggtca
m GAPDH	F- aggccggtgctgagtatgtc, R-tgcctgcttcaccttct
m PTGS2	F- ttccaaatgtcaaaaccgt, R-agtccgggtacagtcacactt),
m Hmox-1	F-tcccagacaccgctcctccag, R-ggatttggggctggtttc),
m GPX4	F-caggagccaggaagtaat, R-cagccgttcttatcaatgag),
m SLC7A11	F-gctcgtaatacgccctggag, R-ggaaaatctggatccgggca).

To analyze real-time quantitative PCR (RT-qPCR), the synthesized cDNA was amplified with SYBR Green qPCR Master Mix on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA).

Table III. qRT-PCR primers-

Genes	Primer sequence
h GAPDH	F-cggcctcaccccatttg, R-gggaagcccatcaccatct
h HO-1	F-caggagctgctgacccatga, R-agcaactgtcgccaccagaa)
h GPX4	F-acaagaacggctgcgtggtgaa, R-gccacacacttgtggagctaga)
h SLC7A11	F-tccctctattcggacccattta, R-ttcttctggtacaacttccagt
m SREBP 1c	F-tgcggctgttgtctaccata, R-tgctggagctgacagagaaa
m PPARg	F- tgtggggataaagcatcagg, R-ccggcagttaagatcacacc
m HSL	F-aggcctcagtgtgaccgcca, R-gccccacgcaactctgggtc
m ATGL	F-gccacagcgctggtcact, R-cctccttggacacctcaataatg
m GAPDH	F-cggcctcaccccatttg, R-gggaagcccatcaccatct
m PTGS2	F-ttccaatccatgtcaaaaccgt, R-agtccgggtacagtcacactt),

m GPX4	F-cctccccagtactgcaacag, R-ggctgagaattcgtgcatgg
m HO-1	F-tcagtcccaaacgtcgcggt, R-gctgtgcaggtgttgagcc).

2.9. Dual luciferase assay analysis

For dual luciferase assay, AML12 cells were grown in a 96-well plate, and then cells were co-transduced. After 72 h, cells were treated with filbertone at concentration (0, 5, 10, 20μM) for 8 h. Treated cells were lysed with passive lysis buffer (Promega, Fitchburg, WI, USA) and mixed with luciferase assay reagents (Promega). The chemiluminescent signal was detected using a SpectraMax L Microplate reader (Molecular Devices, Sunnyvale, CA). Firefly luciferase was normalized to Renilla luciferase in each sample.

2.10. Total glutathione (GSH) assay analysis

AML12 and HepG2 cells were treated with filbertone (20 μM) for 4hrs, ferrostatin 1 (1 μM) for 1hr followed by PA (250 μM) for 24hrs. The cellular GSH levels were assessed using the GSH-GSH/GSSG Ratio Detection Assay Kit (Abcam, ab138881) following the manufacturer's instructions. In brief, the GSH assay mixture was added to the whole-cell lysates for a one-step fluorometric reaction and incubated for 60 min while protected from light. Fluorescence was then monitored at EX/EM wavelengths of 490/520 nm, and GSH was calculated from the standard curve. The GSH concentration in each group was normalized to cell viability.

2.11. Statistical analysis

All experiments were conducted at least three times, and the data are presented as the mean ± standard error of the mean. All the data were analyzed using GraphPad Prism 9 (GraphPad Software, Inc.). Statistical analyses were performed using one-way ANOVA with Tukey's post hoc test, and Shapiro-Wilk test was performed as a test of normality. $P<0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Filbertone reverses cell death induced by RSL3

RSL3 is a vital inducer of ferroptosis. To investigate whether filbertone can inhibit RSL3- induced ferroptosis, we treated AML12 and HepG2 cell lines at indicated concentration of RSL3, in presence or absence of 20 mM filbertone. The results showed that filbertone inhibited RSL3-induced mortality in AML12 and HepG2 cells like cells treatment with ferroptosis inhibitors, ferrostatin-1, liproxstatin-1, and a-tocopherol (Figure 3.1A & 3.1B). GPX4 converts peroxide to the equivalent alcohol and prevents GPX4 from building up a significant amount of lipid peroxide, which is known to be a key contributing mechanism to ferroptosis (85). Several studies demonstrated that GPX4 levels decreased when cells undergo ferroptosis (86). As expected, RSL3 treatment markedly reduced GPX4 protein levels but was rescued by filbertone, whereas cells treated with filbertone decreased protein level of PTGS2, a ferroptosis marker (Figure 3.1C). The ferroptosis inhibitor, ferrostatin-1, was used as a positive control (Figure 3.1C). This study indicates that filbertone protects against RSL-induced ferroptosis.

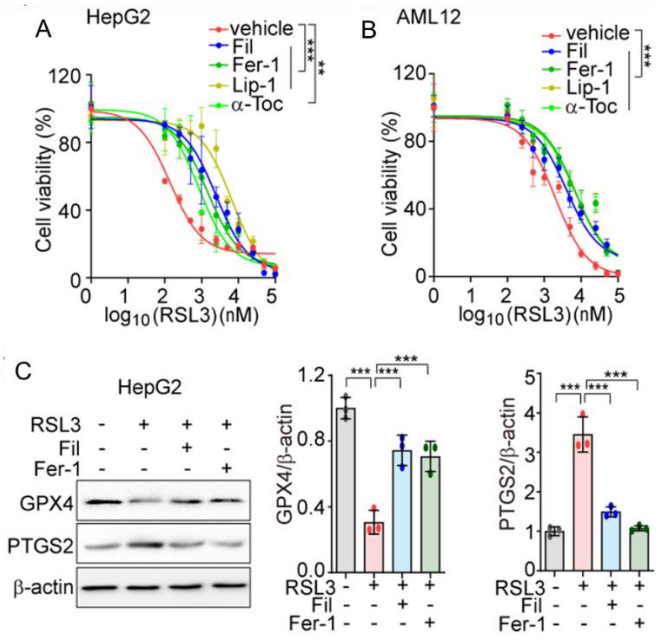

Figure 3.1. Filbertone reverses cell death induced by RSL3. (A, B) To evaluate RSL3-induced ferroptosis, HepG2 (A) and AML12 (B) cells treated with different concentration of RSL3 in the presence of filbertone (20 μM), ferrostatin-1 (1 μM), liproxstatin-1 (2 μM), or a-tocopherol (10 μM). Cell viability was measured by WST- 8 assay. HepG2 were incubated with filbertone (20 μM) or ferrostatin-1 (1 μM) for 1 h, and then treated with RSL3 for 24 h. (C) Quantification of protein levels of GPX4 and PTGS2 by western blotting. Data were expressed as mean ± SD; *p<0.05, **p<0.01, and ***p<0.001.

3.2. Filbertone reverses cell death induced by palmitic acid

Dietary palmitic acid (PA) induces liver inflammation in the early stage of NAFLD (77). Several studies demonstrated that palmitic acid induced ferroptosis (87). In accordance to find the filbertone protection against PA-induced ferroptosis, we tested cell viability using PA in a dose-dependent manner, it was found that cell toxicity increased with dose (0, 100, 250, 500, 1000 μM) in AML12 and HepG2 cells. We found that treatment with PA enhanced the cytotoxic effect in both the cell lines, indicated increase in cell death from 250 μM concentration (Figure 3.2A). Next, we tested cell viability using PA co-treated with filbertone and ferroptosis inhibitors, ferrostatin-1, liproxstatin-1, and a-tocopherol in AML12 and HepG2 cells. We found that filbertone and other inhibitors reduced the cell toxicity, compared PA alone (Figure 3.2B & 3.2C). Collectively, these findings strongly suggest that filbertone protects against PA-induced ferroptosis in AML12 and HepG2 cell lines.

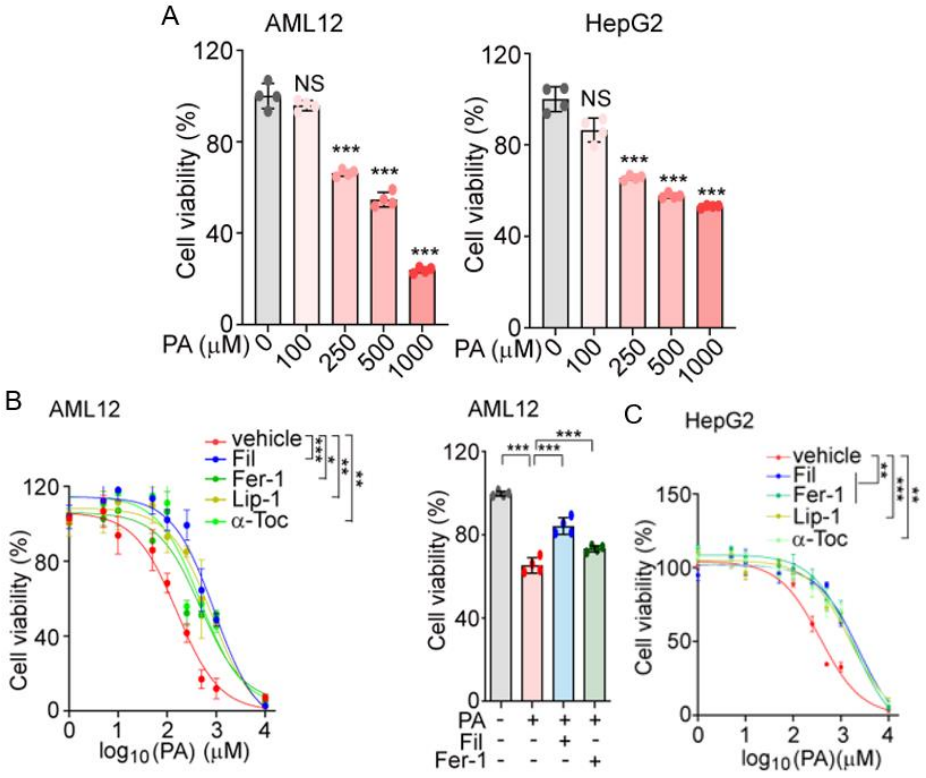

Figure 3.2. Filbertone reverses cell death induced by palmitic acid. (A) AML12 (left) and HepG2 (right) cells were treated with palmitic acid (PA) at the indicated concentrations (0, 100, 250, 500, and 1000 μM) for 24 h, and cell viability was determined by WST-8 assay**.** AML12 (B) and HepG2 (C) cells were pretreated with 20μM filbertone or ferroptosis inhibitor, such as 1 mM ferrostatin-1, 2 μM liproxstatin-1, and 10 μM a-tocopherol, for 1h and then treated with palmitic acid (PA). Cell viability of HepG2 and AML12 cells was measured by WST-8 assay. Data represent mean ± SD; **p*<0.05, ***p*<0.01, and ****p*<0.001.

3.3. Filbertone regulates antioxidant enzyme genes expression in AML12 and HepG2 cells

Our previous study reported that filbertone induces PERK phosphorylation in SH-SY5Y cells (16). Several studies reported that activation of PERK induces Nrf2 activation and transcriptional target genes which involves GPX4, HO-1 and SLC7A11 (84). Our study found that filbertone dose-dependently increased the mRNA and protein expression of ferroptosis-related genes, such as GPX4, HO-1, and SLC7A11 in AML12 and HepG2 cells (Figure 3.3A, 3.3B & 3.3C). Consistent with our previous observation (16), we found that filbertone activated PERK signaling pathway, as determined by increased phosphorylation of PERK and eIF2α, and expression of ATF4 levels in AML12 and HepG2 cells (Figure 3.3D & 3.3E). Altogether, this study showed filbertone upregulates antioxidant genes in hepatocytes.

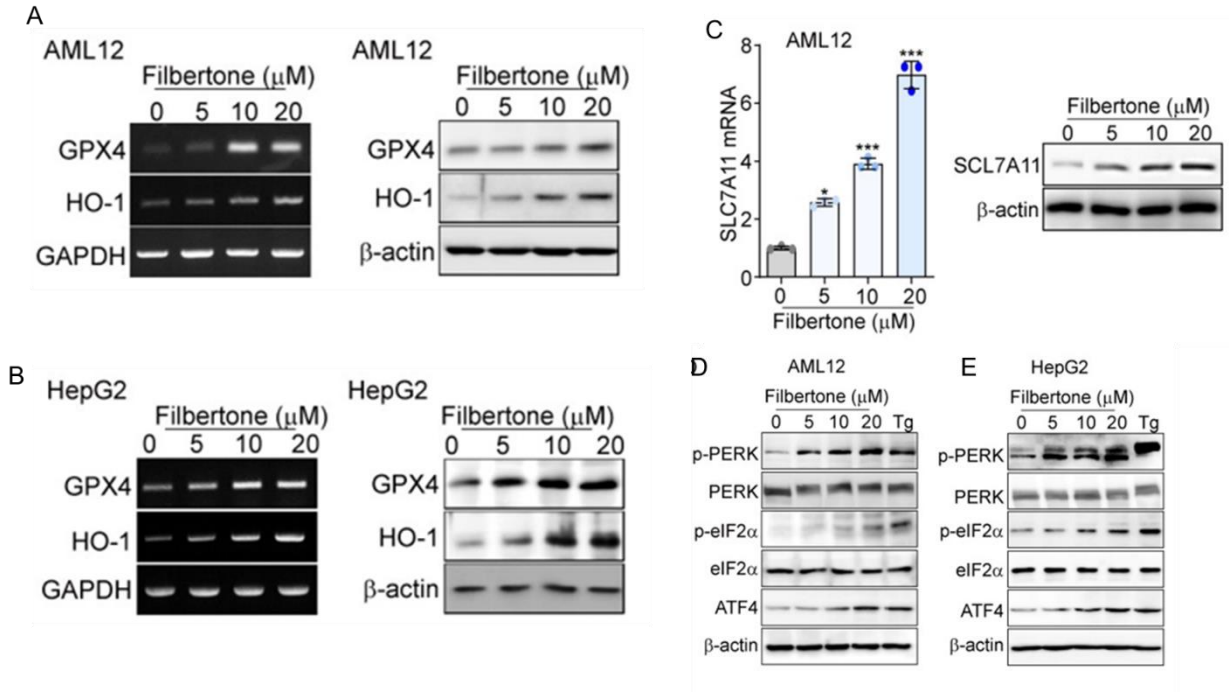

Figure 3.3. Filbertone regulates antioxidant enzyme genes expression in AML12 and HepG2 cells. (A-C) AML12 (A) and HepG2 (B) cells were treated with filbertone (0, 5, 10, and 20 μM) for 4 h. The levels of GPX4 and HO-1 mRNA expression were evaluated by RT-PCR, and the protein expression of GPX4 and HO-1 was measured by western blotting. The levels of mRNA and protein expression of SLC7A11 levels in AML12 cells treated with filbertone were detected by qRT-PCR (*left*) and western blotting (*right*), respectively (C). (D) AML12 (*left*) and HepG2 (*right*) cells were treated with filbertone (0, 5, 10, and 20 μM) for 4 h and 1 μM thapsigargin (Tg) for 1 h. Thapsigargin was used as a positive control. The protein expression of p-PERK, PERK, p-eIF2α, eIF2α, and ATF4 was detected by western blotting. Data represent mean ± SD; *p<0.05 and ***p<0.001.

3.4. Filbertone suppress PA-induced ferroptosis via increasing GPX4 and HO-1 expression in vitro.

Glutathione (GSH) is a potent antioxidant against ferroptosis. GSH is synthesized through the condensation of three amino acids: glutamate (Glu), cysteine (Cys), and glycine (Gly), and serves as the primary cofactor for the enzyme glutathione peroxidase 4 (GPX4) (88, 80). Also, it plays a role in the inhibition of lipid peroxidation driven ferroptosis (88, 89). To investigate the role of filbertone in the expression of glutathione (GSH) in AML12 and HepG2 cells co-treated with PA. As expected, filbertone increased total GSH level decreased by PA alone, ferrostatin-1, used as positive control (Figure 3.4A & 3.4D). Additionally, we evaluated the effect of filbertone co-treated with PA, as expected, the mRNA and protein expression levels of GPX4, HO-1, was increased by filbertone and while downregulating PTGS2 expression in AML12 and HepG2 cells (Figure 3.4B & 3.4C). Moreover, in HepG2 cell filbertone rescued from increased cell toxicity by PA, ferrostatin-1 was used as positive control (Figure 3.4E). These data showed filbertone effectively prevents PA-induced ferroptosis in hepatocytes.

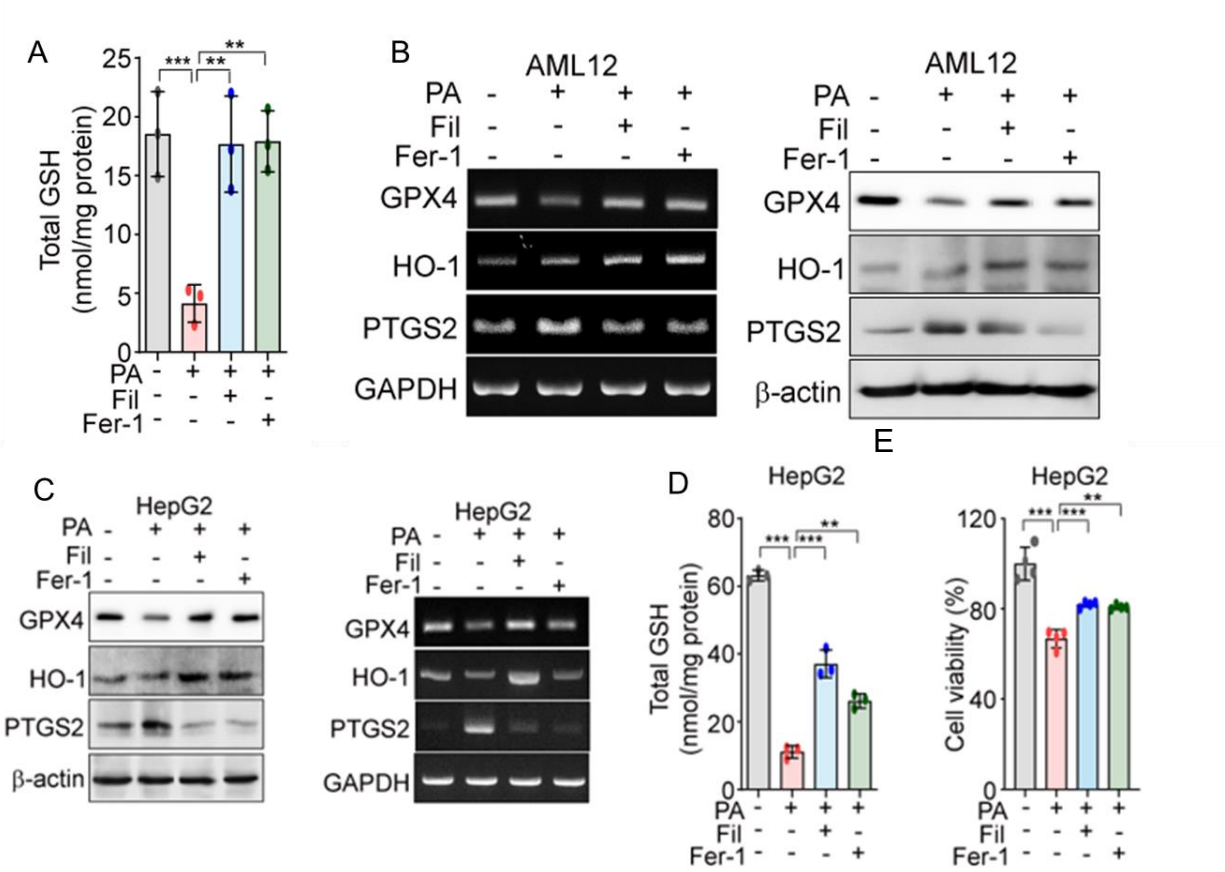

Figure 3.4. Filbertone suppress PA-induced ferroptosis via increasing GPX4 and HO-1 expression in vitro. HepG2 and AML12 cells were pretreated with 20 μM filbertone and 1 μM ferrostatin-1 followed by treated with 250 μM PA. Total GSH level in AML12 (A) and HepG2 (D) cells were measured. (B, C) The mRNA and protein levels of GPX4, PTGS2, and HO-1 were determined by RT-PCR and western blot in AML12 (B) and HepG2 (C). GAPDH and β-actin were used as internal controls. HepG2 (E) cells were pretreated with 20 μM filbertone or ferroptosis inhibitor, such as 1 μM ferrostatin-1, 2 μM liproxstatin-1, and 10 μM a-tocopherol, for 1h and then treated with palmitic acid (PA). Cell viability of HepG2 and AML12 cells was measured by WST-8 assay. Data represent mean ± SD; **p*<0.05, ***p*<0.01, and ****p*<0.001.

3.5. Filbertone requires PERK activation for ferroptosis suppression in hepatocytes

The Protein Kinase R-like Endoplasmic Reticulum Kinase (PERK) serves as an endoplasmic reticulum resident and plays a vital role in enhancing signal transduction mechanisms under Endoplasmic Reticulum (ER stress). Also, it is widely recognized as a key mediator of ER stress (90). Our above data showed filbertone activate PERK phosphorylation and its downstream targets in AML12 and HepG2 cells (Figure 3.5D & 3.5E). To clarify whether the expression of ferroptosis-related genes, such as GPX4, SLC7A11, and HO-1, was related to the activation of PERK by filbertone, we measured the mRNA and protein expression levels of GPX4, SLC7A11, HO-1. Cells were treated with or without filbertone and PERK inhibitor, GSK2606414. The upregulation of GPX4, SLC7A11, and HO-1 genes in response to filbertone was observed, indicating the activation of PERK, whereas, abolished by co-treated with GSK in AML12 (Figure 3.5A & 3.5B) and HepG2 (Figure 3.5C & 3.5D) cells. Taken together, these results demonstrate that filbertone activates PERK in hepatocytes to reduce ferroptosis.

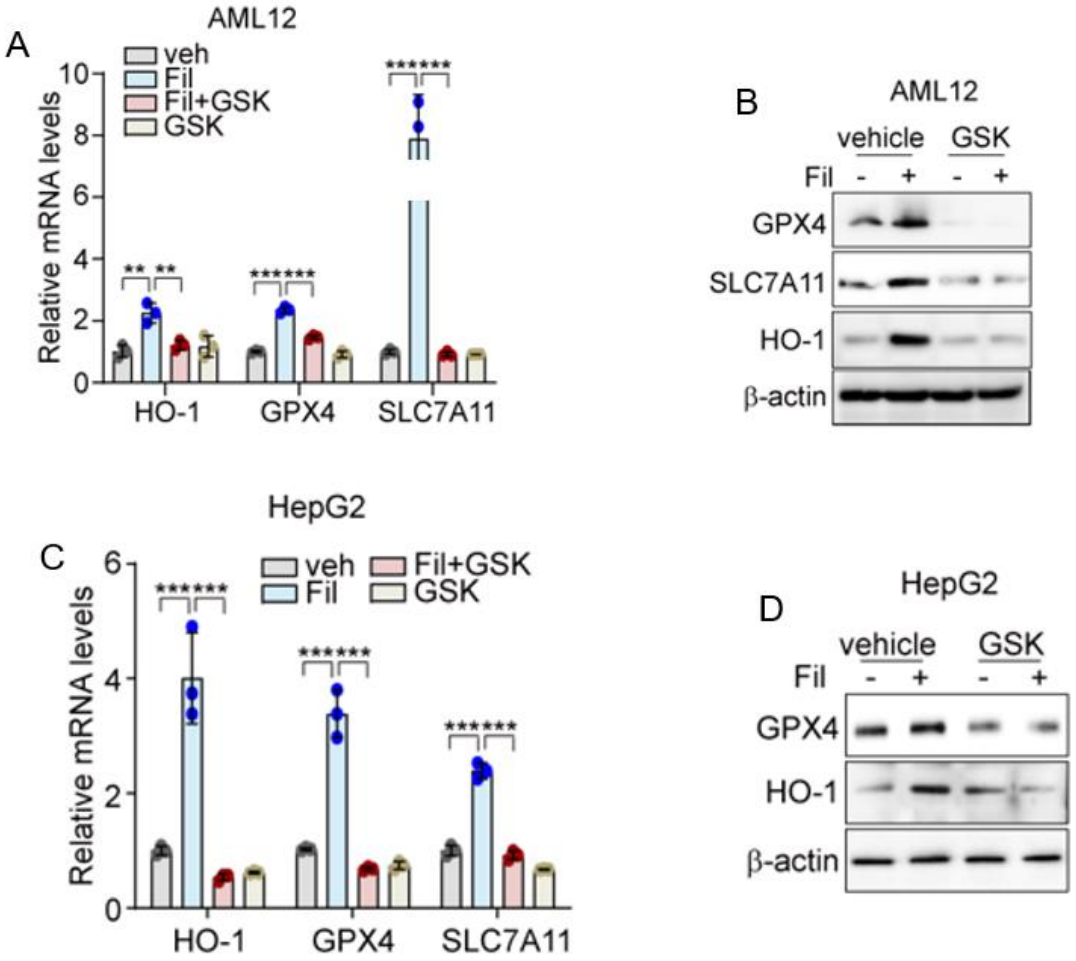

Figure 3.5. Filbertone requires PERK activation for ferroptosis suppression in hepatocytes. To evaluate activation of PERK activation in AML12 (A, B) and HepG2 (C, D) cells were treated with filbertone (20 μM) and GSK2606414 (1 μM) prior to treatment with RSL3 (1 μM) for 24 h. Upon treatment, cell lysates were analyzed by western blotting with the indicated antibodies. The mRNA and protein expression of GPX4, SLC7A11, and HO-1 were measured by qRT-PCR (A) and western blotting (B), respectively, in AML12 cells. The mRNA and protein expression of GPX4, SLC7A11 and HO-1 were measured by qRT-PCR (C) and western blotting (D), respectively, in HepG2 cells. Data represent mean ± SD; $^{**}p<0.01$ and $^{***}p<0.001$.

3.6. Filbertone triggers PERK activation in hepatocytes via mtROS production in hepatocytes

Mitochondria are crucial sources of ROS production in the cellular environment. According to several research, mtROS plays a key role in inducing the Nrf2 signaling pathway and the interaction between mitochondrial stress and ER stress to activate PERK (91, 90). To determine whether filbertone is involved in mtROS production, we treated AML12 and HepG2 cells with the specific mitochondria-targeted antioxidant, Mito Tempo. As shown in figure 3.6A-C, the activation of p-PERK and subsequent increase in mRNA and protein expression levels of ferroptosis-related genes GPX4, SLC7A11, HO-1 were found to be suppressed under treatment with mito tempo. These results suggest filbertone stimulates the activation of PERK through mtROS production.

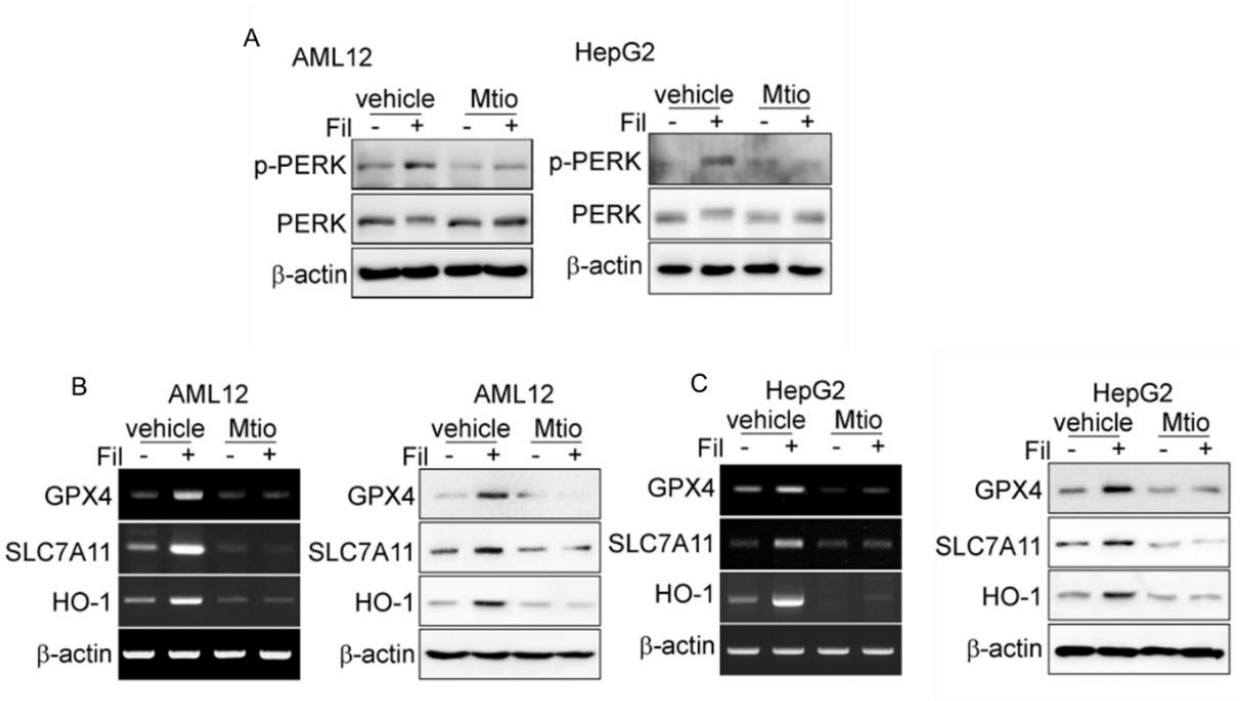

Figure 3.6. Filbertone triggers PERK activation in hepatocytes via mtROS production in hepatocytes. (A - C) AML12 and HepG2 cells were treated for 4 h with filbertone (20 μM) for 4h in the presence or absence of MitoTEMPO (100 nM). (A) Phosphorylated form of PERK was detected by western blotting in AML12 and HepG2 cells. The expression of GPX4, SLC7A11 and HO-1 mRNA and protein levels in AML12 (B) and HepG2 (C) cells. Data represent mean ± SD; **p<0.01 and ***p<0.001.

3.7. Filbertone activates Nrf2 phosphorylation via PERK in hepatocyte

PERK activation can promote Nrf2 nuclear translocation and subsequently increases antioxidant genes expression against ferroptosis (92, 93). We investigated whether Nrf2 activation is regulated by filbertone in a PERK-dependent manner. The activity of ARE luciferase was increased by filbertone in a dose-dependent manner (Figure 3.7A). Likely, filbertone also enhanced the Nrf2 phosphorylation in AML12 and HepG2 cells (Figure 3.7B).

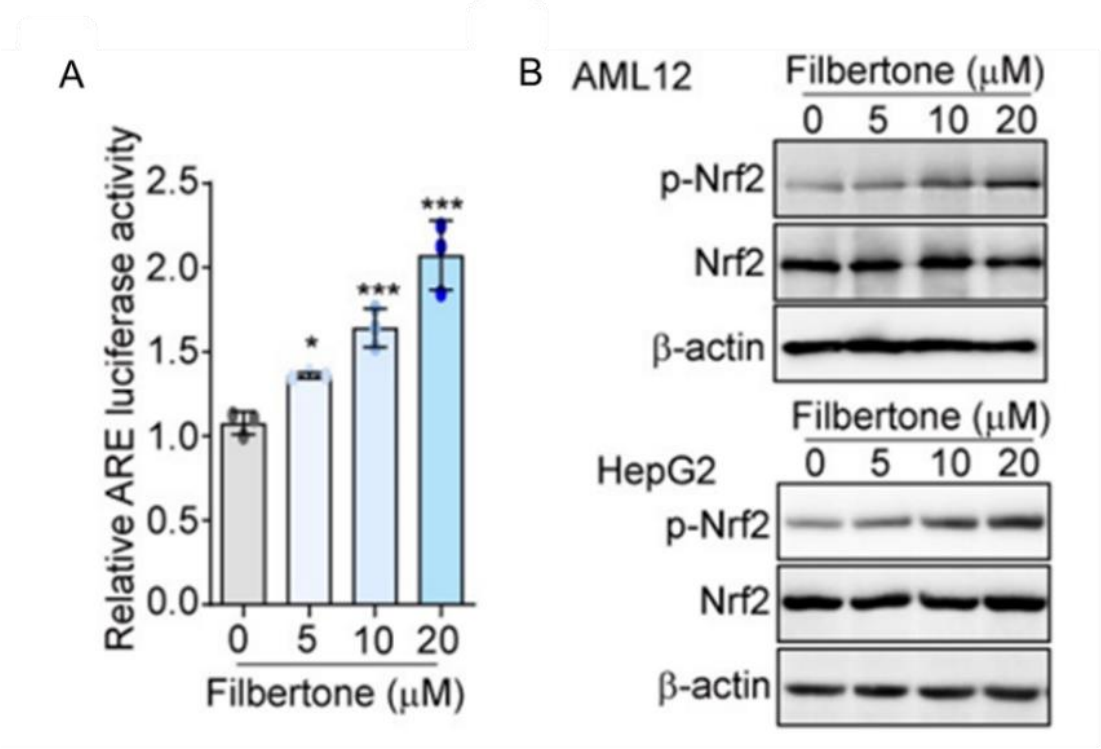

Figure 3.7. Filbertone activates Nrf2 phosphorylation via PERK in hepatocyte. (A) AML12 cells were transfected with pGL4 plasmid containing the antioxidant response element and then treated with filbertone (0, 5, 10, and 20 μM) for 4 h. After 3 h, luciferase activity was determined. (B) AML12 (*upper*) and HepG2 (*lower*) cells were treated with filbertone in the indicated concentrations (0, 5, 10, and 20 μM) for 4 h. The levels of phosphorylated Nrf2 and total Nrf2 in these cells, as determined using western blotting. Data represent mean ± SD; **p*<0.05, ***p*<0.01, ****p*<0.001, and NS, not significant.

3.8. Filbertone-induced Nrf2 phosphorylation reduced through PERK inhibition

To test whether PERK was required as the key mediator of phosphorylation and nuclear translocation of Nrf2, we investigated Nrf2 phosphorylation after filbertone treatment in AML12 and HepG2 cells in presence of PERK inhibitor, GSK2606414. We found that filbertone increased Nrf2 phosphorylation and translocation was inhibited by GSK (Figure 3.8A & 3.8B), indicating filbertone regulates Nrf2 activity through PERK. Altogether, the data confirmed that the protective activity of filbertone in hepatocytes is associated with PERK-Nrf2 pathway.

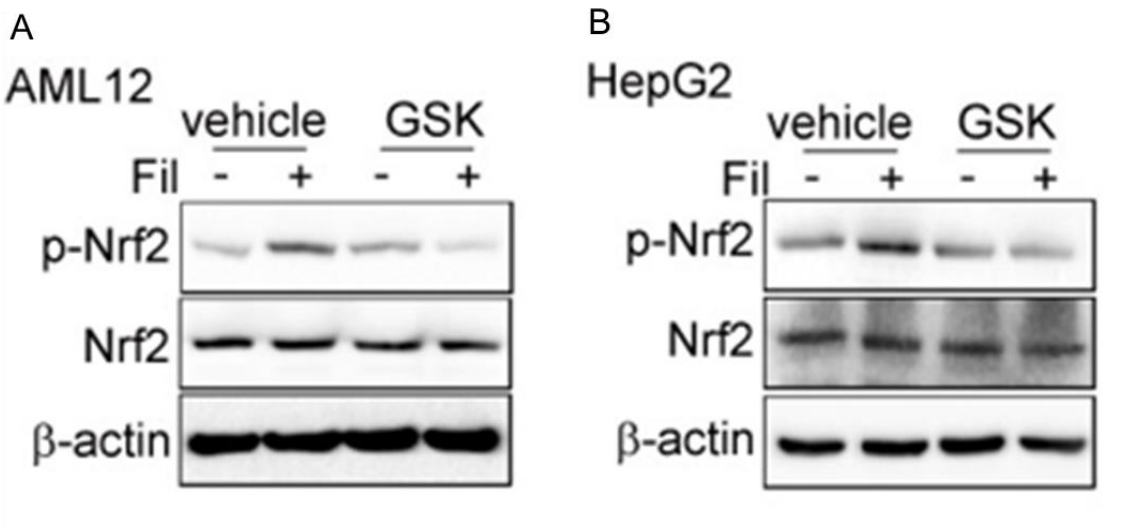

Figure 3.8. Filbertone-induced Nrf2 phosphorylation reduced through PERK inhibition. Cell lysates were evaluated for Nrf2 phosphorylation by western blot. (A) AML12 and (B) HepG2 cells were treated with 20 μM filbertone in the presence or absence of the PERK inhibitor GSK2606414 (1 μM). Phosphorylation of Nrf2 was detected by western blotting. Data represent mean ± SD; **p*<0.05, ***p*<0.01, ****p*<0.001, and NS, not significant.

3.9. Filbertone enhances HO-1, GPX4, and reduces PTGS2, 4-HNE expressions in vivo

To evaluate the effect of filbertone administration on high-fat diet -induced liver damage and ferroptosis. After 16 weeks of feeding, HFD mice had higher body weight than normal diet, whereas HFD+ filbertone fed mice were reduced significantly. Additionally, the HFD-fed mice demonstrated increased liver damage measured by liver injury markers alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which was reduced by filbertone administration (confirmed by other lab members, unpublished). Next, we clarified whether filbertone regulates ferroptosis- related genes, the result showed the mRNA and protein expression level of GPX4, SLC7A11, HO-1 (Figure 3.9A & 3.9B) and protein level of Nrf2 (Figure 3.9B) were upregulated in HFD+ Filbertone group, comparatively to HFD alone. While reduced PTGS2 (a ferroptosis marker) level (Figure 3.9A & 3.9B). Moreover, filbertone administration decreased 4-HNE (a lipid peroxidation end-product) protein expression, indicating filbertone maintains lipid ROS in vivo (Figure 3.9B). Taken together, these results demonstrated that filbertone supplementation improves HFD-fed fatty liver in mice.

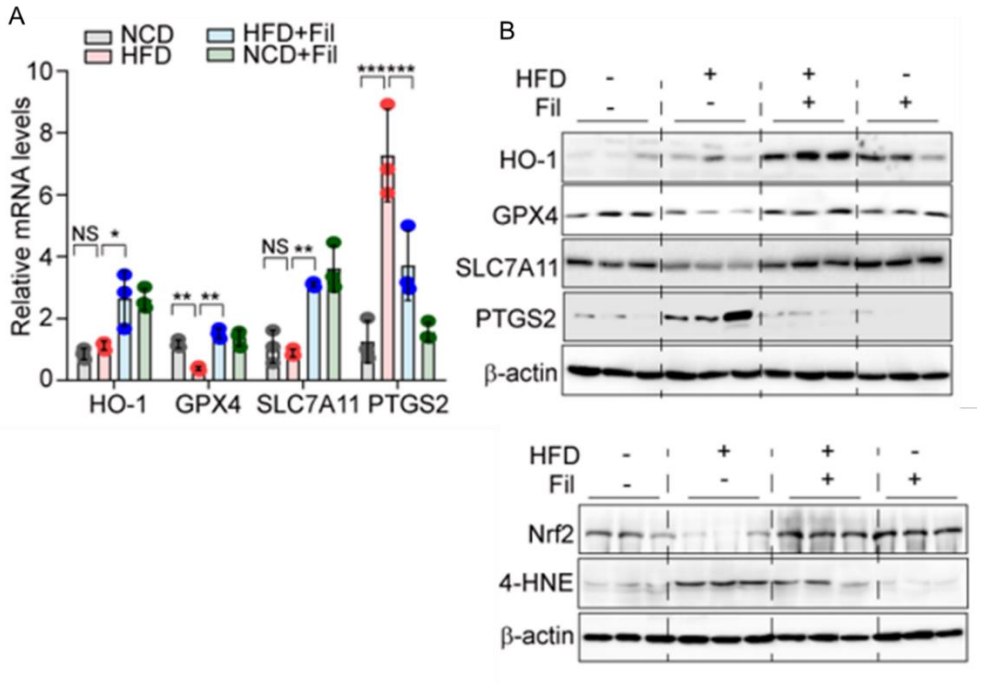

Figure 3.9. Filbertone enhances HO-1, GPX4, and reduces PTGS2, 4-HNE expressions in vivo. To verify NAFLD amelioration exhibited by filbertone, wild type mice (*n*=5) were fed normal chow diet (NCD) or 60% high fat diet (HFD) for 16 weeks in the presence or absence of 0.2% (v/w) filbertone. The mRNA (A) and protein (B) expression levels of HO-1, GPX4, SLC7A11, and PTGS2 in liver tissues were determined by qRT-PCR (A) and western blotting (B). The expression of key regulator of antioxidant gene Nrf2 and ferroptosis marker 4-HNE in liver tissues was detected by immunoblotting. Data represent mean ± SD; **p*<0.05, ***p*<0.01, ****p*<0.001, and NS, not significant.

3.10. Filbertone reduces ferroptosis in MCD-induced NASH in vivo

Fatty liver extends to a variety of conditions, ranging from reversible symptoms to the more severe non-alcoholic steatohepatitis (NASH), which is characterized by the presence of hepatic steatosis. Next, we verified the protective role of filbertone on MCD-diet fed mice model. The protein (Figure 3.10A) and mRNA (Figure 3.10D) expression of PTGS2 were significantly increased in MCD+ RSL3 and MCD+ GSK (PERK inhibitor) groups compared to normal diet, showing induced ferroptosis, while reduced by filbertone administrated group with MCD, RSL3 and GSK. Additionally, filbertone supplementation increased the antioxidant genes GPX4, HO-1 and SLC7A11 Protein (Figure 3.10B) and mRNA (Figure 3.10D) expression level. Moreover, ferroptosis was enhanced synergically by MCD and RSL3 together, confirmed by upregulated PTGS2 protein expression (Figure 3.10C). Altogether our results showed that filbertone is potent to improve the MCD- liver fibrosis in mice.

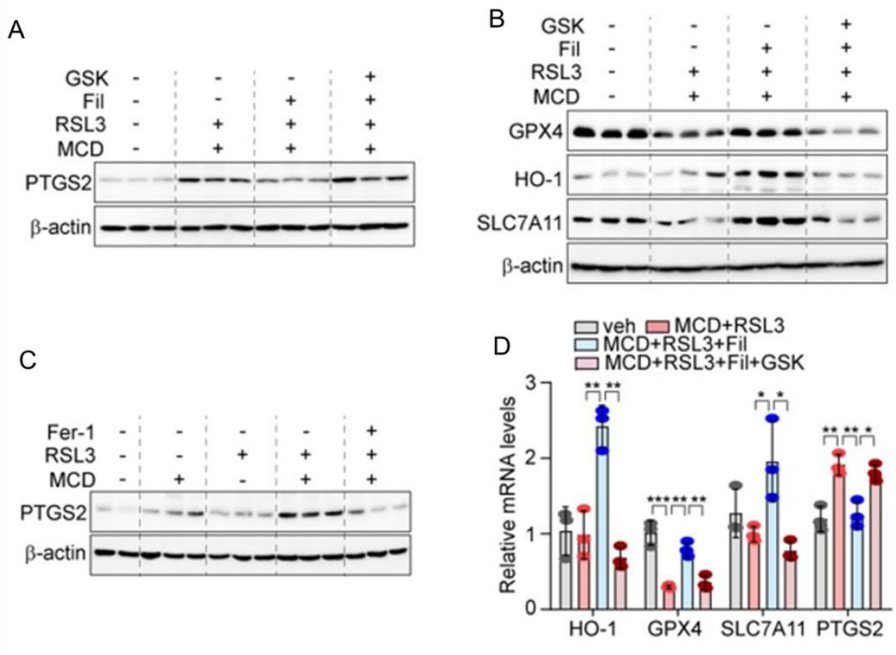

Figure 3.10. Filbertone reduces ferroptosis in MCD-induced NASH in vivo. Wild type mice were fed with NCD or methionine/choline deficient (MCD) diet for 10 days. The MCD-fed mice were administered RSL3 (10 mg/kg) in the presence or absence of filbertone (0.2%, v/w) or PERK inhibitor GSK2656157 (5 mg/kg) for 10 days. The levels of ferroptosis marker PTGS2 protein (A) and mRNA (D) expression were analyzed by qRT-PCR and western blotting. The protein (B) and mRNA (D) levels of antioxidant molecules, GPX4, HO-1, and SLC7A11, were evaluated by western blotting and qRT-PCR. Wild type mice were fed NCD or MCD diet and administrated RSL3 (10 mg/kg) in the presence or absence of ferrostatin-1 (1 mg/kg) for 10 days. (C) The levels of ferroptosis marker PTGS2 protein expression in liver tissue were determined by western immunoblotting. Data represent mean ± SD; *p<0.05, **p<0.01, ***p<0.001, and NS, not significant.

4. Discussion

Ferroptosis, a nonapoptotic necrotic cell death which is modulated and initiated by lipid peroxidation that is dependent on iron. Comparatively to other types of cell death, ferroptosis is defined by specific and fundamental characteristics such as reduced glutathione peroxidase 4 (GPX4) activity, phospholipid peroxidation, and the build-up of redox-active iron. GPX4 is recognized as the primary enzyme that prevents ferroptosis. The inhibition of GPX4 can induce ferroptosis, and the overexpression of GPX4 can inhibit iron-dependent lipid peroxidation induced by RSL3 (GPX4 antagonist) (94) and palmitic acid (87), thus inhibiting ferroptosis. Whereas Heme oxygenase-1 (HO-1), an antioxidant gene of Nrf2 also possesses cytoprotective features against stress-induced factors. In the present study, we found that filbertone mitigates ferroptotic cell death and lipid peroxidation induced by RSL3/PA. Subsequently, filbertone restored the expression level of GPX4, HO-1 and SLC7A11 antioxidants in hepatocytes. These results indicated that filbertone upregulates the antioxidant genes expression, lead to suppress ferroptotic cell death in hepatocytes.

Endoplasmic reticulum (ER) stress activation has been proven to reduce ferroptosis, thereby suppressing liver fibrosis (95,82). It triggers diverse patterns of cell death via three primary transmembrane receptors located on the ER membrane, namely IRE1α, PERK, and activating transcription factor 6 (ATF6). Several studies have demonstrated that phosphorylation of PERK is activated by the production of ROS, subsequently phosphorylate Nrf2, a master regulator of the antioxidant response (96, 97). To sustain cell viability, PERK activation inhibits the translation of common proteins, phosphorylates eIF2 at Ser51 and translates the transcription factor ATF4, therefore, regulates the genes expression involved in amino acid biosynthesis, oxidative stress, and cell death (98).

The ROS production is a core of oxidative stress and regulate cell death (80, 99). Also, mitochondrial dysfunction plays a crucial role in ferroptosis (19, 100), by controlling cell oxidative stress (48) as well as several other metabolic processes. The involvement of mitochondrial ROS (mtROS) production is implicated in the development of hepatic injuries (101, 102). In the canonical ER stress response, mtROS activation of PERK serves as a molecule that mediates the association between the ER and mitochondria. Our previous study found that filbertone activates mtROS-PERK pathway and ameliorates neurodegenerative disease (16). In hepatocyte, we found that filbertone treatment increased mtROS level. Subsequently, filbertone activating PERK phosphorylation and enhanced

antioxidant genes GPX4, SLC7A11 and HO-1, which was further inhibited by mito tempo, mitochondrial ROS inhibitor. These data suggested that filbertone activates the mtROS-PERK pathway accompanied by upregulation of antioxidant genes which lead to reduced hepatic ferroptosis.

Previous studies have also brought new insights into ferroptosis induced by ROS, leading to activating PERK-NRF2 signaling pathway (92), could play an essential role in reducing lipid peroxidation and ferroptosis. Through Nrf2 phosphorylation, PERK can also activate the expression of antioxidant genes including GPX4 (repairs lipids and converts toxic lipid hydroperoxides into non-toxic lipid alcohols), SLC7A11 (System xc- is an amino acid antiporter) and HO-1, which play the vital role in cell survival (67, 43). Importantly, these genes expression reduces the ROS production and improves lipid peroxidation and ferroptosis in several diseases such as, I/R injury, NAFLD, liver fibrosis, acute lung injury (103, 104). In this study, we found that filbertone activates the phosphorylation PERK, eIF2a and ATF4 expression level in PA- induced hepatocytes. Moreover, filbertone inducing upregulation of GPX4, HO-1 and SLC7A11 antioxidant genes expression was reduced by PERK inhibitor, GSK2606414, suggested that filbertone action dependents on PERK activation to inhibit ferroptosis in PA-induced hepatocytes. Also, filbertone promoted Nrf2 translocation to nucleus, and PERK-dependent phosphorylation of Nrf2. We also confirmed, the translocation of Nrf2 by filbertone, promoting antioxidant gene expression GPX4 and HO-1 and further reduced ferroptosis caused by lipid peroxidation (data not shown). Therefore, the results suggest that filbertone action is associated with PERK-Nrf2 phosphorylation and promoting antioxidant gene expression, hence inhibits hepatic ferroptosis.

Hepatic steatosis causes inflammation in the liver and cell injury, leading to the progression of fibrosis and cirrhosis. The hepatocyte impaired oxidative capacity of the mitochondria caused by lipid accumulation, increasing the reduced state of the electron transport chain (ETC) complexes, and stimulating the peroxisomal and microsomal pathways of fat oxidation. As a result, rising the production of reactive oxygen species (ROS) and reactive aldehydic derivatives leads to promote oxidative stress and cell death by, ATP, NAD, and glutathione depletion, DNA, lipid, and protein damage. Also, the presence of oxidative stress stimulates the production of inflammatory cytokines, leading to the development of inflammation and fibrogenic responses. Consequently, this results in the development of nonalcoholic steatohepatitis (NASH) and could trigger the end-stage liver disease.

Our in vitro studies confirmed the protective role of filbertone, against ferroptotic cell death and hepatic cell injury. Next, we questioned whether filbertone could alter hepatic injury in vivo. Excessive accumulation of lipids in hepatocytes lead to the development of ferroptosis, causing liver damage. Previous studies have demonstrated that filbertone reduces lipid accumulation and plasma levels of inflammatory cytokines, such as IL-6, TNF-α in HFD-fed mice (75, 72). The administration of a high-fat diet (HFD) and methionine–choline deficient (MCD) to animals typically leads to the development of obesity and metabolic concerns, particularly liver damage, lead to steatohepatitis. Previous research has provided evidence indicating that an HFD/MCD diet enhances hepatic lipid accumulation and triggers ferroptosis (39, 72). In this study, we investigated whether filbertone reduces the HFD/MCD diet- induced fatty liver induced-hepatic injury via inhibiting ferroptosis.

We observed that HFD/MCD fed mice increased liver injury, via elevating lipid peroxidation and ferroptosis. Moreover, filbertone supplementation improved HFD/MCD-induced lipotoxicity by inhibiting hepatic ferroptosis. Also, we found that filbertone upregulated GPX4, SLC7A11 and HO-1 antioxidant genes expression, while reducing the expression levels of PTGS2 and 4-HNE, ferroptosis marker, suggesting that filbertone potent beneficial effects on HFD/MCD- induced hepatic injury. These findings suggest that filbertone could ameliorate progression of NAFLD and NASH in HFD/MCD fed mice by inhibiting lipid peroxidation and hepatic ferroptosis.

Hepatic fibrosis is associated with lipid peroxidation accumulation in presence of fatty acids and high concentration of iron in hepatocytes (105). Inflammatory response, oxidative stress, lipotoxicity, and dysfunctional organelles causes ballooning and increase the death rate of hepatocyte cells (106). Ferroptosis has been demonstrated to enhance the progression of hepatic fibrosis via iron and ROS accumulation. Previous investigations have shown evidence that the inhibition of ferroptosis leads to improved hepatic injury via reducing lipid ROS level (95, 30) and downregulating fibrotic markers and inflammatory cytokines (75, 72). Although, our study confirmed that filbertone supplementation reduced lipid ROS in MCD-fed mice, however, the suppressive effect of filbertone on fibrotic marker and inflammatory cytokines is required further investigation.

In summary, we demonstrated the beneficial effects of filbertone against HFD/MCD diet induced fatty liver diseases in vivo and in vitro. Filbertone induces the production of mitochondrial ROS, leading to the phosphorylation of PERK and subsequent activation of Nrf2 nuclear translocation in lipid accumulated hepatocytes. Moreover, filbertone triggers mtROS-PERK-Nrf2 pathway and subsequent

upregulating downstream antioxidant genes expression levels, which directly impairs lipid peroxidation, thereby leading to the suppression of hepatic ferroptosis. Taken together, filbertone possesses protective effects to mitigate fatty liver diseases and its progress via impairing hepatic ferroptosis.

5. Conclusion

Our study demonstrated that the bioactive compound filbertone has a potential to protect hepatic injury in obesity induced-fatty liver conditions. In this study, we presented a novel mechanism by which filbertone can stimulate the activation of the PERK signaling pathway, via increasing mtROS, and promote the nuclear translocation of Nrf2 and antioxidant genes expression of GPX4, SLC7A11 and HO-1, which in turn reduced lipid peroxidation and finally decreases hepatic ferroptosis lipid-laden hepatocytes. Additionally, in HFD diet and MCD diet mice model, liver injury was ameliorated by filbertone supplementation. Collectively, we propose that filbertone could potentially serve as an innovative functional food component for the treatment of fatty liver diseases (Figure 6).

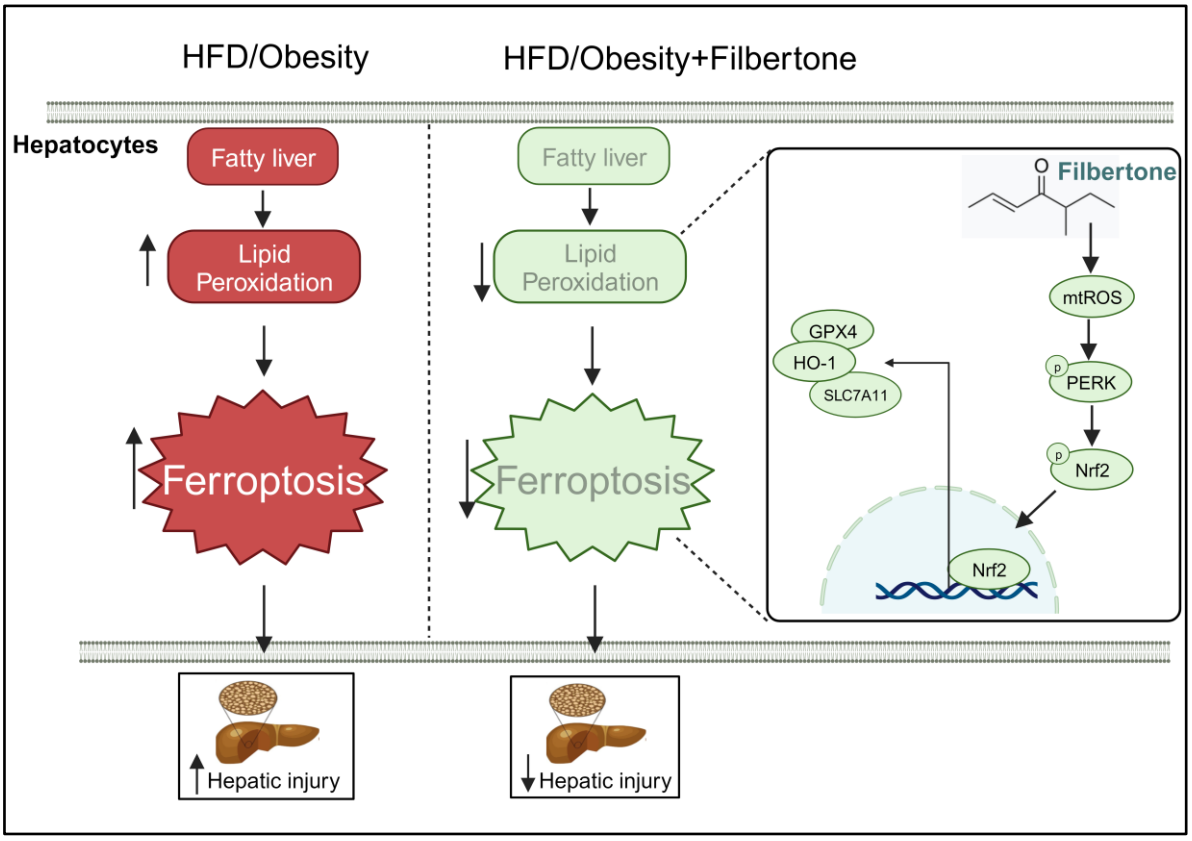

Figure 6. Schematic representation for the protective role of filbertone against ferroptosis in hepatocytes. Filbertone improves fatty liver- induced in HFD fed mice via inhibiting lipid peroxidation and reduces ferroptosis. Filbertone activates Nrf2 and antioxidant genes GPX4, SLC7A11, and HO-1 by regulating mtROS-dependent PERK phosphorylation. Hepatocyte lipid peroxidation is directly inhibited by these antioxidants, preventing ferroptosis.

CHAPTER III

Protective effect of filbertone against hepatocellular carcinoma by stimulating ferroptotic cell death

1.Introduction

Liver fibrosis is a key risk factor for the development of cirrhosis and/or hepatocellular carcinoma (HCC) that has become a major cause of chronic disease worldwide. Hepatocellular carcinoma (HCC) is a prevalent and highly fatal benign tumor and influenced by various underlying causes (107), which leads to abnormal activation of diverse cellular and molecular pathways and disturbances in the homeostasis between the expression and suppression of proto-oncogenes and tumor suppressor genes, respectively. Aside from that, surgical resection, radiofrequency ablation, molecular-targeted systemic therapy, and immunotherapy are employed as therapies for treating hepatocellular carcinoma (HCC) (108). Even so, HCC outlines an immense disease burden, with its incidence of rising trend, and requires further research.

Ferroptosis is an iron-dependent cell death relies on reactive oxygen species (ROS) accumulation (81, 29). It has also drawn interest as a potential inhibitor of many cancer types, such as breast cancer, colon cancer, and liver cancer, hence could act as a crucial approach in cancer treatment. Recent study has elucidated the importance of ferroptosis inducers such as RSL3, via regulating ferroptosis-related antioxidants, SLC7A11 (composed by glutamate cysteine antiport system X-c), in the treatment of hepatocellular carcinoma (109,110). SLC7A11 promotes transport of glutathione (GSH) and functions as an antioxidant within the cells (111, 112). Researchers have found a significant increase in the expression of SLC7A11 in liver cancer tissues compared to para-cancer tissues (113, 114). Whereas the high expression of SLC7A11 in HepG2 cells suggests that inhibiting its activity could potentially serve as an effective clinical treatment for hepatocellular carcinoma (HCC). Recent study has also determined that YAP/SLC7A11 signaling modulates ferroptosis in HCC (45). Moreover, SLC7A11/YAP suppression could serve as a novel biomarker, may improve chemotherapy sensitivity and patient prognosis (115, 116, 47,117), suggested that SLC7A11/YAP might serve as effective strategy for hepatocellular carcinoma treatment.

The National Cancer Institute's (NCCN) hepatobiliary cancer recommendations endorse Sorafenib and Lenvatinib as the optimal initial pharmacological treatment options. These drugs have been found to effectively induce ferroptosis in hepatocellular carcinoma (HCC) (118, 49). Natural bioactive products are also recognized as beneficial assets for the discovery of new drugs, and some of them have demonstrated to have clinical efficacy in treating cancers and neurological diseases, either as dietary supplements or pharmaceutical agents (119). Naturally occurring traditional Chinese medicine, such as

Atractylodin (49) and Saponin formosanin C (19), and several phytochemical compounds including apigenin (120), curcumin (121, 122), (many more) have drawn notable interest as ferroptosis inducers in prevention and treatment of several cancer cells (123). Additionally, increasing studies have shown that some natural compounds, including saponins, flavonoids, and isothiocyanates, possess the potential to either stimulate or inhibit ferroptosis bidirectionally. In this chapter, we investigated the potential role of filbertone in modulating ferroptosis in cancer cells, and its underlying mechanisms involved.

2. Materials and method

2.1. Reagents and chemicals

Filbertone, and Ferrostatin-1, were purchased from Sigma- Aldrich (St Louis, MO, USA). The ferroptosis inducer RSL3 from selleckchem (Houston, TX, USA).

2.2. Cell culture

HepG2 (ATCC; HB-8065) human hepatocyte carcinoma cell lines were cultured in Minimum Essential Medium (MEM, GIBCO, Grand Island, USA), B16F10 (ATCC; CRL-6475) mouse melanoma cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Grand Island, USA), supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin (P/S) (Gibco, Grand Island NY, USA). Cells were grown in humidified incubators, at 37°C with 5% CO2.

2.3. WST-8 assay

To investigate cell viability, Cell counting kit 8 (WST-8/ CCk8) assay was used. After reaching 70% confluence of HepG2 and B16F10 cell lines in 96-well plates, the cells filbertone in concentration manner (5, 10, 20, 40 μM) for 24 hrs. Also, filbertone (40 μM), ferrostatin 1 (1 μM) and RSL3 (1 μM) co-treated for 24hrs. And then, the medium was replaced with medium containing WST-8 (Biomax, Seoul, Korea) for 1h. The optical density of samples was measured at 450nm using spectrophotometer.

2.4. Western blotting

Cells were lysed in RIPA buffer (Thermo Scientific, Waltham, MA, USA) containing phosphatase and protease inhibitors (Sigma-Aldrich), and the total protein concentration was determined by using BCA protein assay reagents (Pierce Biotechnology, Rockford, IL, USA). Prepared lysate sample were boiled in sample buffer containing β-mercaptoethanol for 5 min. An equivalent volume of proteins for all samples was subjected to electrophoresis and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Burlington, MA, USA). The membranes were blocked with 5% skim milk (BD bioscience, San Jose, CA, USA) in PBS containing 0.1% Tween 20 (PBS-T) for 30 min and incubated at 4 °C overnight with primary antibodies, as follows: PTGS2 (1:1000, abcam), SLC7A11 (1:1000, Invitrogen), p-YAP (1:1000, abcam), YAP (1:1000, cell signaling). Membranes were washed with 1X PBS-T thrice for 10 mins and incubated secondary antibodies conjugated with horseradish peroxidase. Antibody binding was visualized by ECL chemiluminescence (Pierce Biotechnology) using Azure

Biosystems C300 analyzer (Azure Biosystems, Dublin, CA, USA).

2.5. RNA isolation and RT-PCR

To analyze real-time quantitative PCR (RT-qPCR), the synthesized cDNA was amplified with SYBR Green qPCR Master Mix on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA). The primer sequence were shown in Table II.

2.6. Measuring Lipid ROS

B16F10 cells were pre-treated with ferrostatin-1 (1 μM) for 1 h and lysophosphatidic acid (LPA, a YAP activator) (20 μM) for 3 hrs, followed by filbertone (40 μM) for 24 hrs. After treatment, cells were stained with 1.5 μM C-11 BODIPY 581/591 (Invitrogen, Carlsbad, CA, USA) at 37^{o}C for 30 min. Lipid ROS was detected by using Olympus FV1200 confocal microscope (Olympus, Tokyo, Japan), followed by fixing cells with formalin solution for 30 min and then washed three times with 1X PBS. The cells were stained with DAPI (Invitrogen).

2.7. Statistical analysis

All experiments were conducted at least three times, and the data are presented as the mean ± standard error of the mean. All the data were analyzed using GraphPad Prism 9 (GraphPad Software, Inc.). Statistical analyses were performed using one-way ANOVA with Tukey's post hoc test, and Shapiro-Wilk test was performed as a test of normality. $P<0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Filbertone induces ferroptotic cell death in HepG2 cells

Recently, multiple studies have accumulated considerable evidence suggesting that ferroptosis serves as a tumor suppressor, with significant influence on the cycle, proliferation, and progression of cancer (125, 10). In our earlier investigation, we proved the protective effects of filbertone (different time and concentration treated) in HepG2 cell line by inhibiting ferroptosis. Consequently, we sought to further investigate and solidify evidence supporting the protective role of filbertone in a tumor model. To confirm whether filbertone induces ferroptosis in HepG2 cell line, using WST-8 assay cell toxicity was examined. We found that filbertone treatment increased cell toxicity (Figure 3.1A), which was then prevented by the administration of ferrostatin-1 (Figure 3.1B). Therefore, the findings of these results demonstrate that filbertone can induce ferroptosis in HepG2 cells.

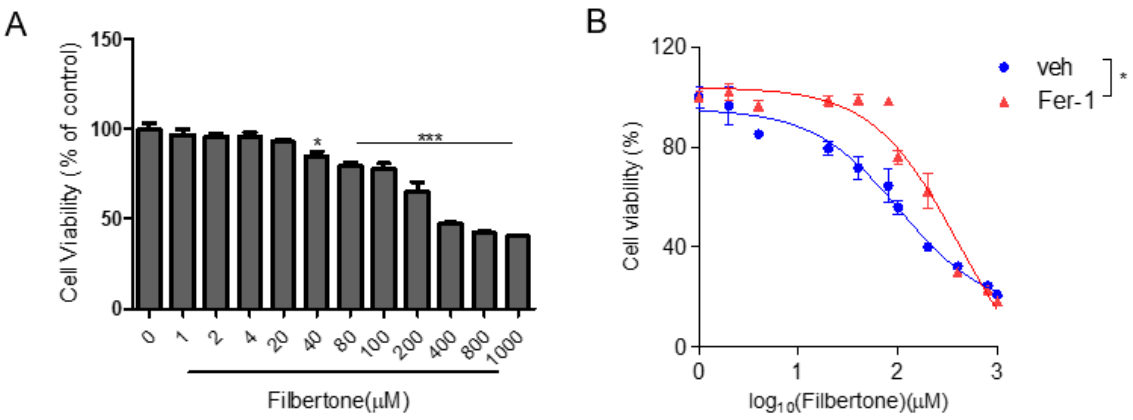

Figure 3.1. Filbertone induces ferroptotic cell death in HepG2 cells. (A, B) To evaluate filbertone-induced ferroptosis, HepG2 cells were treated with filbertone at various concentration and ferrostatin-1(1 μM) for 24 h. Cell viability was measured by WST- 8 assay. Data were expressed as mean ± SD; *p<0.05, **p<0.01, and ***p<0.001.

3.2. Filbertone triggers ferroptotic cell death in B16F10

Next, to define whether filbertone enhance cell death on B16F10 melanoma cell line. We found that cell death enhanced by filbertone was further reversed by ferrostatin-1 (Figure 3.2A), whereas cells co-treated with filbertone and RSL3 induced the cell toxicity (Figure 3.2B). Further, cells were treated with filbertone at various concentration (0, 5, 10, 20, 40 μM) to detect cell viability using WST-8 assay. As shown in Figure 3.2C, no cellular toxicity was observed with the treatment of filbertone at doses until 20μM and increased to 40μM in B16F10. We therefore used filbertone at a concentration of 40μM in subsequent experiments. To identify whether ferroptosis is responsible in the increased cell death observed during filbertone treatment in both cell lines. Further we investigated the cell toxicity with co-treatment with ferrostatin-1, ferroptosis inhibitor. We found the cell toxicity increased by filbertone, was rescued by ferrostatin-1 in B16 F10 (Figure 3.2D). Altogether, these results suggested that filbertone cause cell death in melanoma cell.

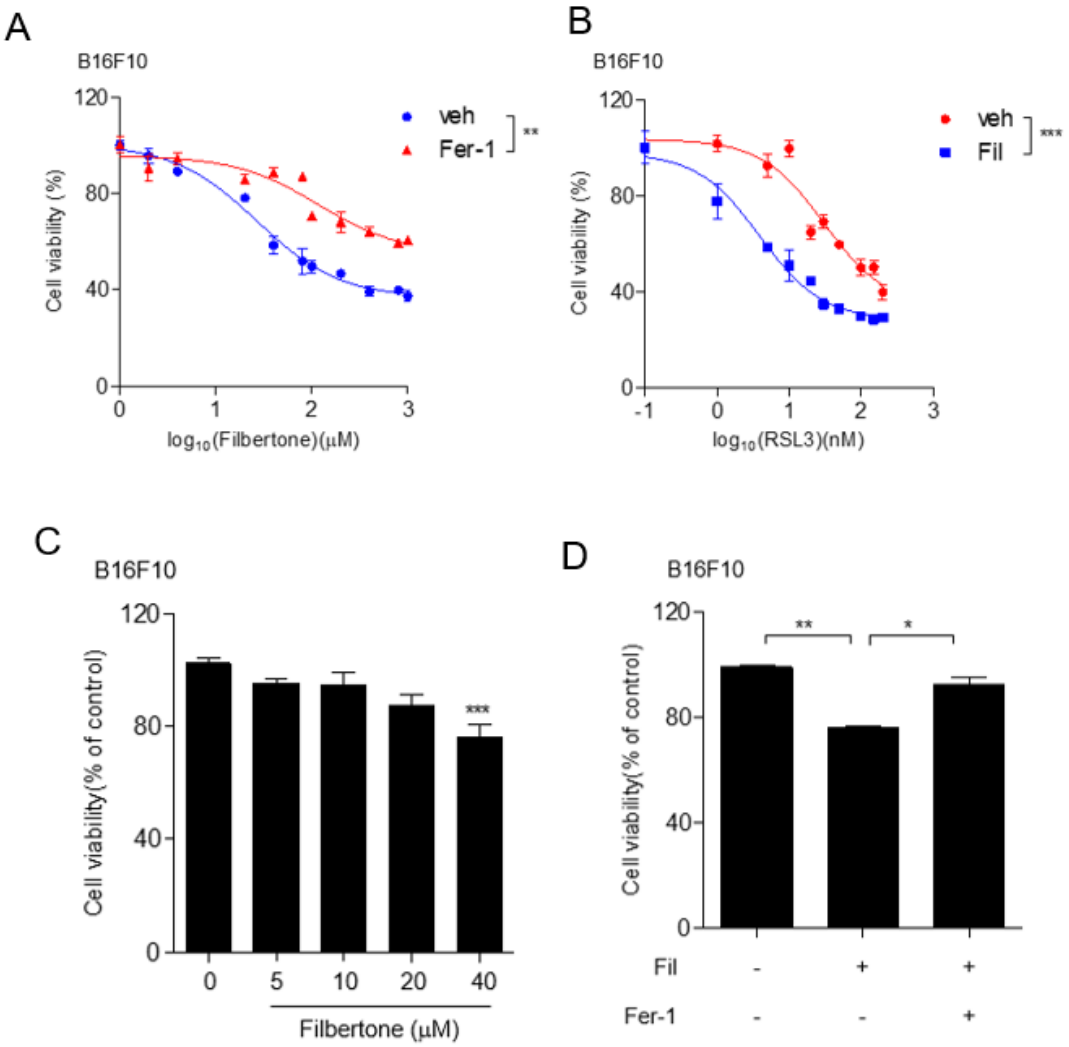

Figure 3.2. Filbertone induces ferroptotic cell death in B16F10 melanoma cells. (A) To examine filbertone-induced ferroptosis, B16F10 cells were treated with filbertone at various concentration and ferrostatin-1(1 μM) for 24 h. (B) To verify ferroptosis induction by filbertone and RSL3 synergically. B16F10 cell was treated with RSL3 at various concentration and filbertone (40 μM) for 24 h. (C) B16F10 cells were treated with filbertone (0, 5, 10, 20, and 40 μM) for 24 h. Cell viability was measured by WST-8 assay.(D) To investigate whether filbertone induces ferroptosis, B16F10 cells were pretreated with ferrostatin1 (1 μM), ferroptosis inhibitor, for 1 h and then treated with filbertone (40 μM) for 24 h. Cell viability was assessed by WST-8 assay. Data were expressed as mean ± SD; *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

3.3. Filbertone activates ferroptosis via inhibiting SLC7A11 expression in HepG2 and B16F10 cells

Ferroptosis is a form of controlled cell death primarily triggered by lipid peroxidation mediated by iron. To investigate the accumulation of lipid reactive oxygen species (ROS) in HepG2 (Figure 3.3B) protein expression 4-HNE, lipid peroxidation end- product was analyzed and in B16F10 cells the C11-BODIPY staining (Figure 3.3C) was used. Cells were treated with filbertone and co-treated with ferrostatin-1. Our observations revealed that the elevated levels of lipid reactive oxygen species (ROS) induced by filbertone were mitigated by the presence of ferrostatin-1 in HepG2 and B16F10 (Figure 3.3B & Figure 3.3C) cells. RSL3 was used as a positive control in the experiment. An excess expression of SLC7A11, cystine-glutamate antiporter system has been suggested to exert a significant influence on the proliferation and spread of cancer cells, and thus considered to be a promising target for stimulating ferroptosis in cancer cells (64), and the protein levels of SLC7A11 and PTGS2, a marker associated with ferroptosis. We observed that filbertone treatment increased PTGS2 protein expression in HepG2 and B16F10 cells (Figure 3.3B & 3.3D), while it decreased the expression levels of SLC7A11 in HepG2 and B16F10 cells (Figure 3.3A & 3.3D). Altogether, the data shown in this study suggest that filbertone triggers the activation of ferroptosis via suppressing SLC7A11.

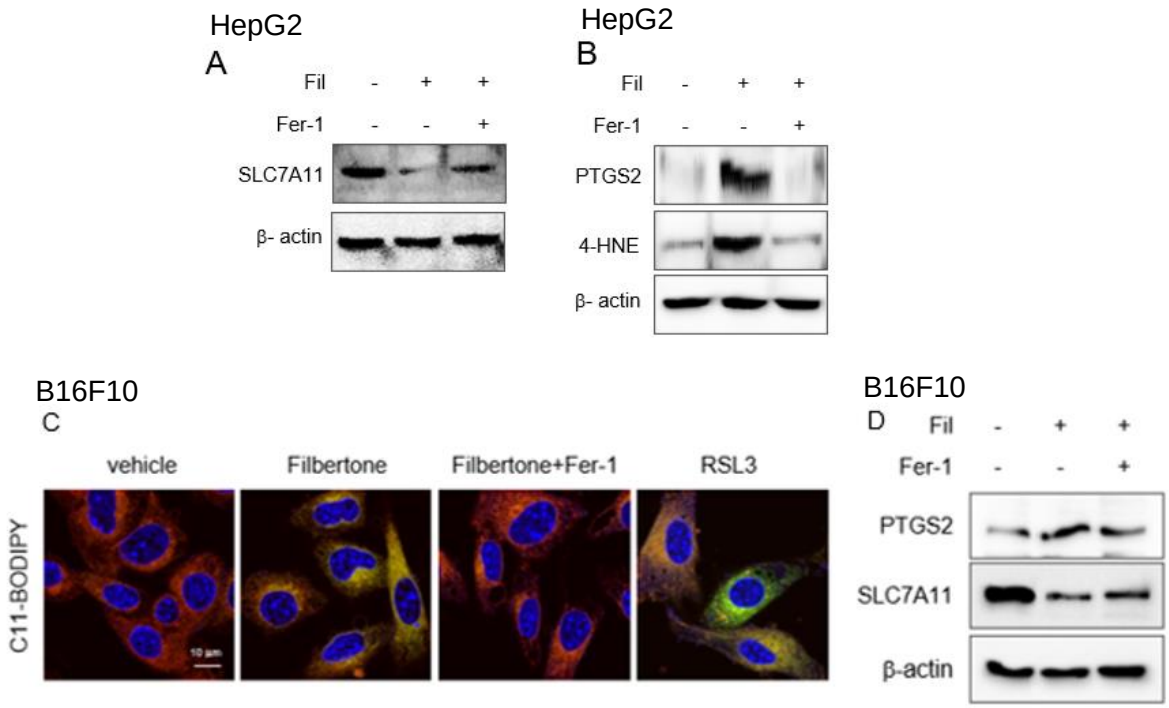

Figure 3.3 Filbertone activate ferroptosis via inhibiting SLC7A11 expression in B16F10 cell. (A, B) B16F10 cells were pretreated with Ferrostatin-1 (1 μM) for 1h and then subsequently treated these cells with filbertone (40 μM) for 24 h. Lipid ROS was measured using C11-BODIPY staining (A). Representative images were obtained by confocal microscopy. RSL3 (1 μM) was used as a positive control. The protein levels of PTGS2 and SLC7A11 were detected by western blotting (B). B16F10 were pretreated with Ferrostatin-1 (1 μM) for 1h and then subsequently treated these cells with filbertone (40 μM) for 24 h. Data were expressed as mean ± SD; *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

3.4. Filbertone downregulates SLC7A11 expression through YAP inhibition in HepG2 cells

It is well known that Hippo signalling pathway transducers YAP/TAZ induce the expression of SLC7A11 (44, 45, 46), which is also associated to cell growth, survival and cell proliferation, size control of tissues and organs, and tumorigenesis by inhibiting ferroptosis (125). To investigate whether filbertone treatment affects the expression of SLC7A11, HepG2 cells were treated with various concentrations of filbertone (0, 5, 10, 20, 40 μM) followed by measurement of SLC7A11 mRNA expression levels. We found that SLC7A11 was significantly decreased by filbertone in a dose dependent manner (Figure 3.4A), indicating that filbertone blocks SLC7A11 antioxidant expression. Next, to verify whether SLC7A11 suppression is related to YAP inhibition, HepG2 cell were treated with lysophosphatidic acid (LPA), YAP activator and we found that filbertone enhanced phosphorylation of YAP, which was suppressed in presence of LPA (Figure 3.4B). Additionally, decreased mRNA expression of SLC7A11 by filbertone further, induced by LPA (Figure 3.4C), suggested filbertone blocks SLC7A11/YAP signaling, could prompt ferroptosis.

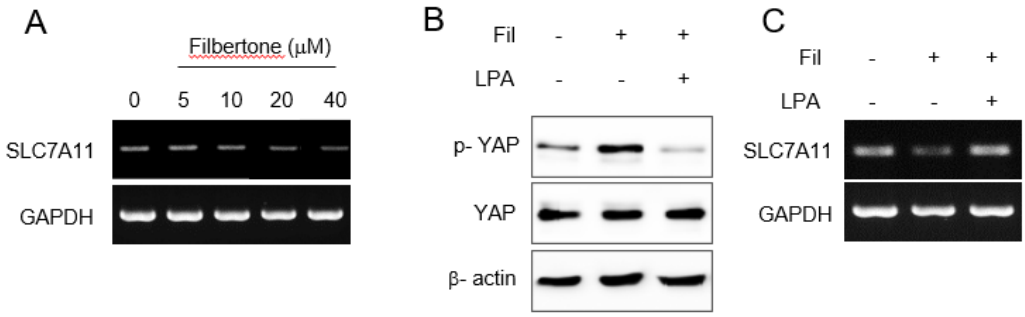

Figure 3.4 Filbertone downregulates SLC7A11 expression through YAP inhibition in HepG2 cells. To verify filbertone-induced ferroptosis by repression of SLC7A11 expression, HepG2 cells were treated with filbertone (0, 5, 10, 20, 40 μM) for 24 h. The mRNA expression level of SLC7A11 is determined by RT-PCR (A). To examine filbertone suppress SLC7A11 expression by YAP inhibition, HepG2 cells were treated with 20 uM lysophosphatidic acid (LPA) and filbertone (40 μM) for 30mins. The protein expression of p-YAP/YAP (B) and mRNA expression level of SLC7A11 (C) were analysed. Data were expressed as mean ± SD; *p<0.05, **p<0.01, ***p<0.001.

3.5. Filbertone downregulates SLC7A11 expression through YAP inhibition in B16F10 cells

Next, we also investigated whether filbertone treatment could downregulate the expression of SLC7A11 in melanoma cells. B16F10 cells were treated with various concentrations of filbertone (0, 5, 10, 20, 40μM) at different time periods (0, 3, 6, 12, 18, 24 h), and followed by measurement of SLC7A11 protein expression levels. We found that both the protein (Figure 3.5B & Figure 3.5C) and the mRNA (Figure 3.5A) expression levels of SLC7A11 were significantly decreased at concentration and time dependent manner. To examine filbertone-induced suppression of SLC7A11 expression by YAP inhibition, B16F10 cells were filbertone and lysophosphatidic acid (LPA), YAP activator. We found phosphorylation of YAP was increased by filbertone, while suppressed by lysophosphatidic acid (LPA), YAP activator (Figure 3.5D). Moreover, increased YAP phosphorylation (Figure 3.5E) and decreased SLC7A11 mRNA (Figure 3.5F) expression were observed by filbertone-treated cancer cells. These data indicated that the inhibition of SLC7A11/YAP signaling by filbertone is associated with ferroptotic cell death.

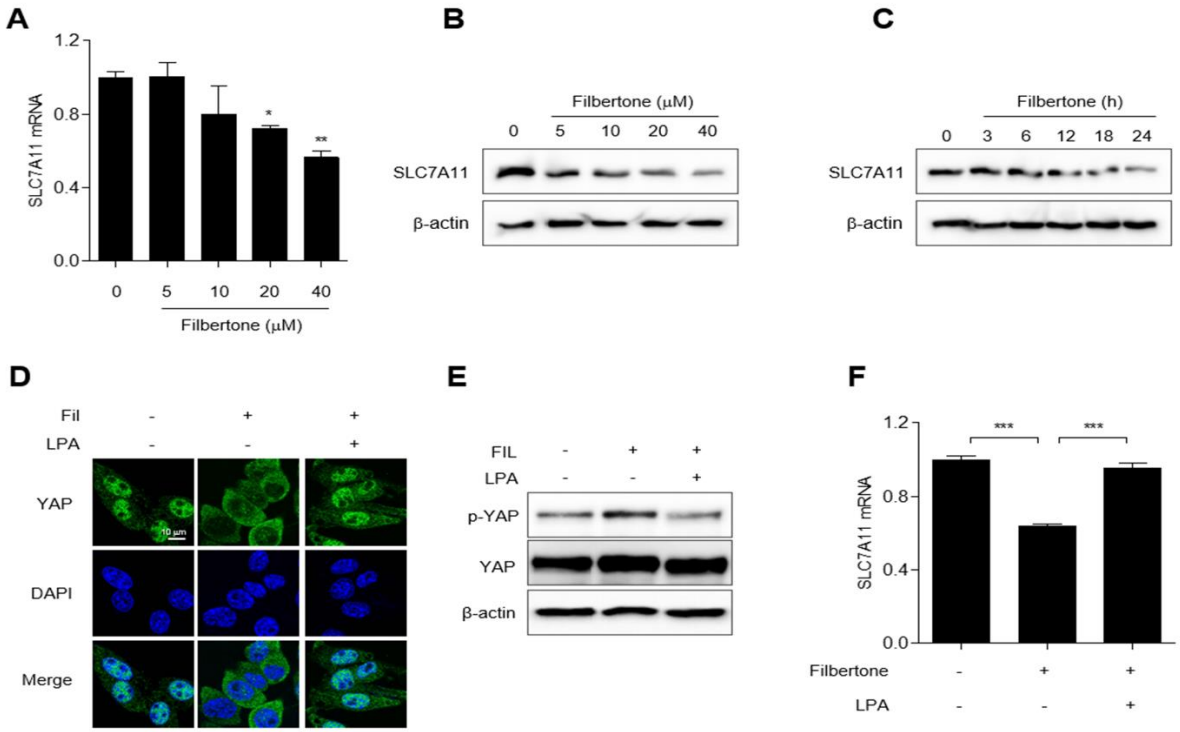

Figure 3.5. Filbertone downregulates SLC7A11 expression through YAP inhibition in B16F10 cells. (A, B) To verify the ferroptosis induction by filbertone via alteration of SLC7A11 expression, B16F10 cells were treated with filbertone (0, 5, 10, 20, 40 μM) for 24 h. The mRNA levels (A) and protein levels (B) of SLC7A11 were analyzed by qRT-PCR and western blotting, respectively. (C) B16F10 cells were treated with different time (3, 6, 12, 18, and 24 h) of filbertone (40 μM). The protein levels of SLC7A11 were detected by western blotting. (D, E) To investigate filbertone-induced suppression of SLC7A11 expression by YAP inhibition, B16F10 cells were pretreated with 20 mM lysophosphatidic acid (LPA) for 3 h and then treated with filbertone (40 μM) for 24 h. The cells were stained with DAPI and immunostained with anti-YAP antibody. Representative image of YAP was obtained by confocal microscopy (D). YAP phosphorylation was detected by western blotting (E). (F) B16F10 cells were treated with LPA and filbertone for 30 min. The mRNA levels of SLC7A11 were analyzed by qRT-PCR. Data were expressed as mean ± SD; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.6. Filbertone-induced ferroptosis is reversed by YAP activation in B16F10 cells

We further examined the impact of filbertone on inhibiting YAP activation and promoting ferroptosis. To analyze the cell viability, B16F10 cell line were treated with filbertone and lysophosphatidic acid (LPA), YAP activator. We found that filbertone- induced cytotoxic effects were mitigated by LPA, YAP activator (Figure 3.6A). In contrast, the accumulation of lipid ROS induced by filbertone was reduced by LPA, a known YAP activator in B16F10 cells (Figure 3.6B). Hence, the data suggest that the inhibition of YAP activation is associated with filbertone- induced ferroptotic cell death.

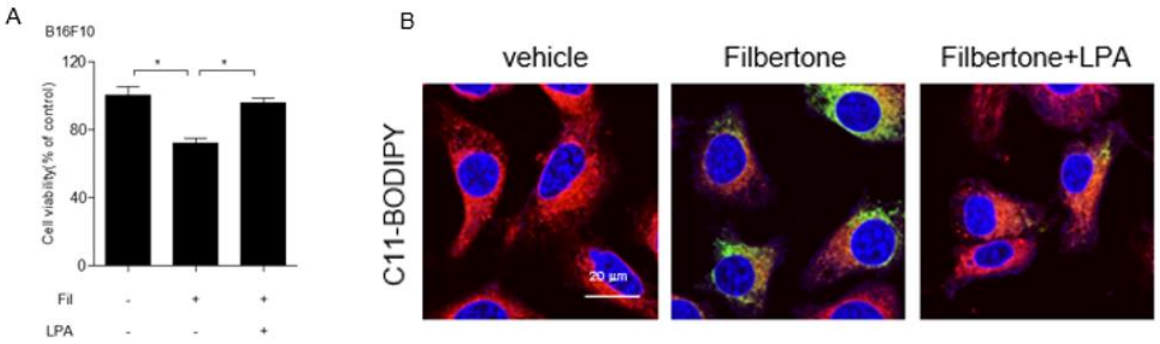

Figure 3.6. Filbertone-induced ferroptosis reversed by YAP activation in B16F10 cells. YAP activation reverses filbertone-induced ferroptosis. (A) To investigate that filbertone induces ferroptosis through YAP inhibition, B16F10 (A) cells were pretreated with LPA (20 μM), YAP activator, for 3 h and then filbertone (40 μM) for 24 h. Cell viability was measured by WST-8 assay. (B) To analyze lipid ROS, B16F10 cells were stained with BODIPY 581/591-C11. Representative images of lipid ROS were observed by confocal microscopy. Data were expressed as mean ± SD; *p<0.05 and **p<0.01.

3.7. Filbertone induces YAP phosphorylation via mtROS production in HepG2 cells

To assess filbertone inhibition of YAP activation, HepG2 cells were treated with filbertone at various time periods (0, 10, 20, 30 mins). We found that in presence of filbertone YAP phosphorylation and downstream target p-MST1/2 and p-LATS protein expression level increased significantly (Figure 3.7A), suggesting that filbertone suppresses YAP activation. Previous studies have shown that YAP phosphorylation is activated by production of mitochondrial ROS (126), thus we examined whether filbertone induces YAP phosphorylation by production of mitochondrial ROS in HepG2 cell. The increased protein expression of p-YAP by filbertone was downregulated by mito-tempo (mtROS inhibitor) co-treatment (Figure 3.7B). Altogether, our data revealed that filbertone promotes mtROS production to suppress YAP activation, lead to ferroptotic cell death in HepG2 cells.

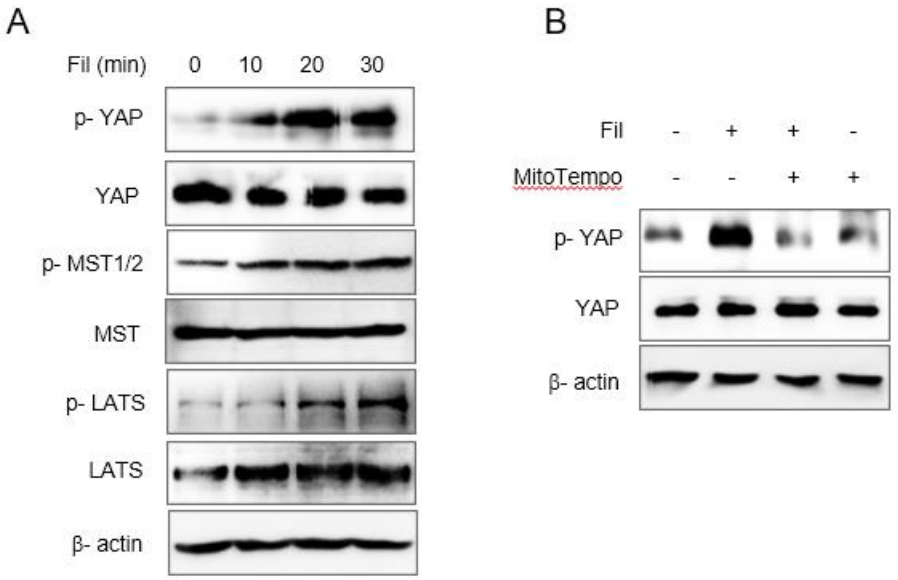

Figure 3.7. Filbertone induces YAP phosphorylation via mtROS production in HepG2 cells. HepG2 cells were treated with different time (10, 20, and 30 min) of filbertone (40 μM). p-YAP/YAP, p-MST1/2/MST and p-LATS/LATS was detected by western blotting (A). To examine whether filbertone induces YAP phosphorylation by mitochondrial ROS, HepG2 cells were pretreated with MitoTEMPO, mtROS scavenger, for 1 h and then treated with filbertone (40 μM) for 30 min. YAP phosphorylation were assessed by western blotting. Data were expressed as mean ± SD; *p<0.05 and **p<0.01.

3.8. Filbertone inhibits YAP nuclear translocation via mtROS-induced YAP phosphorylation in B16F10 cells

Next, we treated B16F10 cells with filbertone at various time durations (0, 10, 20, 30 min). As illustrated in figure 3.8B in B16F10 cell, filbertone treatment enhanced protein expression of YAP phosphorylation, while decreased the nuclear translocation of YAP in a time -dependent manner (Figure 3.8A). We also found that the filbertone increased protein expression of p-YAP and inhibits nuclear translocation of p-YAP, which was reversed by mito-tempo (mtROS inhibitor) (Figure 3.8C & Figure 3.8D). These results indicate that filbertone inhibits YAP nuclear translocation by mtROS-induced YAP phosphorylation to promote ferroptotic cell death.

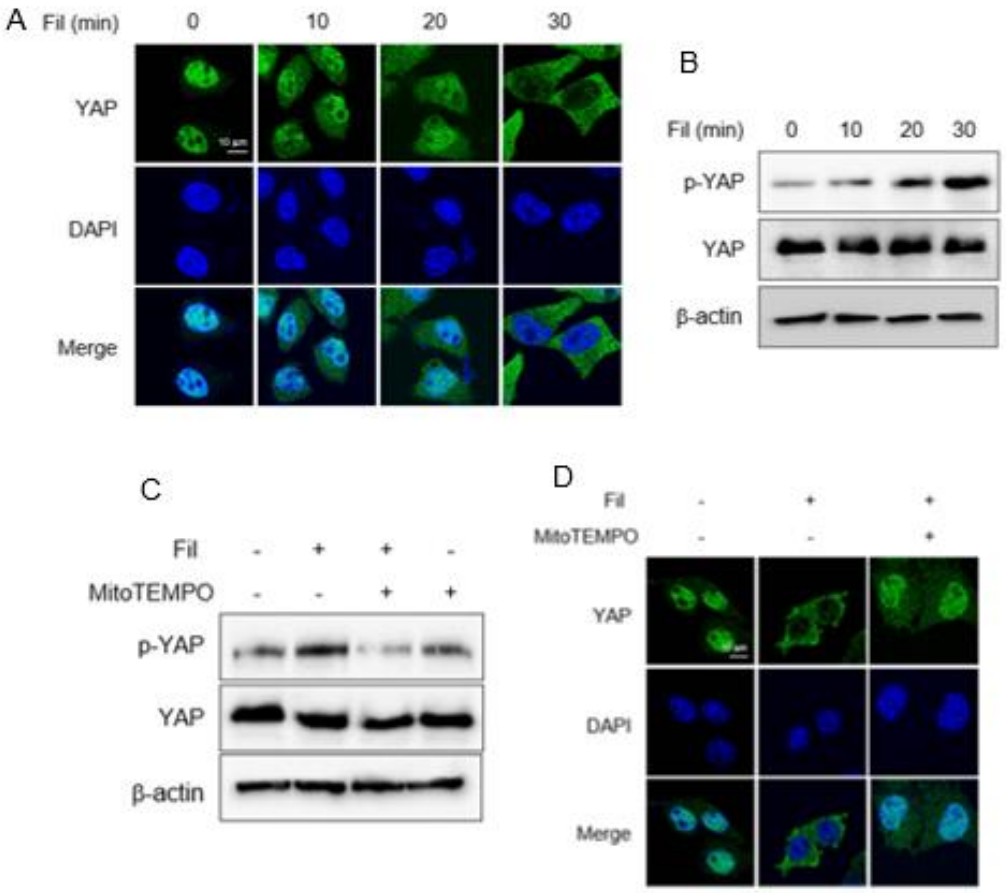

Figure 3.8 Filbertone inhibits YAP nuclear translocation via mtROS-induced YAP phosphorylation in B16F10 cells. (A & B) B16F10 cells were treated with different time (10, 20, and 30 min) of filbertone (40 μM). The cells were stained with DAPI and immunostained with anti-YAP antibody. Representative images of YAP were observed by confocal microscopy (A). YAP phosphorylation was detected by western blotting (B). (D & E) To investigate whether filbertone induces YAP phosphorylation by mitochondrial ROS, B16F10 cells were pretreated with MitoTEMPO, mtROS scavenger, for 1 h and then treated with filbertone (40 μM) for 30 min. YAP phosphorylation (D) and YAP nuclear translocation (E) were assessed by western blotting and immunofluorescence staining, respectively.

4. Discussion

The emergence of non-apoptotic cell death modes, such as ferroptosis, has shown promise in addressing therapy-resistant malignancies, as cancer cells develop resistance to apoptosis. It has been demonstrated that cells exhibiting resistance to conventional therapy or cells that have metastasized in cancer patients have heightened susceptibility to ferroptosis (86). These findings provide insights into potential strategies for cancer treatment via enhancing ferroptosis (127, 128).

Cancer cells frequently exhibit elevated amounts of reactive oxygen species (ROS) (134), and plays a role in the development of tumors, as it triggers survival signaling pathways, causes DNA damage, promotes genetic changes, and facilitates the evasion of cellular senescence by tumor cells (23). Interestingly, it has been shown that, an excessive production of ROS, is associated with ferroptosis in cancer cells, which prevent tumor development and progression by promoting cell death (134, 135). SLC7A11 mediates cystine uptake into the cell, thereby maintains the thiol-containing pool of ROS scavengers, particularly of GSH, which plays a pivotal role in regulating ferroptotic pathway (37, 88, 136, 112).

In the present study, we used HepG2, a hepatoblastoma cell line, which is used in a wide range of studies such as hepatocellular carcinoma-related studies (25, 129). Recent research has yielded evidence of novel treatment implications in HepG2 cells, where further proven that the inhibition of antioxidant pathways could lead to protect cancer cells from oxidative stress, via enhancing ferroptosis (130, 131,127). In the present chapter HepG2 cells were used to investigate the activation of ferroptosis and its pathway. In addition to this, melanoma metastatic cells, a highly malignant form of cancer characterized by a significant chance of metastasis in advanced stages, commonly circulates by hematogenous spread to the liver (132). Hence, the B16F10 cell line, a well-known metastatic melanoma, was also employed in this investigation.

Our findings demonstrate that the expression of SLC7A11 was elevated in both HepG2 and B16F10 cells. Additionally, we observed that the upregulation of SLC7A11 was subsequently reduced by the presence of filbertone. Consistent with these results, downregulation of SLC7A11 by filbertone induced ferroptosis in HepG2 and B16F10 cells. These findings suggest that the downregulation of SLC7A11 by filbertone could contribute to associated to the regulation of reactive oxygen species (ROS), thus leading to enhance ferroptosis.

The Hippo pathway is mainly consist of mammalian serine/threonine (Ste20) like kinases 1/2 (MST1/2), large tumor suppressor 1/2 (LATS1/2), and transcriptional coactivator Yes-associated protein (YAP), and it regulates cell growth, survival, proliferation, and migration; tissue and organ size control; and tumorigenesis and development (137, 138). Other studies have uncovered that the YAP/TAZ, well-known transducers of the Hippo signaling pathway, induces the expression of SLC7A11 (44, 45, 46), which is related to cell growth, survival, and cell proliferation, via inhibiting ferroptosis (125). We found that filbertone treatment increased PTSG2, ferroptosis marker, while reduced SLC7A11 antioxidant gene expression level in HepG2 and B16F10 cell, indicating filbertone induces ferroptotic cell death in HepG2 and B16F10 cell. YAP1 is a novel factor in the pathogenesis of several disorders and has been demonstrated to promote cellular proliferation and regeneration in many research studies. In this study we showed that YAP activation inhibits filbertone-induced ferroptosis, leading to cancer cell growth. These results indicated that inhibition of YAP activation and SLC7A11 expression by filbertone contributes to enhance ferroptosis in HepG2 and B16F10 cells.

The vital role of YAP has been shown in controlling mitochondrial redox homeostasis for obesity-mediated metabolic adaptation and breast tumor progression (116). In this study, we found that filbertone inhibited the translocation of YAP to nucleus in B16F10 cells and increases phosphorylation of YAP and downstream factors p-MST1/2 and p-LATS in HepG2 cells. It was also found, filbertone promoted mtROS level in both cell lines, leading to suppression of SLC7A11 expression, promoting ferroptotic cell death. Whereas mito-tempo, mitochondrial ROS inhibitor, reversed the YAP activation in the cancer cells. Taken together, these data suggest that filbertone-induced mtROS is associated with suppression of YAP activation and thus ferroptosis in cancer cells.

In summary, our study indicates that filbertone is involved in decreasing cell growth of HepG2 and B16F10 cancer cells through enhancing ferroptosis. Inactivating YAP pathway by filbertone downregulated SLC7A11 and led to promote ferroptosis. The protein expression and nuclear translocation of YAP was inhibited by filbertone-induced mtROS. Moreover, LPA, activator of YAP reversed filbertone-mediated suppression of YAP/SLC7A11 in HepG2 and B16F10 cells. Taken together, these data suggest the YAP /SLC7A11 pathway plays an important role in filbertone-induced ferroptotic cell death in HepG2 and B16F10 cells. Filbertone-induced ferroptosis may offer a novel strategy to circumvent cancer cells, which warrants an in vivo investigation.

5. Conclusion

In summary, our data reveals that filbertone has beneficial effect on cancer cell growth. Filbertone stimulates phosphorylation of YAP via mtROS production, resulting in an impairment of nuclear translocation of YAP in HepG2 and B16F10 cells. Filbertone suppresses SLC7A11 expression, triggering ferroptotic cell death. These results support that filbertone acts as potent suppressor of cancer cell growth by promoting ferroptotic cell death.

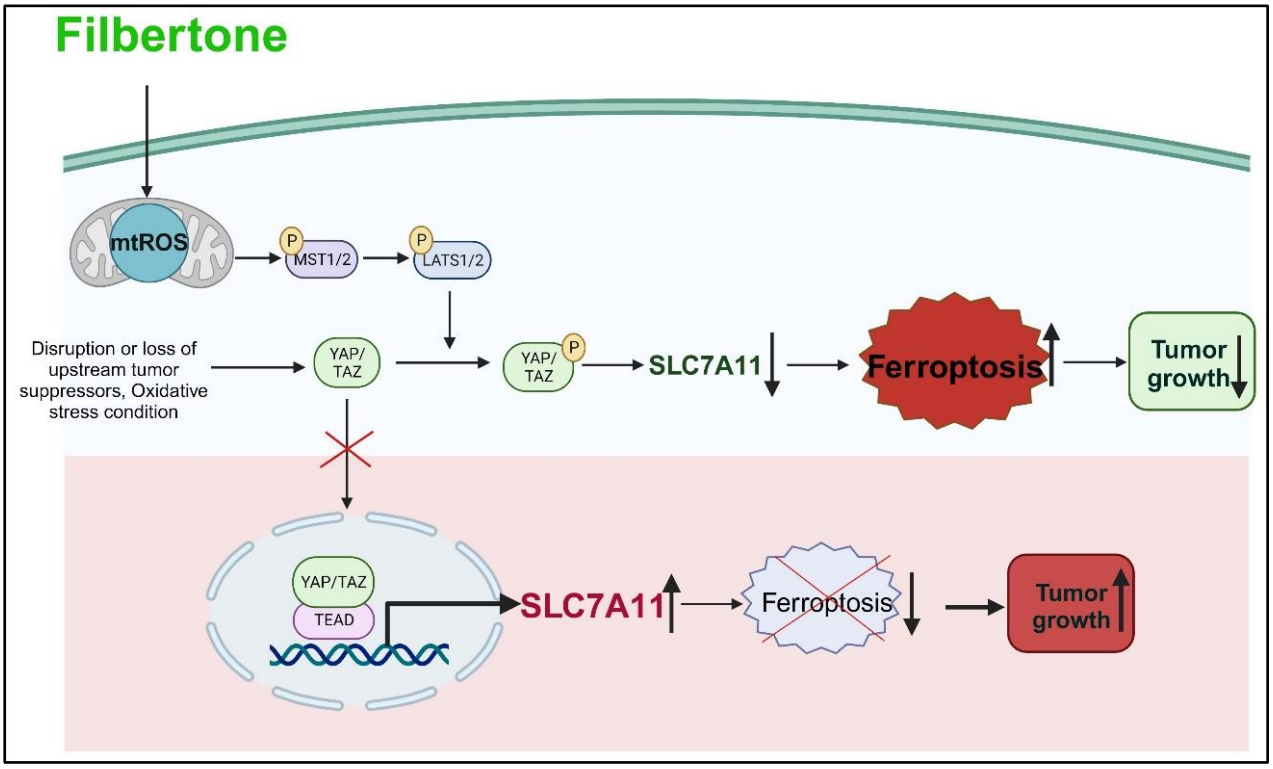

Figure 7. Schematic representation for anti-tumor role of YAP inhibition via ferroptosis accumulation induced by filbertone. Filbertone drives mtROS-dependent YAP phosphorylation, leading to decreased nuclear translocation of YAP. YAP inhibition by filbertone suppresses SLC7A11 expression. Filbertone induces ferroptotic cell death through suppressing YAP/SLC7A11 in hepatocellular carcinoma and melanoma cells. These results suggest that filbertone-induced ferroptosis acts as a potent suppressor for cancer cell growth.

6. Reference

1. Luo, Huasong, and Rui Zhang. "Icariin enhances cell survival in lipopolysaccharide-induced synoviocytes by suppressing ferroptosis via the Xc-/GPX4 axis." Experimental and Therapeutic Medicine 21.1 (2021): 1-1.
2. Marchesini, Giulio, et al. "Obesity-associated liver disease." *The Journal of Clinical Endocrinology & Metabolism* 93.11_supplement_1 (2008): s74-s80.
3. Chen, Si, et al. "The emerging role of ferroptosis in liver diseases." Frontiers in Cell and Developmental Biology 9 (2021): 801365.
4. Rolo, Anabela P., João S. Teodoro, and Carlos M. Palmeira. "Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis." Free radical biology and medicine 52.1 (2012): 59-69.
5. Shum, Michael, et al. "Mitochondrial oxidative function in NAFLD: Friend or foe?." Molecular Metabolism 50 (2021): 101134.
6. Su LJ, Zhang JH, Gomez H, et al. Reactive Oxygen Species-Induced Lipid Peroxidation in Apoptosis, Autophagy, and Ferroptosis. Oxid Med Cell Longev 2019; 2019:5080843.
7. Aizawa, Satoka, Gurmehr Brar, and Hidekazu Tsukamoto. "Cell death and liver disease." Gut and liver 14.1 (2020): 20.
8. Shojaie, Layla, Andrea Iorga, and Lily Dara. "Cell death in liver diseases: a review." International journal of molecular sciences 21.24 (2020): 9682.
9. Luedde, Tom, Neil Kaplowitz, and Robert F. Schwabe. "Cell death and cell death responses in liver disease: mechanisms and clinical relevance." Gastroenterology 147.4 (2014): 765-783.
10. Yang, Wan Seok, et al. "Peroxidation of polyunsaturated fatty acids by lipoxygenases drives ferroptosis." Proceedings of the National Academy of Sciences 113.34 (2016): E4966-E4975.
11. Hu, Wenli, et al. "Ferroptosis and its role in chronic diseases." Cells 11.13 (2022): 2040.
12. Lin, P. L., et al. "Saponin formosanin C-induced ferritinophagy and ferroptosis in human hepatocellular carcinoma cells. Antioxidants (Basel). 2020; 9 (8)." (2020).
13. Liu, Man-ru, Wen-tao Zhu, and Dong-sheng Pei. "System Xc−: A key regulatory target of ferroptosis in cancer." Investigational New Drugs 39.4 (2021): 1123-1131.
14. Bayır, Hülya, et al. "Achieving life through death: redox biology of lipid peroxidation in ferroptosis." Cell chemical biology 27.4 (2020): 387-408.
15. Xie, Wanqing, Shivani Agarwal, and Jindan Yu. "Ferroptosis: the vulnerability within a cancer

monster." The Journal of Clinical Investigation 133.10 (2023).

16. Park, Jeongmin, et al. "Activation of ROS-PERK-TFEB by filbertone ameliorates neurodegenerative diseases via enhancing the autophagy-lysosomal pathway." The Journal of Nutritional Biochemistry 118 (2023): 109325.
17. Shin, Daiha, et al. "Nrf2 inhibition reverses resistance to GPX4 inhibitor-induced ferroptosis in head and neck cancer." *Free Radical Biology and Medicine* 129 (2018): 454-462.
18. Li, Jie, et al. "Ferroptosis: past, present and future." Cell death & disease 11.2 (2020): 88.
19. Jelinek, Anja, et al. "Mitochondrial rescue prevents glutathione peroxidase-dependent ferroptosis." Free Radical Biology and Medicine 117 (2018): 45-57.
20. Gao, X. et al. Ibuprofen induces ferroptosis of glioblastoma cells via downregulation of nuclear factor erythroid 2-related factor 2 signaling pathway. Anticancer Drugs 31, 27–34 (2020).
21. El Hout, Mouradi, et al. "A promising new approach to cancer therapy: targeting iron metabolism in cancer stem cells." Seminars in cancer biology. Vol. 53. Academic Press, 2018.
22. Samant, Hrishikesh, Hosein Shokouh Amiri, and Gazi B. Zibari. "Addressing the worldwide hepatocellular carcinoma: Epidemiology, prevention and management." Journal of gastrointestinal oncology 12.Suppl 2 (2021): S361.
23. Gorrini, C., Harris, I. S. & Mak, T. W. Modulation of oxidative stress as an anticancer strategy. Nat. Rev. Drug Discov. 12, 931–947 (2013).
24. Kajarabille, Naroa, and Gladys O. Latunde-Dada. "Programmed cell-death by ferroptosis: antioxidants as mitigators." *International journal of molecular sciences* 20.19 (2019): 4968.
25. Efferth, Thomas. "From ancient herb to modern drug: Artemisia annua and artemisinin for cancer therapy." *Seminars in cancer biology*. Vol. 46. Academic Press, 2017
26. Chen, D., et al. "ATF4 promotes angiogenesis and neuronal cell death and confers ferroptosis in a xCT-dependent manner." Oncogene 36.40 (2017): 5593-5608.
27. Ayton, Scott, and Peng Lei. "Nigral iron elevation is an invariable feature of Parkinson's disease and is a sufficient cause of neurodegeneration." BioMed research international 2014 (2014).
28. Barbariga, Marco, et al. "Ceruloplasmin functional changes in Parkinson's disease-cerebrospinal fluid." Molecular neurodegeneration 10 (2015): 1-12.
29. Chen, Xin, et al. "Ferroptosis in infection, inflammation, and immunity." Journal of Experimental Medicine 218.6 (2021): e20210518.
30. Latunde-Dada, Gladys O. "Ferroptosis: role of lipid peroxidation, iron and ferritinophagy."

Biochimica et Biophysica Acta (BBA)-General Subjects 1861.8 (2017): 1893-1900.

31. Martin-Sanchez, Diego, et al. "Ferroptosis and kidney disease." nefrologia 40.4 (2020): 384-394.
32. Müller, Tammo, et al. "Necroptosis and ferroptosis are alternative cell death pathways that operate in acute kidney failure." Cellular and Molecular Life Sciences 74 (2017): 3631-3645.
33. Wang, Ying, et al. "Ferroptosis and its role in skeletal muscle diseases." Frontiers in Molecular Biosciences 9 (2022): 1051866.
34. He, Sixiao, et al. "ACSL4 contributes to ferroptosis-mediated rhabdomyolysis in exertional heat stroke." Journal of Cachexia, Sarcopenia and Muscle 13.3 (2022): 1717-1730.
35. Chen, Zhen, et al. "Ferroptosis as a potential target for cancer therapy." Cell death & disease 14.7 (2023): 460.
36. Xu, Feng-li, et al. "SLC27A5 promotes sorafenib-induced ferroptosis in hepatocellular carcinoma by downregulating glutathione reductase." Cell Death & Disease 14.1 (2023): 22.
37. Wang, Haibo, et al. "Silencing of PTPN18 induced ferroptosis in endometrial cancer cells through p-P38-mediated GPX4/xCT down-regulation." Cancer Management and Research (2021): 1757-1765.
38. Wang, Ling, et al. "P53-dependent induction of ferroptosis is required for artemether to alleviate carbon tetrachloride-induced liver fibrosis and hepatic stellate cell activation." IUBMB life 71.1 (2019): 45-56.
39. Luo, Yinli, et al. "Protective effects of ferroptosis inhibition on high fat diet-induced liver and renal injury in mice." International Journal of Clinical and Experimental Pathology 13.8 (2020): 2041.
40. Friedmann Angeli, Jose Pedro, et al. "Inactivation of the ferroptosis regulator Gpx4 triggers acute renal failure in mice." Nature cell biology 16.12 (2014): 1180-1191.
41. Zhou, Linxiang, et al. "Ferroptosis—A New Dawn in the Treatment of Organ Ischemia–Reperfusion Injury." Cells 11.22 (2022): 3653.
42. Lu, Yuzhen, et al. "Ferroptosis as an emerging therapeutic target in liver diseases." Frontiers in Pharmacology 14 (2023): 1196287.
43. Yuan, Yuan, et al. "Kaempferol ameliorates oxygen-glucose deprivation/reoxygenation-induced neuronal ferroptosis by activating Nrf2/SLC7A11/GPX4 axis." Biomolecules 11.7 (2021): 923.
44. Wang, Jin, et al. "YAP1 protects against septic liver injury via ferroptosis resistance." Cell &

Bioscience 12.1 (2022): 163.

45. Chen, Jinsi, et al. "AKR1C3 suppresses ferroptosis in hepatocellular carcinoma through regulation of YAP/SLC7A11 signaling pathway." Molecular Carcinogenesis 62.6 (2023): 833-844.
46. Sun, Tianai, and Jen-Tsan Chi. "Regulation of ferroptosis in cancer cells by YAP/TAZ and Hippo pathways: The therapeutic implications." Genes & diseases 8.3 (2021): 241-249.
47. Gao, Ruize, et al. "YAP/TAZ and ATF4 drive resistance to Sorafenib in hepatocellular carcinoma by preventing ferroptosis." EMBO molecular medicine 13.12 (2021): e14351.
48. Zhou, Xiaoxi, et al. "Ferroptosis in chronic liver diseases: opportunities and challenges." Frontiers in Molecular Biosciences 9 (2022): 928321.
49. He, Yongfei, et al. "Atractylodin may induce ferroptosis of human hepatocellular carcinoma cells." Annals of Translational Medicine 9.20 (2021).
50. Li C, Deng X, Xie X, Liu Y, Friedmann Angeli JP, Lai L. Activation of glutathione peroxidase 4 as a novel anti-inflammatory strategy. Front Pharmacol. 2018;9:1120. doi:10.3389/fphar.2018.01120
51. Basu, Aalok, Thanaphon Namporn, and Pakatip Ruenraroengsak. "Critical Review in Designing Plant-Based Anticancer Nanoparticles against Hepatocellular Carcinoma." Pharmaceutics 15.6 (2023): 1611.
52. Kamel, Fatemah O., et al. "Hepatoprotective effects of bioactive compounds from traditional herb Tulsi (Ocimum sanctum Linn) against galactosamine-induced hepatotoxicity in rats." Frontiers in Pharmacology 14 (2023).
53. Lv, Jinchang, et al. "The relationship between ferroptosis and diseases." Journal of Multidisciplinary Healthcare (2022): 2261-2275.
54. Zhou, Zheng, Jiye Li, and Xiaochuan Zhang. "Natural Flavonoids and Ferroptosis: Potential Therapeutic Opportunities for Human Diseases." Journal of Agricultural and Food Chemistry 71.15 (2023): 5902-5916.
55. Hong, Mu-Keng, et al. "6-Gingerol ameliorates sepsis-induced liver injury through the Nrf2 pathway." International Immunopharmacology 80 (2020): 106196.
56. Zhou, Ling, et al. "Chrysin induces autophagy-dependent ferroptosis to increase chemosensitivity to gemcitabine by targeting CBR1 in pancreatic cancer cells." Biochemical pharmacology 193 (2021): 114813.

57. Han, Shangting, et al. "HO-1 contributes to luteolin-triggered ferroptosis in clear cell renal cell carcinoma via increasing the labile iron pool and promoting lipid peroxidation." Oxidative Medicine and Cellular Longevity 2022 (2022).
58. Wen, Rui-jia, et al. "Baicalin induces ferroptosis in osteosarcomas through a novel Nrf2/xCT/GPX4 regulatory axis." Phytomedicine 116 (2023): 154881.
59. Han, Dongxu, et al. "Apigenin ameliorates di (2-ethylhexyl) phthalate-induced ferroptosis: The activation of glutathione peroxidase 4 and suppression of iron intake." Food and Chemical Toxicology 164 (2022): 113089.
60. Huang, Tongwen, et al. "Quercetin Alleviates Acrylamide-Induced Liver Injury by Inhibiting Autophagy-Dependent Ferroptosis." Journal of Agricultural and Food Chemistry 71.19 (2023): 7427-7439.
61. Li, Danlei, et al. "Fisetin attenuates doxorubicin-induced cardiomyopathy in vivo and in vitro by inhibiting ferroptosis through SIRT1/Nrf2 signaling pathway activation." Frontiers in pharmacology 12 (2022): 808480.
62. Wu, Yangyang, et al. "Protective effect of rutin on ferroptosis-induced oxidative stress in aging laying hens through Nrf2/HO-1 signaling." Cell biology international 47.3 (2023): 598-611.
63. Duan, Wentao, et al. "Silibinin Inhibits Cell Ferroptosis and Ferroptosis-Related Tissue Injuries." Antioxidants 12.12 (2023): 2119.
64. Liu, Guofang, et al. "Wogonoside attenuates liver fibrosis by triggering hepatic stellate cell ferroptosis through SOCS1/P53/SLC7A11 pathway." Phytotherapy research 36.11 (2022): 4230-4243.
65. Jiang, Zhe, et al. "The natural flavone acacetin protects against high-fat diet-induced lipid accumulation in the liver via the endoplasmic reticulum stress/ferroptosis pathway." Biochemical and Biophysical Research Communications 640 (2023): 183-191.
66. Lo, Yi-Hsin, et al. "Nobiletin alleviates ferroptosis-associated renal injury, inflammation, and fibrosis in a unilateral ureteral obstruction mouse model." Biomedicines 10.3 (2022): 595.
67. Feng, Senling, et al. "Nobiletin induces ferroptosis in human skin melanoma cells through the GSK3β-mediated Keap1/Nrf2/HO-1 signalling pathway." Frontiers in genetics 13 (2022): 865073.
68. Choi, Jiwon, Hyewon Choi, and Jayong Chung. "Icariin Supplementation Suppresses the Markers of Ferroptosis and Attenuates the Progression of Nonalcoholic Steatohepatitis in Mice

Fed a Methionine Choline-Deficient Diet." International Journal of Molecular Sciences 24.15 (2023): 12510.

69. Luo, Huasong, and Rui Zhang. "Icariin enhances cell survival in lipopolysaccharide-induced synoviocytes by suppressing ferroptosis via the Xc-/GPX4 axis." Experimental and Therapeutic Medicine 21.1 (2021): 1-1.
70. Xie, Jiangbo, et al. "Dihydromyricetin attenuates cerebral ischemia reperfusion injury by inhibiting SPHK1/mTOR signaling and targeting ferroptosis." Drug Design, Development and Therapy (2022): 3071-3085.
71. Jauch, Johann, et al. "Isolation, synthesis, and absolute configuration of filbertone–the principal flavor component of the hazelnut." Angewandte Chemie International Edition in English 28.8 (1989): 1022-1023.
72. Mutsnaini, Luthfiyyah, et al. "Filbertone protects obesity-induced hypothalamic inflammation by reduction of microglia-mediated inflammatory responses." Biotechnology and Bioprocess Engineering 26 (2021): 86-92.
73. Puchl'ová, Eva, and Peter Szolcsányi. "Filbertone: a review." Journal of agricultural and food chemistry 66.43 (2018): 11221-11226.
74. Emberger, Roland, et al. "Use of 5-methyl-hept-2-en-4-one as a fragrance and/or flavor." U.S. Patent No. 4,654,168. 31 Mar. 1987.
75. Moon, Youna, et al. "Filbertone ameliorates adiposity in mice fed a high-fat diet via activation of cAMP signaling." Nutrients 11.8 (2019): 1749.
76. Kim, Hyemee, and Byungyong Ahn. "Filbertone, (2E)-5-methyl-2-hepten-4-one, regulates thermogenesis and lipid metabolism in skeletal muscle of a high-fat diet fed mice." Applied Biological Chemistry 66.1 (2023): 1-10.
77. Han Zhang,1 Enxiang Zhang,2,* and Hongbo Hu1,* Role of Ferroptosis in Non-Alcoholic Fatty Liver Disease and Its Implications for Therapeutic Strategies. Biomedicines. 2021 Nov; 9(11): 1660.
78. Mundi, Manpreet S., et al. "Evolution of NAFLD and its management." Nutrition in Clinical Practice 35.1 (2020): 72-84.
79. Gaschler MM, Stockwell BR. Lipid peroxidation in cell death. Biochem Biophys Res Commun 2017; 482:419-25.
80. Dixon, Scott J., and Brent R. Stockwell. "The role of iron and reactive oxygen species in cell

death." Nature chemical biology 10.1 (2014): 9-17.

81. Dixon, Scott J., et al. "Ferroptosis: an iron-dependent form of nonapoptotic cell death." cell 149.5 (2012): 1060-1072.
82. Lee, Young-Sun, et al. "Ferroptosis-induced endoplasmic reticulum stress: crosstalk between ferroptosis and apoptosis." Molecular cancer research 16.7 (2018): 1073-1076.
83. Wang, Qiujie, et al. "GSTZ1 sensitizes hepatocellular carcinoma cells to sorafenib-induced ferroptosis via inhibition of NRF2/GPX4 axis." Cell death & disease 12.5 (2021): 426.
84. Dodson, Matthew, Raul Castro-Portuguez, and Donna D. Zhang. "NRF2 plays a critical role in mitigating lipid peroxidation and ferroptosis." Redox biology 23 (2019): 101107.
85. Sun, Yitian, et al. "The emerging role of ferroptosis in inflammation." Biomedicine & Pharmacotherapy 127 (2020): 110108.
86. Liu, Meng, et al. "The critical role and molecular mechanisms of ferroptosis in antioxidant systems: a narrative review." Annals of translational medicine 10.6 (2022).
87. Kuang, Hao, et al. "Palmitic acid-induced ferroptosis via CD36 activates ER stress to break calcium-iron balance in colon cancer cells." *The FEBS Journal* (2023).
88. Li, Feng-Jiao, et al. "System Xc−/GSH/GPX4 axis: An important antioxidant system for ferroptosis in drug-resistant solid tumor therapy." Frontiers in Pharmacology 13 (2022): 910292.
89. Ma, Tianyu, et al. "GPX4-independent ferroptosis—a new strategy in disease's therapy." Cell death discovery 8.1 (2022): 434.
90. Liu, Z., et al. "Protein kinase R-like ER kinase and its role in endoplasmic reticulum stress-decided cell fate." Cell death & disease 6.7 (2015): e1822-e1822.
91. Hartwick Bjorkman, Sarah, and Renata Oliveira Pereira. "The interplay between mitochondrial reactive oxygen species, endoplasmic reticulum stress, and Nrf2 signaling in cardiometabolic health." Antioxidants & redox signaling 35.4 (2021): 252-269.
92. Cullinan, Sara B., et al. "Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival." *Molecular and cellular biology* 23.20 (2003): 7198-7209.
93. Wei, Ruiran, et al. "Tagitinin C induces ferroptosis through PERK-Nrf2-HO-1 signaling pathway in colorectal cancer cells." International journal of biological sciences 17.11 (2021): 2703.
94. Imai, Hirotaka, et al. "Lipid peroxidation-dependent cell death regulated by GPx4 and ferroptosis." Apoptotic and Non-apoptotic Cell Death (2017): 143-170.
95. Kuo, Chan-Yen, et al. "Chrysophanol attenuates hepatitis B virus X protein-induced hepatic

stellate cell fibrosis by regulating endoplasmic reticulum stress and ferroptosis." Journal of pharmacological sciences 144.3 (2020): 172-182.

96. Wu, Shenglin, et al. "6-Gingerol alleviates ferroptosis and inflammation of diabetic cardiomyopathy via the Nrf2/HO-1 pathway." Oxidative Medicine and Cellular Longevity 2022 (2022).
97. Gottlieb, Yehonatan, et al. "Endoplasmic reticulum anchored heme-oxygenase 1 faces the cytosol." Haematologica 97.10 (2012): 1489.
98. Siwecka, Natalia, et al. "Dual role of endoplasmic reticulum stress-mediated unfolded protein response signaling pathway in carcinogenesis." International journal of molecular sciences 20.18 (2019): 4354.
99. Spahis, Schohraya, et al. "Oxidative stress as a critical factor in nonalcoholic fatty liver disease pathogenesis." Antioxidants & redox signaling 26.10 (2017): 519-541.
100. Gao, Minghui, et al. "Role of mitochondria in ferroptosis." Molecular cell 73.2 (2019): 354-363.
101. Simões, Inês CM, et al. "Mitochondria in non-alcoholic fatty liver disease." The international journal of biochemistry & cell biology 95 (2018): 93-99.
102. Nassir, Fatiha, and Jamal A. Ibdah. "Role of mitochondria in nonalcoholic fatty liver disease." International journal of molecular sciences 15.5 (2014): 8713-8742.
103. Li, Meng-Die, et al. "Arsenic induces ferroptosis and acute lung injury through mtROS-mediated mitochondria-associated endoplasmic reticulum membrane dysfunction." Ecotoxicology and Environmental Safety 238 (2022): 113595.
104. Yamada, Naoya, et al. "Iron overload as a risk factor for hepatic ischemia-reperfusion injury in liver transplantation: potential role of ferroptosis." American Journal of Transplantation 20.6 (2020): 1606-1618.
105. Friedman, Scott L., et al. "Mechanisms of NAFLD development and therapeutic strategies." Nature medicine 24.7 (2018): 908-922.
106. Afonso, Marta B., Rui E. Castro, and Cecilia MP Rodrigues. "Processes exacerbating apoptosis in non-alcoholic steatohepatitis." Clinical Science 133.22 (2019): 2245-2264.
107. Dhar, Debanjan, et al. "Mechanisms of liver fibrosis and its role in liver cancer." Experimental Biology and Medicine 245.2 (2020): 96-108.
108. Rodríguez, Macías, and Infante Hernández JM. "Risk factors for hepatocellular carcinoma in patients with liver cirrhosis." Revista Espanola de Enfermedades Digestivas: Organo Oficial de

la Sociedad Espanola de Patologia Digestiva 92.7 (2000): 458-469.

109. Feng, Hao, et al. "AdipoR1 regulates ionizing radiation-induced ferroptosis in HCC cells through Nrf2/xCT pathway." Oxidative Medicine and Cellular Longevity 2022 (2022).
110. Criscuolo, Daniela, Francesco Morra, and Angela Celetti. "A xCT role in tumor-associated ferroptosis shed light on novel therapeutic options." Exploration of Targeted Anti-tumor Therapy 3.5 (2022): 570.
111. Koppula, Pranavi, et al. "Amino acid transporter SLC7A11/xCT at the crossroads of regulating redox homeostasis and nutrient dependency of cancer." Cancer Communications 38 (2018): 1-13.
112. Koppula, Pranavi, Li Zhuang, and Boyi Gan. "Cystine transporter SLC7A11/xCT in cancer: ferroptosis, nutrient dependency, and cancer therapy." Protein & cell 12.8 (2021): 599-620
113. Bi, Fangfang, et al. "METTL9-SLC7A11 axis promotes hepatocellular carcinoma progression through ferroptosis inhibition." Cell Death Discovery 9.1 (2023): 428.
114. Liang, Yimin, et al. "Ferroptosis regulator SLC7A11 is a prognostic marker and correlated with PD-L1 and immune cell infiltration in liver hepatocellular carcinoma." Frontiers in Molecular Biosciences 9 (2022): 1012505.
115. Yang, Wen-Hsuan, et al. "The Hippo pathway effector YAP promotes ferroptosis via the E3 ligase SKP2." Molecular Cancer Research 19.6 (2021): 1005-1014.
116. Dai, Jia-Zih, et al. "YAP dictates mitochondrial redox homeostasis to facilitate obesity-associated breast cancer progression." Advanced Science 9.12 (2022): 2103687.
117. Huang, Long Shuang, et al. "Sphingosine kinase 1/S1P signaling contributes to pulmonary fibrosis by activating Hippo/YAP pathway and mitochondrial reactive oxygen species in lung fibroblasts." International journal of molecular sciences 21.6 (2020): 2064.
118. Iseda, Norifumi, et al. "Ferroptosis is induced by lenvatinib through fibroblast growth factor receptor-4 inhibition in hepatocellular carcinoma." Cancer Science 113.7 (2022): 2272-2287.
119. Zheng, Kai, et al. "Regulation of ferroptosis by bioactive phytochemicals: Implications for medical nutritional therapy." Pharmacological Research 168 (2021): 105580.
120. Ashrafizadeh, Milad, et al. "Apigenin as tumor suppressor in cancers: Biotherapeutic activity, nanodelivery, and mechanisms with emphasis on pancreatic cancer." Frontiers in Chemistry 8 (2020): 829.

121. Jiao Y, Wilkinson J, Di X, et al. [1]Curcumin, a cancer chemopreventive and chemotherapeutic agent, is a biologically active iron chelator. Blood. 2009;113(2):462–469. doi:10.1182/blood-2008-05-155952.
122. Kose, Tugba, et al. "Curcumin and (−)-epigallocatechin-3-gallate protect murine MIN6 pancreatic beta-cells against iron toxicity and erastin-induced ferroptosis." Pharmaceuticals 12.1 (2019): 26.
123. Li, Hang-Yu, et al. "Plant-based foods and their bioactive compounds on fatty liver disease: Effects, mechanisms, and clinical application." Oxidative medicine and cellular longevity 2021 (2021).
124. Wu, Shuang, et al. "Ferroptosis and cancer: complex relationship and potential application of exosomes." Frontiers in Cell and Developmental Biology 9 (2021): 733751.
125. Qin, Yifei, et al. "Oncogenic activation of YAP signaling sensitizes ferroptosis of hepatocellular carcinoma via ALOXE3-mediated lipid peroxidation accumulation." Frontiers in cell and developmental biology 9 (2021): 751593.
126. Park, Jeongmin, et al. "YAP inhibition by CO promotes ferroptosis via ATF4-dependent REDD1 increase in melanoma." The Journal of Immunology 210.1_Supplement (2023): 245-09.
127. Liang C, Zhang X, Yang M, et al. Recent Progress in Ferroptosis Inducers for Cancer Therapy. Adv Mater 2019;31: e1904197.
128. Lu, Bin, et al. "The role of ferroptosis in cancer development and treatment response." Frontiers in pharmacology 8 (2018): 992.
129. Donato, María Teresa, Laia Tolosa, and María José Gómez-Lechón. "Culture and functional characterization of human hepatoma HepG2 cells." Protocols in in vitro hepatocyte research (2015): 77-93.
130. Lippmann, Jana, et al. "Redox modulation and induction of ferroptosis as a new therapeutic strategy in hepatocellular carcinoma." Translational oncology 13.8 (2020): 100785.
131. Huang, Dan, et al. "Steroidal saponin SSPH I induce ferroptosis in HepG2 cells via regulating iron metabolism." Medical Oncology 40.5 (2023): 132.
132. Obrador, Elena, et al. "Melanoma in the liver: Oxidative stress and the mechanisms of metastatic cell survival." Seminars in Cancer Biology. Vol. 71. Academic Press, 2021.

133. Sosa, V. et al. Oxidative stress and cancer: an overview. Ageing Res. Rev. 12, 376–390 (2013).
134. Wang, Haiyan, et al. "Emerging mechanisms and targeted therapy of ferroptosis in cancer." Molecular Therapy 29.7 (2021): 2185-2208.
135. Zhang, Chen, et al. "Ferroptosis in cancer therapy: a novel approach to reversing drug resistance." Molecular cancer 21.1 (2022): 47.
136. Hong, Ting, et al. "PARP inhibition promotes ferroptosis via repressing SLC7A11 and synergizes with ferroptosis inducers in BRCA-proficient ovarian cancer." Redox biology 42 (2021): 101928.
137. Xiang, Jiangxia, Mengmeng Jiang, and Xing Du. "The role of Hippo pathway in ferroptosis." Frontiers in Oncology 12 (2023): 1107505.
138. Magesh, Suchitra, and Danfeng Cai. "Roles of YAP/TAZ in ferroptosis." Trends in cell biology 32.9 (2022): 729-732.

7. 국문요약

연구배경: 비만으로 인해 간의 비정상적인 지방 축적의 발생은 간의 손상과 심각한 합병증을 일으키는 것으로 알려져 있다. 간에 과도한 지방의 축적으로 인해 발생하는 간지방증은 간 섬유화의 진행을 야기하여 간의 비가역적 손상을 발생시켜, 심한 경우 간경화 및 간암의 발병으로 이어진다. Ferroptosis는 glutathione peroxidase 4 (GPX4)의 불활성화와 지질 과산화 작용을 특징으로 하는 철 의존성 산화적 세포예정사의 한 형태이다. 필버톤은 헤이즐넛에서 발견되는 천연 화합물로 항비만 활성 및 지질 항상성을 조절하는 것으로 알려져 있다. 그러나 필버톤이 간질환에 대한 잠재적 효과와 이와 관련한 분자적 메커니즘에 대해서는 잘 알려져 있지 않다.

실험목적: 본 연구에서 우리는 필버톤이 ferroptosis를 조절하여 간질환에 대해 보호작용을 나타낼수 있는지에 대해 조사하였다.

결과: 제 2장에서는 필버톤3669이 ferroptosis 억제를 통해 지방간으로 유도된 간 손상으로부터 간을 보호할 수 있는지 조사하였다. HFD/MCD 식이 투여 쥐에게서 발생한 간 손상이 필버톤 투여를 통해 유의적으로 감소하였으며, 이는 ferroptosis를 억제함으로써 나타나는 현상임을 확인하였다. 필버톤이 ferroptosis를 조절하는 메커니즘을 조사하기 위해, *in vivo* 및 *in vitro* 실험에서 Nrf2, GPX4, HO-1 및 PTGS2 와 같은 ferroptosis 관련 단백질의 발현 수준을 측정하였다. 또한, 필버톤이 PERK-Nrf2 활성화를 통해 ferroptosis를 억제한다는 것을 확인하였다.

제3 장에서는필버톤이 암세포에 미치는 영향을 조사하고자 WST-8 분석을 통해 세포 생존율을 측정하였다. 필버톤은 B16F10 및 HepG2 세포의 생존율을 감소시키는 것을 관찰하였다. 필버톤이 암세포에 미치는 영향에 대한 메커니즘을 분석하였을 때, 필버톤이 YAP 활성 억제와 ferroptosis를 유도한다는 것을 확인하였다. 또한, 필버톤에 의한 YAP 활성의 억제는 미토콘드리아 ROS 생성을 통한 것으로 확인되었다. 따라서, 필버톤은 미토콘드리아 ROS 생성을 통해 YAP을 억제하며, 이는 SLC7A11 발현의 하향 조절 이끌어 ferroptosis를 유도하는 것으로 여겨진다.

결론: 종합하자면, 필버톤은 지질 과산화 억제를 통해 ferroptosis를 억제함으로써 지방간으로 인해 유도된 간손상을 보호하는 효과를 지닌 것으로 나타났다. 또한 필버톤은 ferroptosis를 유도하여 암세포 증식을 차단하는효과가 있음이 확인되었다. 따라서, 필버톤은 비만으로 유도되는 지방간 질환과 암세포 증식을 억제하는 효능을 가진 기능성 식품성분일 것으로 기대된다.