Effects of liposomes encapsulating ferulic acid on CCl_4-induced oxidative liver damage in a rat model

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List of abbreviations

AST	Aspartate aminotransferase
APF	Amino phenyl fluorescein
ALT	Alanine transaminase
CCl_4	Carbon tetra chloride
CAT	Catalase
C_{18}	Octadecyl silyl
DPPH	2,2-Diphenyl-1-picrylhydrazil
DMEM	Dulbecco's modified Eagle's medium
DHE	Dihydroethidium
EPC	Egg phosphatidyl choline
Ferulic-lipo	Liposome encapsulating ferulic acid
FBS	Fetal bovine serum
H&E	Hematoxylin and eosin
HPLC	High performed liquid chromatography
H_2O_2	Hydrogen peroxide
HepG2	Hepatoblastoma cell line
I.P	Intraperitoneal
I.V	Intravenous
IU/L	International units per liter
OCT	Optimal cutting temperature
γ-Ory-lipo	Liposome containing γ-Oryzanol
OH	Hydroxyl radical
PDI	Polydispersity index
PD-10	Protein desalting columns
PBS	Phosphate-buffered saline
ROS	Reactive oxygenase species
SOD	Superoxide dismutase
UV-Vis	Ultraviolet-visible

ABSTRACT

Abstract

Oxidative stress is well known as one of the causative agents of liver diseases. Antioxidants are helpful for the treatment of oxidative stress-mediated liver damage. A naturally occurring antioxidant, γ- oryzanol, is rapidly hydrolyzed to its active hydrophobic metabolite, ferulic acid, inside the body. As a hydrophobic drug, ferulic acid has several limitations, such as poor solubility and low bioavailability associated with minimal drug delivery in the body. Limitations associated with the hydrophobicity of ferulic acid can be overcome by encapsulating it in a liposomal formulation. As intravenously administered nanoparticles (including liposomes) can effectively reach the liver, such systems may be suitable drug delivery carriers to treat liver injury. Therefore, in this study, I prepared a liposomal formulation of ferulic acid (ferulic-lipo) and examined its effects on liver damage induced by CCl_4.

Ferulic-lipo was prepared by lipid hydration method, size was ~100 nm, and drug encapsulation efficiency was about 92%. At first, I measured the hydroxy radical scavenging capability of ferulic-lipo and compared it with a known antioxidant α-tocopherol. I found that ferulic-lipo showed higher radical scavenging than α-tocopherol liposomes. As ferulic-lipo showed a significant antioxidative effect, I examined whether ferulic-lipo exhibited protective effects against CCl_4-mediated cytotoxicity in human hepatocarcinoma (HepG2) cells. Ferulic-lipo showed significant improvement in the viability of HepG2 cells (~30%) against CCl_4-induced toxicity. Based on this finding, next, I applied ferulic-lipo for *in vivo* study by preparing a liver-injured rat model to assess its potentiality against CCl_4-induced oxidative liver damage. Then, I examined the serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, which indicate the severity of liver damage in the rat model of liver injury. I found a marked elevation in serum ALT and AST levels in the CCl_4-treated group,

demonstrating the development of a liver-injured rat model. With the intravenous administration of ferulic-lipo, serum ALT & AST levels significantly reduced (30% & 35%) dose-dependently indicating hepatoprotective effects of ferulic-lipo. Next, I studied the impact of ferulic-lipo on ROS production in the liver. Administration of CCl_4 results in a high level of ROS generation in the liver. Contrary, the liver's ROS was markedly reduced (~50%) by intravenous administration of ferulic-lipo. To further confirm ferulic-lipo's hepatoprotective effects, I performed histopathological observation of liver tissues and found that ferulic-lipo significantly reduced CCl_4-mediated hepatocyte damage in the treatment group.

Next, I found that the stability of ferulic acid was not so high in ferulic-lipo, so I focused on improving the stability of ferulic acid. I hypothesized that γ- oryzanol would be a prodrug of ferulic acid because it tends to metabolize into ferulic acid inside the body rapidly. Then, I prepared a liposomal formulation of γ- oryzanol (γ-ory-lipo) and checked its chemical stability. Regarding the stability of γ-oryzanol in the liposome, I found γ-ory-lipo showed high stability. Additionally, I measured the antioxidant activity of γ-ory-lipo, but the activity was lower than ferulic-lipo. Next, I checked the conversion of ferulic acid to γ- oryzanol and found that γ-oryzanol was successfully and immediately converted into the ferulic acid in the cultured cells (HepG2 cells) and rat liver analyzed by HPLC. Based on these findings, it was suggested that γ-ory-lipo would be a good prodrug formulation of ferulic acid. The overall results of this study indicate that ferulic-lipo exhibited potent antioxidative capacity and was suggested to be an effective formulation for preventing oxidative damage of liver tissue.

Chapter I

INTRODUCTION

Chapter I: Introduction

1.1. Oxidative stress

Reactive oxygenase species (ROS), also known as free radicals (hydroxyl, superoxide, hydrogen peroxides), are highly reactive molecules generally produced by living cells as a metabolic byproduct [1]. Mitochondria is the primary source of endogenous ROS (superoxide) during oxidative phosphorylation [2]. Leakage of electrons during electron transport in mitochondria facilitates their subsequent binding to oxygen molecules resulting in the generation of ROS. Other sources of mitochondria ROS generation are NADPH oxidases, xanthine oxidase, peroxidase, glucose oxidase, and lipoxygenase present on the membranes of endothelial cells and phagocytes. On the other hand, environmental pollutants, pesticides, ionizing and non-ionizing radiant, heavy metals (lead, arsenic, mercury, chromium, and cadmium), foods, drugs, and xenobiotic compounds generate exogenous ROS. Growing evidence suggests ROS acts as a signaling molecule for cell proliferation and survival [3]. However, excessive production of ROS results in an imbalance between the production of reactive molecules and the biological system's ability to detoxify them, a phenomenon known as oxidative stress [4]. Due to high reactivity, ROS leads to oxidative damage to cellular components like nucleic acid and proteins and promotes cell death by inducing apoptosis, necrosis, and autophagy. Numerous scientific studies have revealed ROS as the main component in the progression of various life-threatening diseases in humans, like cancer, ischemia-reperfusion, cardiovascular diseases, diabetes, and other metabolic disorders [5].

Studies demonstrated that elevated ROS helps cancer initiation by inducing oxidative damage of DNA together with genetic instability and oncogene activation [6]. Besides, high production of ROS associate with impaired cellular signaling and DNA mutation that accelerates the process of aging and carcinogenesis. Literature shows increased intracellular

ROS leads to mitochondrial dysfunction and cellular structure and function alternation, resulting in oxidative stress-mediated aging [7]. Many studies revealed the involvement of oxidative stress in the pathogenesis of diabetes mellitus. Elevated ROS is associated with reduced pancreatic β-cell function and insulin resistance in type 2 diabetes mellitus [8]. Moreover, Bulua et al. [9] and Zhou et al. [10] indicated oxidative stress as a key factor for advancing cardiovascular diseases by facilitating inflammatory cytokine secretions and vascular endothelium dysfunctions. Oxidation of the low-density lipoprotein (LDL) by ROS help in the progression of atherosclerosis by modulating matrix metalloproteinase (MMPs) degradation and altering vasomotor activity [11].

Additionally, Youn et al. demonstrated that vascular smooth muscle cells (VSMC) generated ROS has a role in the development of obesity by inducing leptin resistance and glucose intolerance [12]. Several studies suggested that ROS also contributes to the generation of neurological disorders like Alzheimer's, Parkinson's, multiple sclerosis, and memory loss [13]. In the case of Alzheimer's disease, ROS produces toxic β-amyloid peptides, disrupting the homeostasis of several metallic ions associated with mitochondrial dysfunction. ROS has also been reported to be linked with lung diseases like asthma and chronic obstructive pulmonary disorders, where ROS accelerates inflammation by activating different signaling pathways (NF-kappa B and AP-1) [14]. Rheumatoid arthritis, an inflammatory condition, is characterized by chronic inflammation of joints and surrounding tissues, also shown to be initiated and progressed by ROS[15]. Additionally, ROS plays a central role in the pathogenesis of kidney diseases by affecting renal apparatus, such as glomerular- and tubule-interstitial nephritis, renal failure, proteinuria, and uremia [16].

Thus, it can be stated that oxidative stress and ROS are the confirmed reason for the pathogenesis of many life-threatening human diseases that subsequently affect different organs, tissues, and systems. Maintaining intracellular ROS levels at the ambient level is crucial for

cellular homeostasis and biological functions. Increased ROS favors the onset of different diseases, causing deleterious effects on human health. Therefore, it is emerging to minimize elevated intracellular ROS to treat oxidative stress and its associated diseases.

1.2. Antioxidant's defense against oxidative stress

Antioxidants gained much attention in clinical studies because of their beneficial role in fighting against oxidative stress and its mediated diseases. These compounds are stable molecules that provide first-line defense against ROS by preventing free radical formation and autoxidation. Growing evidence suggests that antioxidative compounds containing phenolic and aromatic rings donate electrons to free radicals and stabilize them by the resonance delocalization of the electron in the aromatic ring [17]. It's been reported that antioxidants exert their biological functions by interacting with enzymatic and non-enzymatic antioxidant defense systems [18]. The enzymatic antioxidant defenses include superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT). These antioxidants reduce free radicals into the oxidative product (hydrogen peroxides), which is further metabolized (by catalase) in the presence of a cofactor in water. In contrast, non-antioxidant defense systems contain ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH), β-carotene, and vitamin A that help in terminating free radical chain reactions. Other defense strategies of antioxidants include metal ion chelation, genomic modulation, co-antioxidants, and electron-donating. A well-known underlying mechanism by which antioxidants exhibit their protective effects involves the activation of the Nrf2 (NF-E2-related factor 2) signaling pathway that enhances antioxidative enzymes levels modulating the expression of genes and their products that results in detoxification and elimination of reactive oxidants through conjugative reactions [19].

Because of combating free radicals, antioxidants are widely used to manage many incurable diseases, such as cancer, diabetes, coronary heart disease, liver & kidney diseases,

and renal toxicity. Antioxidants are potent anticancer agents because they can inhibit cancer growth by scavenging free radicals. It's reported that vitamin c prevents melanoma of the skin by improving the endogenous antioxidant system and accelerating the ROS elimination system of the body [20]. Likewise, β-carotene has an inhibitory effect against UV-light-mediated cell damage and cancer progression [21]. Besides, vitamin E also treats tumorigenesis and immune competence by regulating immune functions [22]. Another antioxidant, Resveratrol, is known to have potent anti-inflammatory effects by inhibiting proinflammatory cytokines IL-1 and TNF- α which are the primary mediators of inflammation [23].

Additionally, curcumin and quercetin are demonstrated as potent antidiabetic compounds because of their ability to control hyperglycemia by preventing glucose oxidation during Diabetes Mellitus [24]. Antioxidants, including flavonoids, play a vital role in ameliorating eye defects and the progression of cataracts [25]. Extensive studies mentioned the pivotal role of antioxidants in managing chronic liver diseases mediated by alcohol, xenobiotics, and drugs. Preclinical research with silymarin has confirmed its potent hepatoprotective effects against oxidative stress-mediated liver damage [26]. Moreover, antioxidant treatments provide a better therapeutic approach for rheumatoid arthritis. Vitamin B, vitamin E-alpha tocopherol, and green tea containing the polyphenolic compound catechin were shown to reduce arthritis in a mice model [27]. As a potent antioxidative compound, lycopene has also been shown to protect against osteoporosis, male infertility, and cardiovascular diseases reported by numerous *in vivo* and *in vitro* studies [28]. Similarly, antioxidants help prevent neurological disorders mediated by oxidative stress. L-carnitine protects cells from oxidative damage through its antioxidative potentialities. Previous studies mentioned that L-carnitine could cross the blood-brain barrier readily and, therefore, beneficial for treating oxidative damage-mediated neuronal loss [29]. Based on these findings, antioxidants are the best candidates for preventing oxidative stress-related diseases. And

antioxidants are crucial for maintaining the body's homeostatic condition, which helps in scavenging elevated ROS.

1.3. Ferulic acid as a potent antioxidant

Ferulic acid (Fig-1.1), also known as 4-hydroxy-3-methoxy cinnamic acid, is the most common member of cinnamic acid derivatives, generally found in whole grains (rice, wheat, oats, cereal seeds, barley), fruits, and vegetables. As a phenolic acid, ferulic acid possesses strong antioxidative properties. It's been postulated that the presence of the phenolic hydroxyl group in the chemical structure of ferulic acid is mainly responsible for its strong antioxidative properties [30]. The antioxidative mechanism of ferulic acid is related to inhibiting the formation of reactive oxygen species and neutralizing free radicals [31]. The structural characteristic of ferulic acid is also related to antioxidative potentialities like the carboxylic acid group-containing unsaturated c-c double bond and phenolic nucleus form resonance stabilized phenoxy radicals that aid in neutralizing reactive radicals [32]. Besides, ferulic acid acts as a metal chelating agent by binding transition metals (iron and copper), preventing lipid peroxidation [33].

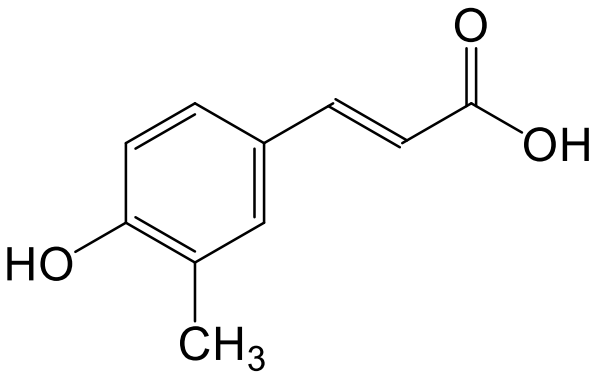

Fig. 1.1. Chemical structure of Ferulic acid

Literature showed ferulic acid has many positive effects on human health. The anti-inflammatory effects of ferulic acid are related to the decreasing level of several inflammatory mediators, such as Prostaglandin E2 and tumor necrosis factor-alpha in lipopolysaccharide-stimulated Raw 264.7 cells [34]. Based on previous studies, ferulic acids are also effective in preventing cancer because of their potential to quench ROS and protect cellular molecules (DNA, protein). According to stich et al., treatment with ferulic acid significantly reduced urinary N-nitrosoproline levels in humans, representing ferulic acid's inhibitory effects against carcinogen nitrosamines [35]. Moreover, *In vivo* study conducted with ferulic acid also reported that ferulic acid significantly reduced azoxymethane-induced colon carcinogenesis in F344 rats [36]. Numerous studies showed that ferulic acid could ameliorate elevated blood glucose levels induced by streptozotocin in the animal model [37]. Sultana et al. demonstrated ferulic acid's neuroprotective effect that helps to reduce oxidative stress-mediated beta-amyloid toxicity [38]. Apart from this, ferulic acid is recognized as a potent hepatoprotective agent against different liver damage. Administration of ferulic acid showed significant hepatoprotective effects against alcohol-induced hepatic damage and improved hepatic enzymes level, alkaline phosphatase, alanine transaminase, and aspartate transaminase dose-dependently [39]. Based on the above discussion, ferulic acid is one of the potent antioxidants with diverse physiological functions and can be used as a pharmaceutical ingredient for the treatment of oxidative stress-mediated non-communicable diseases.

1.4. Limitation associated with ferulic acid delivery

The solubility of a drug is a crucial factor for its desired therapeutic potentiality on the target site [40]. About 60% of our bodies are made up of water, so a drug must have certain degrees of solubility for good clinical efficacy. The low water solubility of pharmaceutically active drug molecules offers the greatest obstacle to drug development and successful drug

delivery [41]. The poor aqueous solubility of the drug is also associated with limited bioavailability resulting in suboptimal drug concentration after administration. Additionally, a drug with low bioavailability generally fails to reach the effective concentration required for its therapeutic actions. Numerous studies reported that poor aqueous solubility and low bioavailability of ferulic acid are major limitations for its successful drug delivery [42]. Despite having potent antioxidative effects, the instability of ferulic acid has restricted its application in pharmaceutical industries [43]. Previous studies showed that ferulic acid easily decomposes through decarboxylation and forms 4-hydroxy-3-methoxy styrene, 4-ethyl guaiacol, acetovanillone, vanillin, vanillic acid, and other dimers of 4-hydroxy-3-methoxy styrene that reduce its efficacy [44]. Jittraporn et al. demonstrated that ferulic acid proneness deteriorates in heat, light, and humidity [45]. The application of ferulic acid to treat insomnia is restricted because of its low concentration in the brain after drug delivery [46]. Moreover, *in vivo* studies with ferulic acid revealed its limited pharmacokinetics properties with the shortest plasma half-life after intravenous administration [47]. Based on these findings, it can be concluded that the wide application of ferulic acid has been restricted due to its hydrophobicity, low bioavailability, and phytochemical instability. Therefore, it is vital to design a methodology to solve these problems of ferulic acid for its better therapeutic efficiency.

1.5. Liposome as a suitable drug delivery carrier

In recent years, nanotechnology-based drug-delivery systems received much attention because of their potential to overcome challenges associated with the insolubility of hydrophobic drugs [48]. It reported that incorporating poorly soluble drugs into nanoformulations offers enhanced bioavailability, better stability, and improved therapeutic efficacy [49]. Lipid-based nanoparticles such as micelles, liposomes, dendrimers, nanoemulsions, and solid lipid nanoparticles are generally used as a carrier for drug delivery

[50]. Among them, liposomes (Fig-1.2) are considered a suitable alternative carrier for drug delivery, bearing beneficial effects, including superior therapeutic effects, sustainable drug release, specific site-targeting, and protection of drugs from degradation [51]. Additionally, compared to other lipid base nanoparticles, liposomes can encapsulate and protect drugs being denatured in the gastrointestinal tract and thus help in improving drug bioavailability and stability [52,53].

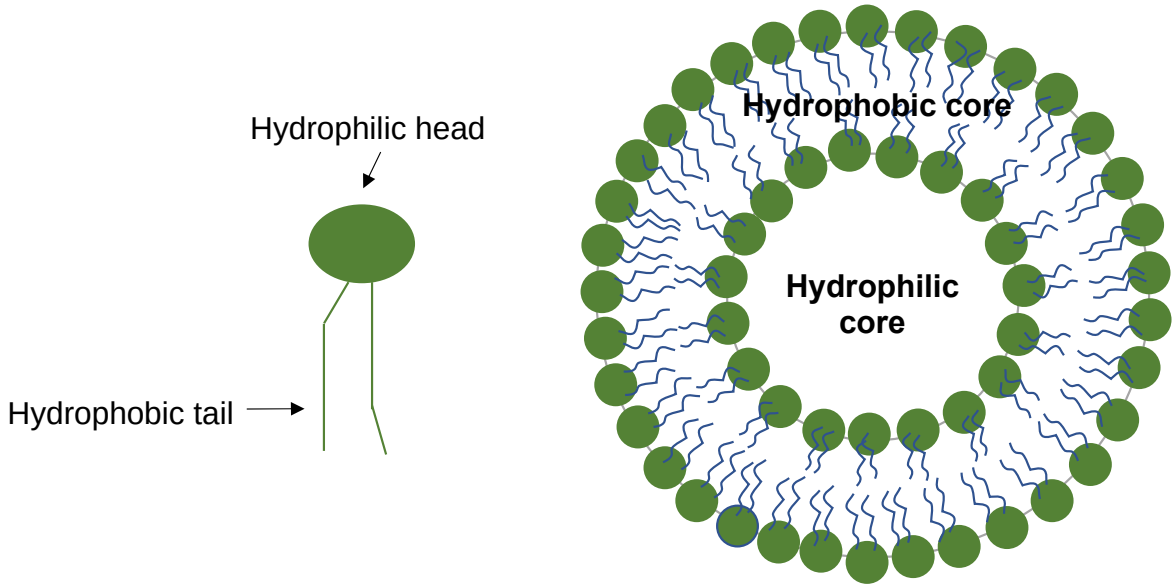

Fig. 1.2. Structure of liposome

It's been demonstrated that liposomes can solubilize water-insoluble drugs into the lipid bilayer and help in modulating *in vivo* behavior of drugs [54]. In addition, the similarity of liposomes with bio-membrane also enhanced their application as drug delivery carriers [55]. It's been evidenced that liposomes can carry relatively higher doses of drugs with maximum therapeutic index due to their structural compositions. Carvedilol is used for the treatment of cardiovascular diseases. However, its low water solubility and rapid metabolism result in low bioavailability (25-30%), which limits its clinical potential. Ghassemi et al. prepared

carvedilol-loaded liposomes to enhance carvedilol's bioavailability and found relatively higher carvedilol bioavailability in liposomal formulations compared to a free drug suspension[56]. Besides, Tian et al. demonstrated that liposome encapsulating liposomes showed increased solubility and bioavailability due to being protected from the harsh environment of the gastrointestinal tracts compared to curcumin suspension [57]. Based on this information, liposomes could be a suitable formulation for improving the limitation associated with the hydrophobicity and bioavailability of the hydropic drug ferulic acid.

Therefore, in this study, I focused on liposomes to solve ferulic acid hydrophobicity and stability problems and selected a liver damage model to assess the hepatoprotective effect of ferulic acid-containing liposomes.

Chapter II

Hepatoprotective effects of ferulic-liposome against CCl_4-induced liver injury

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Chapter II: Hepatoprotective effects of ferulic-liposome against CCl_4-induced liver injury

2.1. Introduction

Liver diseases are the 10th most common cause of death worldwide, with 2 million individuals dying from liver diseases each year [58]. As the liver plays a central role in metabolism, it is the major target of toxicity of various drugs, xenobiotics, and oxidative stress [59]. Oxidative stress is known to be one of the causative agents responsible for liver injury [60]. Reactive oxygen species (ROS) generated by the metabolism of lipids, carbohydrates, proteins, various drugs, and pesticides induce necrosis and apoptosis in the liver by disrupting cellular macromolecules. Although some remarkable drug developments have occurred in the field of medical science in recent years, there remains an unmet need for a dependable synthetic drug for the treatment of the liver injury. Antioxidants are widely used to prevent various diseases mediated by oxidative stress. Dry eye diseases induced by oxidative stress are found to be ameliorated with the treatment of the liposomal formulation containing antioxidant astaxanthin [61]. Antioxidative treatments also seemed to be effective in preventing liver diseases mediated by oxidative stress [62,63]. Vitamin E has previously shown as promising source for the treatment of different liver diseases [64]. In addition, other antioxidants such as vitamin C, N-acetyl cysteine and silymarin are known to act as potent hepatoprotectives agents [60].

Similarly, γ-oryzanol exhibits antioxidative properties, and is one of the most common natural compounds used for the treatment of various diseases. γ-Oryzanol is insoluble in water and consists of ferulic acid esters of triterpene alcohols. While γ-oryzanol acts as a potent antioxidative therapeutic agent, it has a number of limitations. Specifically, γ-oryzanol is

rapidly metabolized inside the body after consumption [65], which limits its clinical application. Herein, we instead focused our attention on ferulic acid (4-hydroxy-3-methoxy cinnamic acid), which is an active metabolite of γ-oryzanol. Ferulic acid exhibits various therapeutic effects against a number of different diseases, including cancer, diabetes, bacterial infection, hepatic injury, and cardiovascular and neurodegenerative disorders [66]. The broad range of preventive effects associated with ferulic acid are attributed to its potent antioxidative properties. The hydroxyl group attached to phenyl ring of ferulic acid is mainly responsible for its antioxidative properties by donating an electron to scavenge free radicals [67,68]. Ferulic acid is a methoxylated derivative of cinnamic acid that is widely used in the pharmaceutical, food and cosmetics industries because of its low toxicity. Moreover, compounds that exhibit an -OCH_3 group in their structure (e.g., ferulic acid) also have a direct effect on the activity of hepatic antioxidant enzymes (e.g., superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and paraoxonase), maintenance of oxidative stress conditions, and inhibition of inflammation leading to apoptosis of liver cells [69,70]. Thus, ferulic acid is expected to be a potent antioxidant and reliable hepatoprotective agent to combat oxidative stress mediated liver injury.

Solubility is of great importance for drug discovery and formulation development [71]. The potential therapeutic applications of ferulic acid are limited by its poor solubility and stability [72]. Encapsulation of active therapeutics into proper formulations (such as liposomes, micelles, dendrimers, and metal-based nanoparticles) is a suitable approach to overcome stability and solubility problems [73,74]. In particular, liposomal formulations are useful for encapsulating hydrophobic drugs into the lipid bilayer to improve stability and solubility of the loaded drugs. Liposomes are suitable carriers for drug delivery because of their structural similarity to cellular membranes and the resultant stability of encapsulated drugs [75,76]. The

liver is one of the most targeted organs for nanoparticles following intravenous administration [77,78]. Thus, liposomes are suitable for use as carriers to deliver ferulic acid to injured liver.

In the present study, I employed a CCl_4-mediated liver injury model. CCl_4 is one of the most commonly used xenobiotics applied in animal model studies to initiate lipid peroxidation mediated tissue injuries. Numerous studies have demonstrated that CCl_4 is metabolized in the endoplasmic reticulum of hepatocytes by the phase I cytochrome P450 system to highly reactive trichloromethyl radicals ($\cdot CCl_3$) and peroxy trichloromethyl radicals ($\cdot OOCCl_3$) to induce oxidative damage in the liver [79]. These highly reactive molecules subsequently bind to macromolecules in hepatocytes such as DNA, proteins, and lipids in hepatocytes, leading to lipid peroxidation reactions. The CCl_4-induced liver injury model is therefore commonly employed to evaluate the hepatoprotective effects of antioxidative compounds both in *vivo* and *in vitro* [80].

Here, I prepared a liposomal formulation containing ferulic acid as a means to improve the solubility and stability of ferulic acid. I examined the anti-oxidative activity of ferulic acid as a liposomal formulation and its therapeutic potential against ROS induced by CCl_4 both *in vivo and in vitro*.

2.2. Materials and Methods

2.2.1. Materials

Ferulic acid was purchased from LKT Laboratories, Inc (Tokyo, Japan) and Egg phosphatidyl choline (EPC) was obtained from NOF Corporation (Tokyo, Japan). Amino phenyl fluorescein (APF) was purchased from Goryo Chemical (Sapporo, Japan). CCl_4 was brought from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). All reagents used in this study were high grade commercially available products. Seven-week old male Wistar rats (weighing 180 to 200 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Ethical permission and approval of the animal experiments was obtained by the Animal and Ethics Review Committee of Tokushima University.

2.2.2. Determination of DPPH radical scavenging capacity of ferulic acid

2,2-Diphenyl-1-picrylhydrazil (DPPH) radical scavenging activity of ferulic acid was measured according to an established method with some modifications [81]. Under oxidizing conditions, DPPH radicals form a purple color in ethanol solution, which becomes pale yellow upon reaction with antioxidants. 1ml of 500 µM ferulic acid (final concentration: 20 µM), 14 ml of 10 mM Tris/HCl (final concentration: 5.6 mM), 10 ml of 125 µM DPPH solution (final concentration: 50 µM) were added to an Erlenmeyer flask. After mixing with a stirrer, the absorbance was measured spectrophotometrically at 517 nm. α-Tocopherol was used as a positive control. DPPH radical scavenging activity (%) was calculated using the following equation:

% DPPH radical scavenging activity = $\{(A_0 - A_1) / A_0\} \times 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance of the extractives/standard.

2.2.3. Preparation of liposomes encapsulating ferulic acid

Liposomes were prepared using EPC and ferulic acid by conventional lipid film hydration methods. A chloroform solution containing 100 µl of 200 µM EPC (final concentration: 20 µM) and 10 µl of 20 µM ferulic acid (final concentration: 0.2 µM) was dried using a rotary evaporator (N-1000, EYELA, Tokyo, Japan) to form thin lipid films. Hydration of the dried lipid films was carried out by addition of 1ml of phosphate-buffered saline (PBS) at room temperature. The liposomal suspension then underwent freeze-thaw treatment for a total of 3 times. A polycarbonate membrane filter with 100 nm pores (Nuclepore, Cambridge, MA) was used to extrude the liposomal suspension. Size (diameter in nm), polydispersity index and zeta potential (mV) of the liposomes (Table 1) were determined using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK).

2.2.4. Measurement of encapsulation efficiency of ferulic acid containing liposomes

Encapsulation efficiency of the ferulic acid containing liposomes (ferulic-lipo) was measured by ultraviolet-visible (UV-Vis) spectrophotometric methods. Ferulic-lipo (0.5 µM/ml) was prepared following the method described above, and the suspension was treated with Triton X-100 (Sigma Science, Albuquerque, NM) to lyse the lipid vesicles. Free ferulic acid was separated from the liposomes by gel filtration using a PD-10 column (GE Healthcare Biosciences KK, Tokyo, Japan). Briefly, ferulic-lipo (1 ml) was loaded onto a PD-10 column and eluted with PBS to obtain the liposomal filtration (1.8 ml). Then, 500 µl of the filtered liposomes solution (final concentration: 0.14 µM), 500 µl of 10% Triton X-100 (final concentration: 1%), and 4ml PBS were added to a test-tube and incubated for 15 min at 65^{o}C. After vortexing the solution, the absorbance of ferulic acid was measured at 322 nm using a

UV-Vis spectrophotometer (DU-800, Beckman Coulter, Indianapolis, IN). A Standard calibration curve was generated by preparing various solutions of ferulic acid at known concentrations. Encapsulation efficiency (EE%) was calculated using the following equation:

$$EE\ (\%) = (Weight_{\text{total drug}} - Weight_{\text{free drug}}) / Weight_{\text{total drug}} \times 100\ \%$$

2.2.5. Evaluation of scavenging activity of liposomes against hydroxyl radicals

Hydroxyl radicals are generated by a chemical reaction known as the Fenton reaction [82]. As follows:

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

APF acts as an indicator of reactive oxygen species (ROS) generation. This fluorescein remains non-fluorescent until reaction with a hydroxyl radical; after reacting with a free radical APF becomes fluorescent. The fluorescence intensity of APF was measured to evaluate hydroxyl radical production [83]. To generate hydroxyl radicals, 165 µl of DDW, 30 µl of a 100 µM of APF solution (final concentration: 10 µM), 45 µl of a 10 mM liposomal suspension (final lipid concentration: 1.5 mM), 30 µl of a 10 mM H_2O_2 solution (final concentration: 1 mM), and 30 µl of a 1 mM $FeSO_4$ solution (final concentration: 100 µM) were added to microplate well and mixed. The fluorescence intensity (excitation: 490 nm; emission: 515 nm) was measured using an Infinite M200 microplate reader (Tecan Group Ltd., Switzerland) within 10 s.

2.2.6. Estimation of CCl_4-mediated cytotoxicity *in vitro*

HepG2 cells (2 $x10^5$ cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C and maintained in 5% atmospheric

CO_2 for 72 h. After removing the medium, DMEM containing 75 µM of CCl_4 was added to the cells. The cells were then incubated with the medium containing CCl_4 for 30 min at 37ºC. Cells were then further treated with DMEM containing 50 µM, 75 µM or 100 µM of 50 mM ferulic acid liposomes and incubated at 37ºC for 12 h. Following incubation, DMEM was removed, and the cells were washed with PBS. After treatment with trypsin, cells were collected and stained with trypan blue (0.4%), and the number of viable cells and total cells were counted.

2.2.7. Preparation of CCl_4-induced liver injury rat model

The CCl_4- induced liver injury rat model was constructed based on previously published reports with some modifications [84]. Anesthetization of rats was carried out using a small animal anesthetizer (Bio Machinery, Japan). Hepatic injury was induced by intraperitoneal administration of 10% CCl_4 (1 ml/kg B.W) diluted in liquid parafilm (1:9 ratio). CCl_4-induced liver injured rats were used as a model of acute live injury in this study.

2.2.8. Determination of hepatoprotective effects of ferulic-lipo

To determine the hepatoprotective effects of ferulic-lipo, rats were administered ferulic-lipo intravenously at 30 min after CCl_4 administration. The injected dose of EPC and ferulic-lipo were 25 µmol/kg and 2.5 µmol/kg, respectively. After 24 h of liposome administration, rats were sacrificed, and blood was collected from rat hearts under anesthetic conditions. Blood samples were subsequently incubated for 3 h at room temperature and then centrifuged at 1000 x g for 10 min at 4ºC for serum collection. The activities of hepatic marker enzymes aspartate transaminase (AST) and alanine aminotransferase (ALT) were measured using a commercially available kit (transaminase CII-Test Wako).

2.2.9. Histopathological observation of liver damage.

Liver tissue samples from sacrificed rats were collected and fixed into optimal cutting temperature (OCT) compound (Sakura Finetek, Tokyo, Japan) and finally stored under frizzing conditions using dry ice/ethanol. The frozen liver tissue samples were cut into 10 µm thick tissue sections using a cryostat (CM3050S, Leica Biosystems, Tokyo, Japan). The sections were then mounted into MAS (strong hydrophilic adhesive)-coated glass slides with Perma Flour Aqueous Mounting Medium (Thermo Fisher Scientific, Waltham, MA). Hematoxylin and eosin (H & E) staining of liver tissues were performed to observe morphological changes in treated rats. Briefly, sections were enclosed with a PAP pen to prepare a hydrophobic membrane. After embedding in 4% paraformaldehyde for 10 min, the sections were washed with PBS. The sections were then stained with Mayer's Hematoxylin solutions (Fuji Film Wako Pure Chemical, Osaka, Japan) and incubated for 10 min at room temperature, and subsequently stained with 1% eosin (Fuji Film Wako Pure Chemical) for 1 min at room temperature. Ethanol (80% to 100%) was used to dehydrate the sections, which were then cleared with xylene. A hydrophobic mounting agent (Entellan® New, Merck Millipore, Burlington, MA) was used for mounting the sections. Finally, sections were observed using a fluorescence phase contrast microscope (BZ-9000, Keyence, Osaka, Japan).

2.2.10. Observation of CCl_4-induced free radical production.

Dihydroethidium (DHE) was used as a fluorescent probe to evaluate ROS generation induced by CCl_4. Frozen liver tissue sections (10 µm) were used for this experiment and prepared according to the method described above. Briefly, prepared tissue sections were washed with PBS and then incubated in 50 µM DHE (dissolved in methanol) at 37^{o}C for 30

min under conditions to avoid exposure to light. Slides containing the sections were then washed with PBS and treated with 4% paraformaldehyde for 10 min at room temperature. After washing with PBS, the slides were mounted. The sections were then incubated overnight at room temperature. Finally, fluorescence of DHE in the tissue sections was visualized by confocal laser scanning microscopy (LSM700, Carl Zeiss, Jena, Germany).

2.2.11. DNA fragmentation assay

DNA was isolated from the liver tissue according to the protocol provided by the kit manufacturer (NucleoSpin Tissue Kit, Macherey-Nagel, Germany). Briefly, 25 mg of liver tissue was collected and homogenized using 50 µl of PBS (-). Prior to lysing, 180 µl of Buffer T1 (pre-lyse buffer) and 25 µl of protein kinase was added. After mixing, the solution was incubated at 56ºC for 2 h. After vortexing, 200 µl of Buffer B3 (lyse buffer) was added and the solution was further incubated at 70ºC for 20 min for lysis. To adjust for DNA binding conditions, 210 µl of ethanol was added to the sample and the sample was then vigorously vortexed. To allow for DNA binding, the sample was transferred onto a tissue column in a collection tube and centrifuged at 11000 x g for 1 min. After transferring the tissue column to a new collection tube, 500 µl of Buffer BW (wash buffer) was added and the sample was centrifuged at 11000 x g for 1 min. Another wash was then carried out using 600 µl of Buffer B5 (wash buffer). Residual ethanol was removed by centrifuging the column for 1 min at 11000 x g. Elution Buffer BE (elution buffer, 100 µl) was then added to elute pure DNA. The DNA concentration was measured using a Nanodrop 8000 (Thermo Fisher Scientific). For gel electrophoresis, 2% agarose gel was used, and isolated DNA (600 ng) was applied onto the prepared gel for each of the following groups: control group, CCl_4 group, and CCl_4 + ferulic acid group. The supplied DNA ladder was used as a marker DNA. Agarose gel electrophoresis was carried out at 100 mA for 40 min. Following electrophoresis, staining was performed using

gel red. Finally, the DNA band was observed using a lumino image analyzer (LAS- 4000 mini, Fujifilm Life Science, Tokyo, Japan).

2.2.12. Statistical analysis

Statistical analysis was carried out using one-way ANOVA followed by the Tukey post-hoc test. Student's t-test was also used to determine differences between the two groups. Data were reported as means ± standard deviations (S.D.). *p*-values <0.05 were considered to be significant.

2.3. Results

2.3.1. DPPH radical scavenging potency of ferulic acid

First, I measured the DPPH radical scavenging potency of ferulic acid in ethanolic solution to assess its antioxidative properties. DPPH radical scavenging activity was evaluated in a time-dependent manner (0 min, 15 min, 30 min, 60 min, 90 min, and 120 min) and compared with the well-known antioxidant α-tocopherol (Fig-2.1). Here, α-tocopherol showed higher DPPH radical scavenging potency than ferulic acid (Fig-2.1A). As shown in Fig-2.1B, particularly at 15 min, DPPH radical scavenging activity of 20 μM α-tocopherol was higher than that of 20 μM ferulic acid.

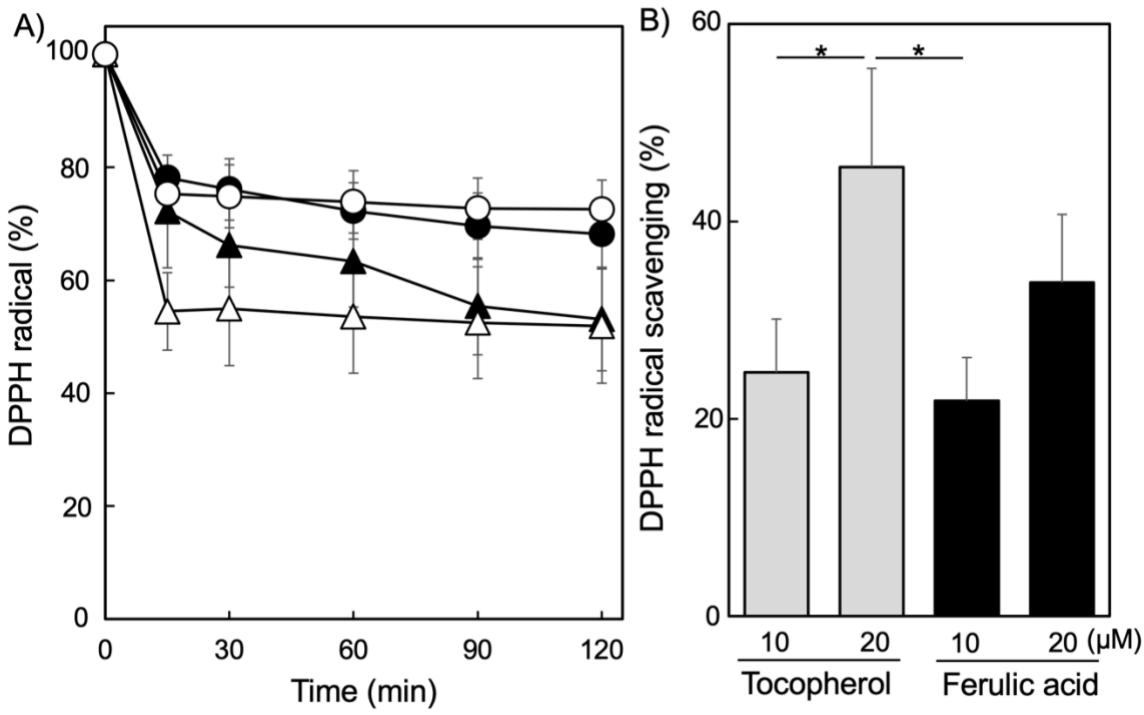

Fig. 2.1. Effects of ferulic acid and α- tocopherol on DPPH radicals

(A) Time dependent changes of DPPH radical by addition of ferulic acid at 10 μM (●) and 20 μM (▲) or α- tocopherol at 10 μM (○) and 20 μM (△) were assessed by measuring absorbance at 517 nm. (B) Percentage of DPPH radical scavenging by ferulic acid (black column) and α- tocopherol (grey column) measured at 15 min after addition of the compounds. Data are means ± S.D. (n=3). *p< 0.05.

2.3.2. Characteristics of liposomal formulations

To overcome the issue of low solubility of ferulic acid in water, I prepared liposomes containing ferulic acid (ferulic-lipo) and evaluated their physiochemical properties. The particle sizes of ferulic-lipo and control liposomes were approximately 100-110 nm (Table 2.1.1). The Zeta potential of ferulic-lipo was -7.37 mV (Table 2.1.1). The polydispersity index (PDI) values for ferulic-lipo and EPC-lipo were 0.16 and 0.14, respectively (Table 2.1.1). The encapsulation efficiency of ferulic acid in liposomes was about 92%.

Table 2.1.1. Physiochemical properties of Ferulic-liposomes

Sample	Size (nm)	Polydispersity index	Zeta potential (mV)
EPC-lipo (control)	112.5 $\pm$ 2.9	0.14 $\pm$ 0.03	-10.5 $\pm$ 0.35
Ferulic-lipo	109.3 $\pm$ 5.2	0.16 $\pm$ 0.02	-7.37 $\pm$ 0.67

Data are means $\pm$ SD of three samples prepared on different days.

2.3.3. Effect of ferulic-lipo on hydroxyl radical scavenging

After preparing ferulic-lipo, I first checked whether the liposomal formulation exhibited hydroxyl radical scavenging activity. Ferulic-lipo exhibited significant preventive effects (20 μM) compared to EPC-lipo based on fluorescence intensity measurement of hydroxyl radical generation (Fig-2.2). Tocopherol-containing liposomes also showed significant preventive effects compared to EPC-lipo. Further, ferulic-lipo showed a greater tendency to scavenge hydroxyl radicals than tocopherol-containing liposomes.

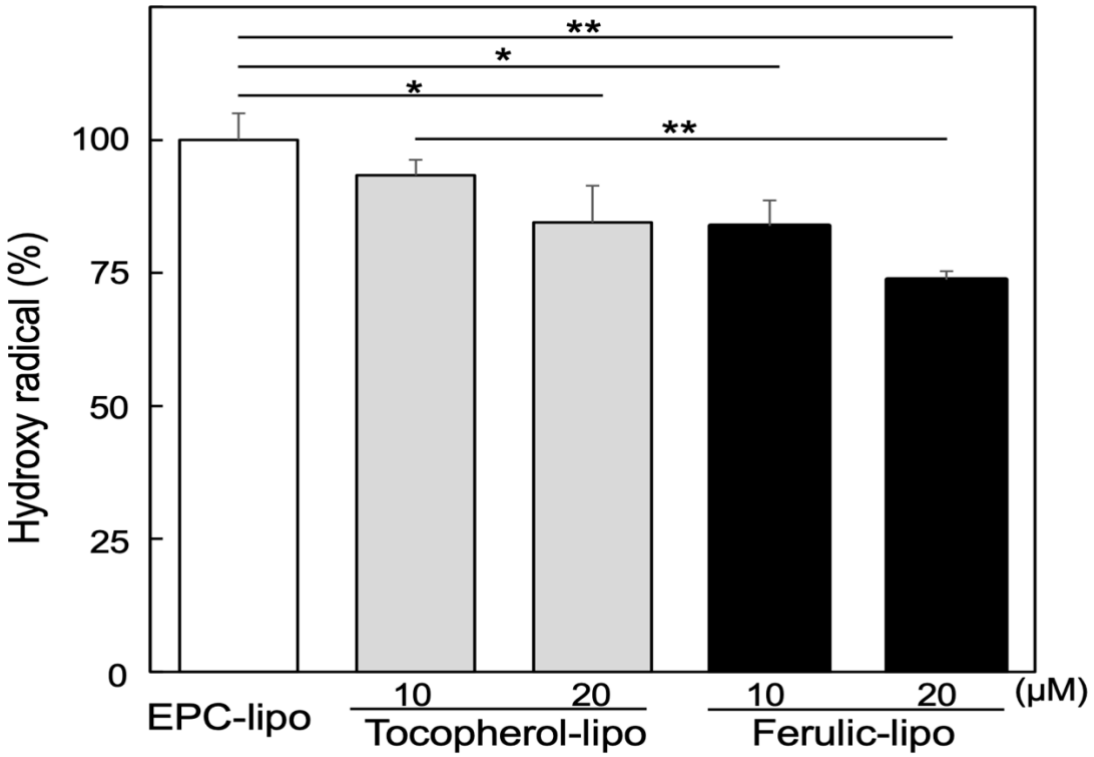

Fig. 2.2. Effects of liposomes encapsulating ferulic acid or α- tocopherol on hydroxyl radicals

Hydroxyl radicals generated via Fenton reaction in the presence of liposomes encapsulating ferulic acid (black column) or α- tocopherol (grey column) were evaluated by the fluorescence intensity of APF. Data are means ± S.D. (n=3). *p< 0.05, **p<0.01.

2.3.4. Ferulic-lipo protects against CCl_4-induced cytotoxicity in HepG2 cells

As ferulic-lipo showed significant antioxidative effects, I examined whether ferulic-lipo also exhibited protective effects against CCl_4-induced toxicity in human hepatocarcinoma (HepG2) cells. As shown in Fig-2.3, treatment with CCl_4 markedly reduced the viability of HepG2 cells (~ 50%) compared to the control group. Moreover, ferulic-lipo at a concentration of 50 μM did not improve cell viability relative to the CCl_4-treated groups. However, at a concentration of 75 μM, ferulic-lipo significantly improved cell viability (by almost 20%) but was not so potent. Furthermore, ferulic-lipo at a concentration of 100 μM showed a highly significant improvement in cell viability (~ 30%).

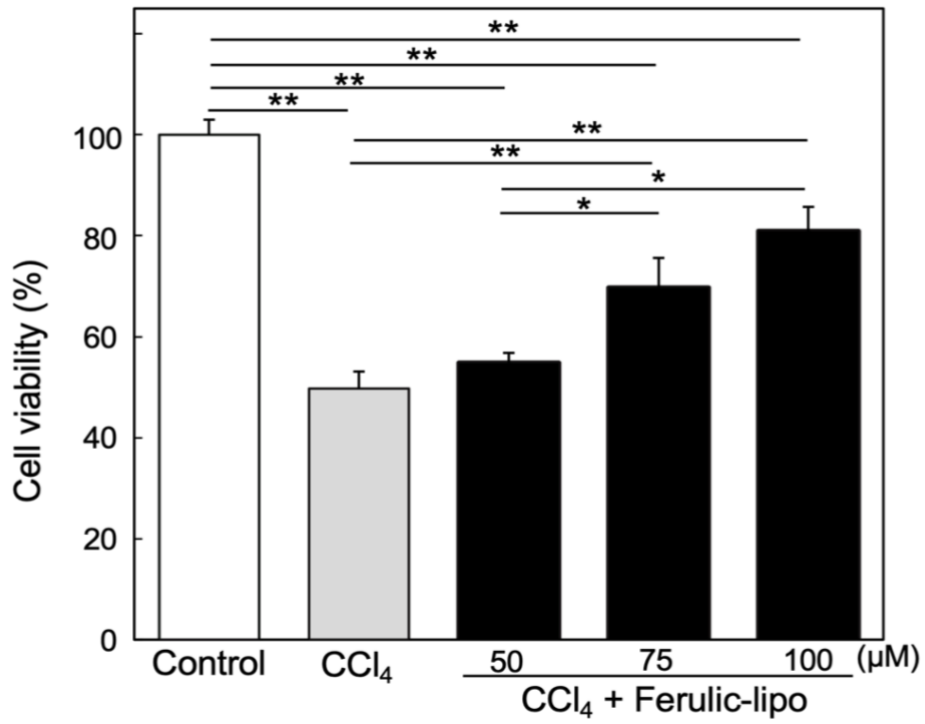

Fig. 2.3. Cell viability of HepG2 treated with CCl_4 in the presence of liposomes encapsulating ferulic acid

Cells were treated with 75 μM of CCl_4; liposomes were added 30 min after CCl_4 treatment. Cell viability was determined after 12 h. Control (white column), CCl_4 treatment (grey column) and CCl_4 + ferulic-lipo (black column). Data are means ± S.D. *p<0.01, **p<0.001.

2.3.5. Effect of ferulic-lipo on CCl_4-induced liver injury *in vivo*

Based on the inhibitory effects of ferulic-lipo against CCl_4-mediated cytotoxicity found in HepG2 cells *in vitro*, I examined the effects of ferulic-lipo *in vivo* in a CCl_4-induced rat model of liver injury. As the extent of liver damage can be evaluated based on the leakage of liver enzymes into the blood stream, levels of the hepatic enzymes AST and ALT were measured. After treatment with CCl_4, serum AST and ALT levels were greatly increased to approximately 500 IU/L and 200 IU/L (Fig-2.4), respectively, indicating severe liver damage. Here, Ferulic-lipo was administrated intravenously to CCl_4-treated rats. Contrary to expectations, ferulic-lipo at a concentration of 0.025μmol/kg did not reduce elevated serum enzyme levels. After increasing the administered amount of ferulic-lipo to 0.25 μmol/kg, I found that serum AST and ALT levels were reduced by 35% and 30%, respectively. However, ferulic-lipo administered at 0.25 μmol/kg was not potent enough to prevent CCl_4-induced hepatotoxicity. I increased the amount of administered ferulic-lipo further to 2.5 μmol/kg, which showed very potent preventive effects on CCl_4-induced hepatotoxicity.

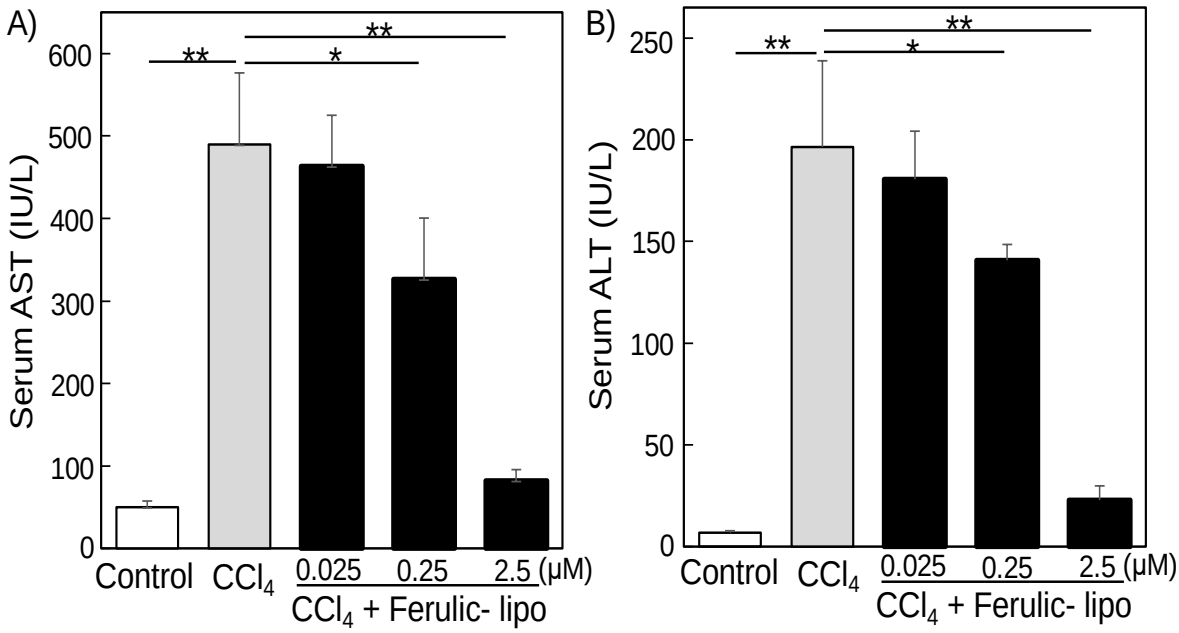

Fig. 2.4. Effect of ferulic-lipo on CCl_4-induced liver injury in rats

Liver injury was induced by CCl_4. 30 min after CCl_4 treatment, ferulic-lipo were intravenously administrated. Serum was collected after 24 h, and the enzymatic activities of AST and ALT were determined. Control (white column), CCl_4 treatment (grey column) and CCl_4 + ferulic-lipo (black column). Data are means $\pm$ S.D. (n=3). *p<0.05, **p< 0.001.

2.3.6. Effect of ferulic-lipo on oxidative stress induced by CCl_4

It is well known that CCl_4-induced hepatotoxicity depends on production of ROS in the liver [86]. Therefore, I measured hepatocyte ROS generation in the liver after CCl_4 administration. As shown in Fig-2.5A, fluorescence indicating ROS generation was clearly observed in CCl_4 treated group, confirming the presence of CCl_4-induced ROS generation in the liver. On the other hand, fluorescence in the liver section after administration of ferulic-lipo was markedly reduced, as shown in CCl_4+Ferulic-lipo treated group. Furthermore, fluorescence intensity, as quantified by image analysis of liver tissue treated with ferulic-lipo was significantly lower (~ 50%) than that of CCl_4-treated liver tissue (Fig-2.5B).

These results suggest that intravenous administration of ferulic-lipo can effectively inhibit oxidative stress mediated by CCl_4 in the liver.

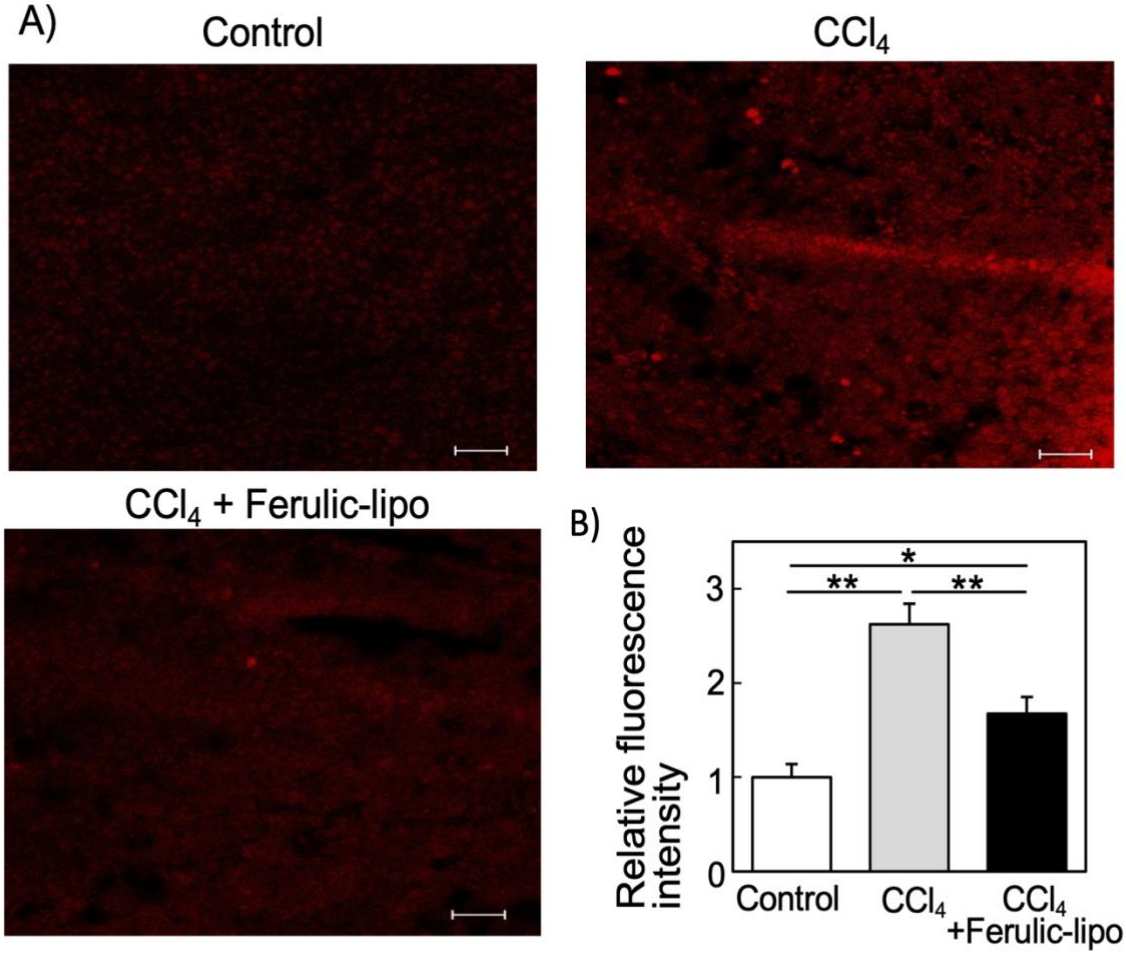

Fig. 2.5. Effect of ferulic-lipo on CCl_4-induced ROS generation in the liver

(A) Confocal laser scanning microscopic images of liver sections stained with dihydroethidium (DHE) represented as control liver tissue, CCl_4-treated liver tissue and CCl_4-treated liver tissue after intravenous administration of ferulic-lipo. (B) Quantified relative fluorescence intensity indicating ROS generation in the liver. Data are means $\pm$ SD (n=3). *p<0.01, **p<0.001. Scale bar = 100 μM.

2.3.7. Effect of ferulic-lipo on CCl_4-induced histopathological damage.

Liver damage was further assessed by histologic observation of liver sections stained with hematoxylin & eosin. Hepatocyte damage was clearly observed in CCl_4-treated liver tissue near the central vein region (C) compared to the control group (Fig-2.6). As shown in Fig-2.6A, there was a white section in the liver tissue treated with CCl_4, which may represent necrotic cell death according to previously published reports. The white section was significantly decreased (by almost 50%) following treatment with ferulic-lipo (Fig-2.6B). Taken together, these results, suggest that ferulic-lipo significantly prevent CCl_4-induced tissue damage in the liver.

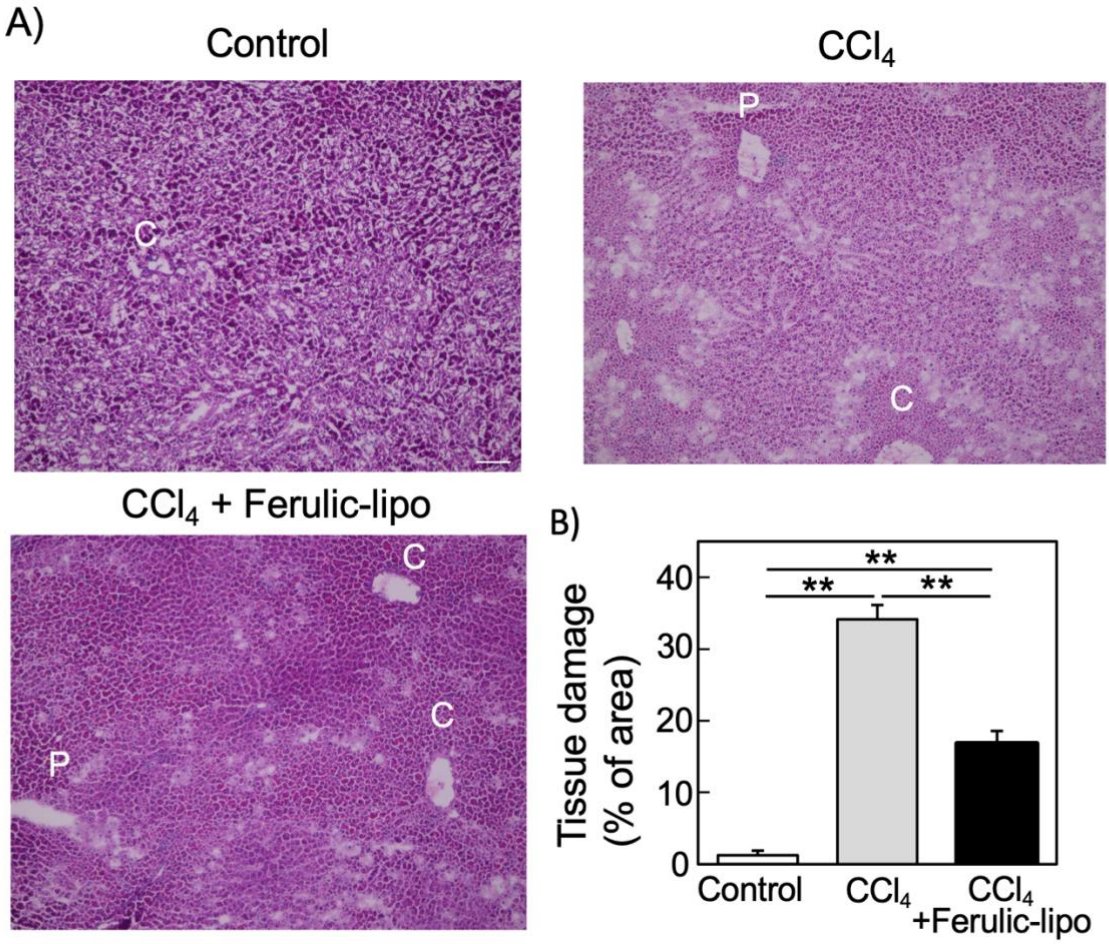

Fig. 2.6. Effect of ferulic-lipo on CCl_4-induced histological changes in liver tissue

(A) Microscopic images of liver sections stained with hematoxylin-eosin (H&E) represented as control liver tissue, CCl_4-treated liver tissue, and CCl_4-treated liver tissue after intravenous administration of ferulic-lipo. Here, the portal vein and the central vein were denoted by P and C. (B) quantitative estimation of tissue damage in the liver. Data are means ± SD (n=3). **p<0. 001. Scale bar = 100 µM.

2.3.8. Effect of ferulic-lipo on CCl_4-induced DNA fragmentation.

It is well known that fragmentation of the double strand of the DNA is a hallmark of apoptosis induced by ROS [88]. Therefore, I assessed CCl_4-induced DNA fragmentation in liver tissue using agarose gel electrophoresis. I observed that CCl_4 treatment induced a high degree of DNA fragmentation in the liver (Fig-2.7). On the other hand, DNA fragmentation was significantly reduced following administration of ferulic-lipo. These results suggest that ferulic-lipo treatment can inhibit CCl_4-induced apoptosis.

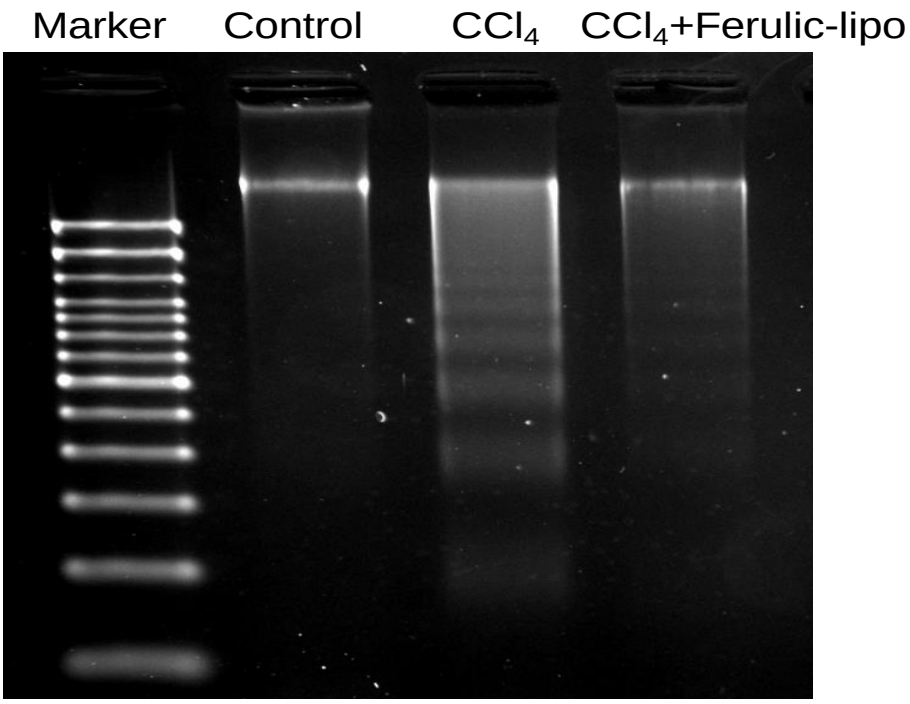

Fig. 2.7. Effect of ferulic-lipo on CCl_4-induced DNA fragmentation

DNA extracted from the liver tissue of untreated rats (control), CCl_4-treated rats (CCl_4), and CCl_4-treated rats after intravenous administration of ferulic-lipo (CCl_4 + ferulic-lipo) were subjected to agarose gel electrophoresis.

2.4. Discussion

The hydrophobicity and instability of ferulic acid are major obstacles to its wide application as a potent antioxidative compound. In this study, I prepared a liposomal formulation of ferulic acid (ferulic-lio) to improve its insolubility problem and examined its effects against the CCl_4-induced liver-injured model (*in vitro & in vivo study*). First, I checked the DPPH radical scavenging property of free ferulic acid in an ethanolic solution and compared it with a standard antioxidant α-tocopherol. It reported that tocopherols react with DPPH radicals by donating a hydrogen ion from the hydroxyl group on the chroman ring [85]. As ferulic acid is a known antioxidant, I expected the scavenging potency for DPPH radicals to be high. However, the addition of α-tocopherol immediately decreased the absorbance of the DPPH radical dose-dependently (Fig-2.1A). Contrary to expectations, the absorbance of DPPH radical gradually decreased by addition of ferulic acid. Especially, at 15 min after addition of both compounds, DPPH radical scavenging activity of 20 μM α-tocopherol was higher than that of 20 μM ferulic acid (Fig-2.1B). Based on the chemical structures of α-tocopherol (Fig-2.8) and ferulic acid, the DPPH radical scavenging activity of these two compounds was expected be similar since they both contain the same number of phenolic hydroxyl groups. However, the results of this study suggest that the methoxy group of ferulic acid may result in relatively larger steric hindrance than the methyl group of α-tocopherol while reacting with DPPH radicals. Thus, the lower probability of interaction with DPPH radicals may be responsible for the lower radical scavenging activity of ferulic acid compared to α-tocopherol.

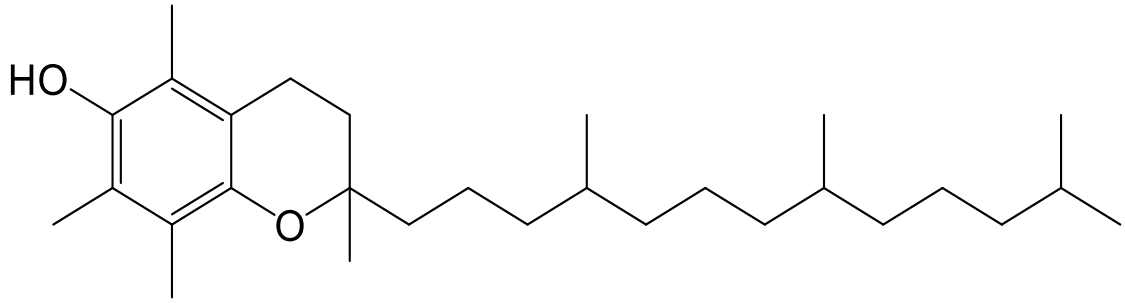

Fig. 2.8. Chemical structure of α-tocopherol

After preparing liposomal formulation of ferulic acid (ferulic-lipo), I assessed its physiochemical characteristics. Physiochemical properties (Table 2.1.1) results demonstrated the successful preparation of a liposomal formulation of ferulic acid with a high encapsulation efficiency (92%). Next, I evaluated ferulic lipo's hydroxyl radical scavenging capacity and compared it with the liposomal formulation of α-tocopherol (Tocopherol-lipo). Ferulic-lipo showed relatively higher hydroxyl radical scavenging capability than tocopherol-lipo (Fig-2.2). According to chemical structure of α-tocopherol, it is assumed that the active site comprised of a phenolic hydroxyl moiety might exist at the interface between the hydrophobic and hydrophilic regions of the liposomes. On the other hand, the active site of ferulic acid might exist on the surface of the liposomes as a result of its two polar hydroxyl groups. The opportunity for liposomal formulations to react with hydroxyl radicals depends on the target antioxidants' chemical structure and its position in the lipid bilayer environment. Therefore, based on the results of the current study, it is suggested that the higher hydroxyl radical scavenging activity observed for ferulic-lipo (compared to α-tocopherol-containing liposomes)

is due to the increased opportunity of ferulic acid in the liposomes to interact with hydroxyl radicals generated in aqueous solution, as a result of the location of the active phenolic hydroxyl moiety. These results demonstrate that ferulic acid's liposomal formulation exhibits a relatively higher hydroxyl radical scavenging activity than α-tocopherol. Based on these findings, I checked the protective effects of ferulic-lipo against CCl_4-induced toxicity in HepG2 cells (Fig-2.3). I observed that ferulic-lipo significantly improves the viability of HepG2 cells in a dose-dependent manner. It was assumed that significant preventive effects of ferulic-lipo against CCl_4-induced toxicity as a result of its antioxidative capacity. Subsequently, I examined the inhibitory effects of ferulic-lipo against the CCl_4-induced rat model of liver injury based on the results of *in vitro* study. First, I examined serum AST and ALT levels to assess hepatic damage after treatment with CCl_4. Serum enzyme level markedly increased with the CCl_4 treatment, as shown in Fig-2.4. Intravenous administration of ferulic-lipo (2.5 μmol/kg) significantly reduced serum enzyme level dose-dependently (Fig-2.4). Based on these results, ferulic-lipo possesses potent hepatoprotective effects against CCl_4-induced hepatotoxicity *in vivo* due to its antioxidative activity. As CCl_4-induced hepatoxicity is related to ROS production, I checked the effect of ferulic-lipo in preventing ROS generation in the liver. Interestingly, ferulic-lipo significantly prevents (~50%) ROS generation in the liver, as shown in Fig-2.5A & 2.5B. The hepatoprotective effects of ferulic-lipo were further assessed by performing H &E staining. It has been recognized that CCl_4-mediated liver tissue damage is characterized by happening surrounding the central vein region [87]. As shown in (Fig-2.6A), hepatocyte damage was observed near the central vein region after treatment with CCl_4 and was significantly reduced (~50%) with ferulic-lipo administration (Fig-2.6B). These results suggest that ferulic-lipo's antioxidant activity effectively inhibits CCl_4-induced tissue damage and ROS generation in the liver. After that, I assessed CCl_4-induced DNA fragmentation in the

liver (Fig-2.7). DNA fragmentation was significantly reduced following the administration of ferulic-lipo might be due to its potent antioxidative effects.

2.5. Conclusion

The results of the present study demonstrate that liposomes encapsulating ferulic acid exert potent antioxidative activity. Ferulic-lipo significantly reduced cytotoxicity induced by CCl_4 *in vitro*. Moreover, intravenous administration of ferulic-lipo effectively attenuated CCl_4-induced hepatotoxicity, ROS generation, and tissue damage in an *in vivo* rat model. Results of this study suggest that liposomal formulations containing ferulic acid may offer a promising approach for the treatment of oxidative stress-related diseases of the liver.

Chapter III

Improvement of stability of Ferulic acid-liposomal formulation using γ-oryzanol as a prodrug

Chapter III: Improvement of stability of Ferulic acid-liposomal formulation using γ-oryzanol as a prodrug

3.1. Introduction

In the second part of this study, I found that ferulic lipo can react with reactive oxygens species. Therefore, the liposomal formulation of ferulic acid can be oxidized before reach to the target site. So, a more stable formulation of ferulic acid would be required for its potential inhibitory effects against oxidative stress. Besides, it's reported that γ- oryzanol (Fig-3.1) proneness rapidly hydrolyzed into ferulic acid inside the body [80]. Therefore, I hypothesized that γ- oryzanol would be a prodrug of ferulic acid.

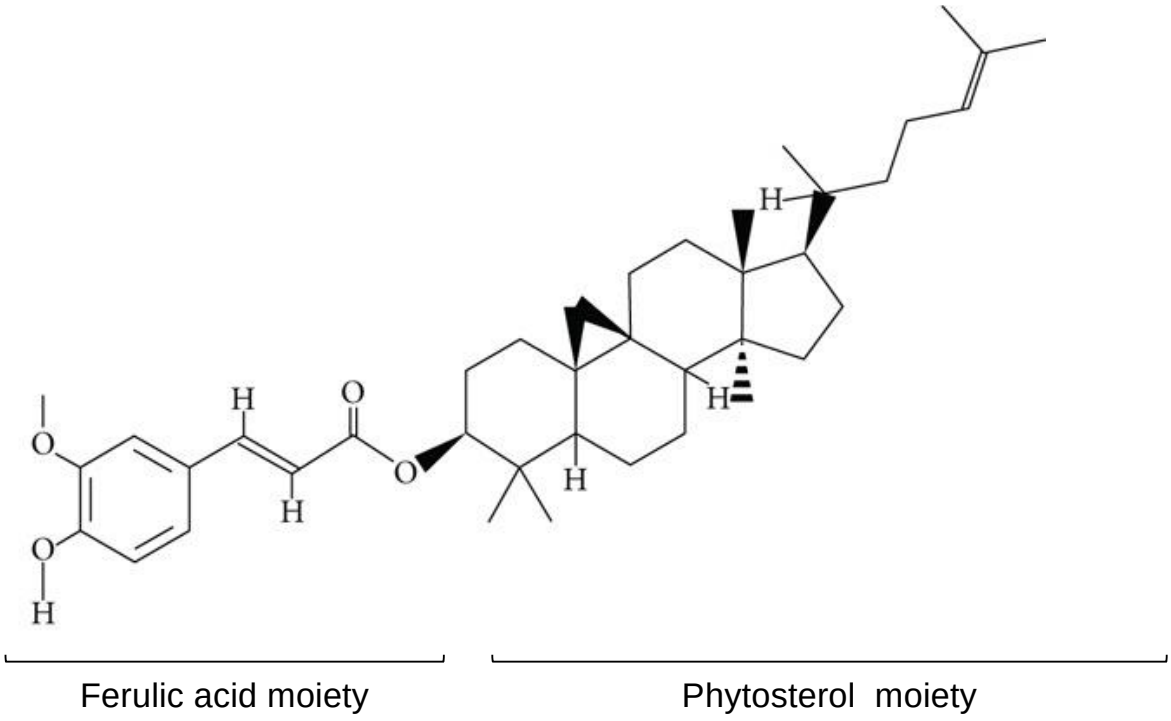

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Fig. 3.1. Chemical structure of γ- oryzanol

Generally, prodrugs are pharmacologically inactive compounds that undergo biotransformation in the body and become converted to their active constituents through enzymatic or chemical cleavages [89]. Prodrug approaches are used when a pharmacologically active drug suffers from poor solubility, poor permeability and instability, short half-life, incomplete absorption, and poor site specificity problems [90]. To date, the number of prodrugs designed to improve the physicochemical and biopharmaceutical properties of parent's drugs. Previously some publications showed prodrug's role in improving stability and solubility problems. Likewise, curcumin digluratic acid, a prodrug formulation of curcumin delineated to show enhanced water solubility and oral bioavailability than free curcumin for treating neuropathy [91]. 5-Fluorouracil is used for the treatment of solid malignant tumors. However, its broad application is restricted due to poor distribution and extensive fast metabolism. Capecitabine (Xeloda), a prodrug of 5-Fluorouracil, was reported to show improved physiochemical and pharmacokinetics properties than 5-Fluorouracil [92]. Besides, resveratrol triacetate, a prodrug of resveratrol, exhibited significantly higher plasma concentration than resveratrol in a rat model [93]. Furthermore, Quercetin pentabenzensulfonate, a prodrug of quercetin, demonstrated higher solubility than quercetin in apolar solvent and methanol [94]. Thus, the prodrug strategy is effective in solving instability, insolubility, and low bioavailability of biologically active drugs.

Moreover, it's reported that the formulation of prodrugs into proper formulations, like liposomes, offers several benefits, such as improved pharmacokinetics, enhanced pharmacological half-life, and increased absorption [95]. It has also been demonstrated that liposomes increase the therapeutic efficiency of encapsulated drugs compared to free drugs and protect them from enzymatic degradation and hydrolysis [96]. Based on these findings, liposomes could be a suitable drug delivery carrier for γ- oryzanol making it a more stable and administrable formulation.

Regarding ferulic acid, γ-oryzanol may be a natural prodrug formulation of ferulic acid because it rapidly hydrolyzes to ferulic acid in the body. Therefore, in this study, I focused on γ-oryzanol as a ferulic acid prodrug to improve ferulic acid's stability. For this, I prepared a liposomal formulation of γ-oryzanol (γ-ory-lipo) and examined its chemical stability. Furthermore, I assessed the antioxidant activity of γ-ory-lipo and compared it with ferulic-lipo against CCl_4-induced oxidative liver damage in both *in vitro* and *in vivo* conditions.

3.2. Material and methods

3.2.1. Materials and animals:

γ-Oryzanol has purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), and Egg phosphatidylcholine (EPC) was purchased from NOF Corporation (Tokyo, Japan). CCl_4 and transaminase CII-Test Wako were brought from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Amino phenyl fluorescein (APF) was purchased from Goryo Chemical (Sapporo, Japan). C_{18} (octadecyl silyl) HPLC column (TSK gel ODS-80 TM) was brought from Tosoh Corporation (Tokyo, Japan). HPLC-grade methanol acetonitrile was used for HPLC analysis. All other reagents and solvents were high-grade, commercially available analytic samples. Seven- week old male Wistar rats (weighing 180 to 200 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan). The Animal and Ethics Review Committee of Tokushima University obtained ethical permission and approval for the animal experiments.

3.2.2. Preparation of liposomes encapsulating γ-oryzanol

γ-Ory-lipo was prepared using EPC, and γ-oryzanol by lipid hydration method. Briefly, the chloroform solution having 100 μl of 200 μM EPC (final concentration: 20 μM) and 10 μl of 20 μM γ-oryzanol (final concentration: 0.2 μM) was taken in the Erlenmeyer flask and dried using a rotary evaporator (N-1000, EYELA, Tokyo, Japan) to form lipid films. Then 1ml of PBS was added for the hydration of lipid films. After freeze-thaw treatment, the liposomal suspension was extruded using a poly-carbonate membrane filter with 100nm pores (Nuclepore, Cambridge, MA). Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK)

was used for the determination of liposomes Size (diameter in nm), polydispersity index, and zeta potential (mV).

3.2.3. Measurement of chemical stability of γ-ory-lipo by HPLC

The HPLC analysis system included C_{18} analytical column having 5 μm particle size and 4.6 mm I.D x 15cm column size at 25ºC. For ferulic acid, the mobile phase consists of methanol and water (48:52 v/v) adjusted to pH 3.0 using o.1 N orthophosphoric acid with an isocratic flow rate of 1.0 ml/min. The sample size was 20 μl, the detection wavelength 321nm, and the run time was 10 min. For γ-oryzanol, the mobile phase containing acetonitrile and methanol (60:40 v/v) with a flow rate of 1.5 ml/min. The wavelength used to detect γ-oryzanol was 325 nm with injecting volume of 20 μl with a run time was 25 min. For analyzing chemical stability, γ-Ory-lipo (2 μM/ml) was prepared following the above-described procedure. After removing free γ-oryzanol using a PD-10 column (2^{nd} part material and methods), γ-ory-lipo (0.1 μM/ml) was incubated with PBS at 37^0C for 24h. After incubation, the sample was analyzed using HPLC (Shimadzu, Kyoto, Japan). The chemical stability of γ-ory-lipo was determined by measuring the peak area and subsequently compared with free γ-oryzanol suspension (0.1 μM/ml), maintaining the same conditions.

3.2.4. Evaluation of hydroxyl radical scavenging property of γ-ory-lipo

It is well known that Hydroxyl radicals are produced by the Fenton reaction [82]. APF acts as a marker of reactive Oxygenase species generation. After reacting with ROS, APF

became fluorescent, and fluorescence intensity was measured to evaluate hydroxyl radical formation. Briefly, 165 µl of DDW, 30 µl of a 100 µM of APF solution (final concentration: 10 µM), 45 µl of a 10 mM liposomal suspension containing γ-oryzanol (final lipid concentration: 1.5 mM), 30 µl of a 10 mM H_2O_2 solution (final concentration: 1 mM), and 30 µl of a 1 mM $FeSO_4$ solution (final concentration: 100 µM) were mixed for the generation of hydroxyl radicals. The fluorescence intensity was measured using an Infinite M200 microplate reader (Tecan Group Ltd., Switzerland).

3.2.5. Assessment of cell viability

HepG2 cells (2×10^5 cells) were cultured at 37°C with 5% atmospheric CO_2 for 72 h. After getting 80-90% confluency, the cells were treated with DMEM containing 100 µM of γ-ory-lipo, and ferulic-lipo, respectively, and incubated at 37°C for 1h, 3h, and 6h. After incubation, cells were washed with PBS and treated with trypsin. Finally, cell viability was measured using trypan blue staining methods. The Percentage of cell viability was assessed by dividing the number of staining cells by the total number of cells.

3.2.6. Analysis of conversion of γ-oryzanol to ferulic acid (*in vitro & in vivo*)

For *in vitro* study, 2×10^5, HepG2 cells were seeded in 35mm petri dish. After 72 h, cells were treated with DMEM containing 100 µM of γ-ory-lipo and incubated at 37°C for 1h. After that, the cells were washed with PBS and treated with lysis buffer (25 mM Tris-HCl [pH-6.5], 1% glycerol [v/v], 1% SDS [v/v], 5% 2-mercaptoethanol and phosphatase inhibitor cocktail PhosSTOP). Next, collected cells were treated with protein removal solution consisting of $HCLO_4$ (60%): acetonitrile: methanol with volume (5:30:65), and the used ratio

was 2:3 (collected cells: protein solution) and incubated at room temperature for 30 min. After incubation, solutions containing the protein removal mixture were centrifuged at 1500G for 15 min at 4^{o}C. The supernatant was collected and analyzed using HPLC (see the chromatographic condition for oryzanol & ferulic acid above 3.2.3 section). Regarding the assessment of *in vivo* conversion of γ-oryzanol to ferulic acid, the same procedure was followed with some modifications. Briefly, γ-ory-lipo at a concentration of 2.5 μmol/kg was intravenously administered to the Wistar rats and incubated for 1h. After incubation, rats were sacrificed, and the liver was collected. Next, 0.5 gm of liver tissue was added to the 15 ml falcon tube containing 4.5 ml 1X PBS and incubated for 30 min in the ice box. After incubation, the solution containing liver tissue was homogenized in the presence of ice. The homogenized solutions were centrifuged at 1000G for 10 min at 4^{o}C. The supernatant was collected and incubated with the protein removal mixture for 30 min. After centrifugation, the supernatant was collected and used for HPLC analysis.

3.2.7. Estimation of the effect of γ-ory-lipo on CCl_4-mediated cytotoxicity

HepG2 cells (2×10^5 cells) were cultured in standard conditions like DMEM containing 10% FBS at 37°C and 5% atmospheric CO_2 for 72 h. For pre-treatment of γ-ory-lipo, the cells were firstly treated with DMEM having 100 μM of 50 mM γ-ory-lipo for 1h. Afterward, 75 μM of CCl_4-contained DMEM solution was added to the cells. After completing treatment, cells were maintained at 37°C for 12 h. For post-treatment of γ-ory-lipo, the cells were first treated with DMEM containing 75 μM of CCl_4 for 30 min. After incubation, cells were treated with DMEM containing 100 μM of 50 mM γ-ory-lipo and maintained at 37°C for 12 h. After that, cells were washed with PBS and treated with trypsin for detachment. Finally, cell viability was counted using the trypan blue exclusion method.

3.2.8. Evaluation of hepatoprotective effect of γ-ory-lipo *in vivo*

To induce liver injury, Wistar rats were injected with 10% CCl_4 (1 ml/kg B.W), diluted 1:9 in liquid parafilm based on previously published reports with some modifications [84]. Briefly, rats were injected with γ-ory-lipo intravenously at a dose of 2.5 μmol/kg and incubated for 1h. Then, rats were treated with 10% CCl_4 and sacrificed 24h of treatment. Finally, blood was collected and incubated for 3h to collect serum. After incubation, serum was collected by centrifugation at 1,000 × *g* for 10 min at 4°C. Serum ALT and AST levels were measured using transaminase CII-Test Wako.

3.2.9. Statistical analysis

Statical analysis was performed using one-way ANOVA with post-hoc Tukey test for more than two groups and student's t-test for comparing two groups. P values <0.05 are considered significant.

3.3. Results

3.3.1. Physiochemical properties of γ-ory-lipo

To improve the stability problem of ferulic acid, I prepared liposomal formulations of γ-oryzanol (γ-Ory-lipo**)** and assessed the physiochemical characteristics of γ-ory-lipo. The particle size of γ-ory-lipo and EPC-lipo was 110-120 nm (Table 3.1.1.). The Polydispersity index (PDI) value of γ-ory-lipo and EPC-lipo was 0.15 and 0.16, respectively. The zeta potential of γ-ory-lipo was -5.92 mV. These findings indicate the successful preparation of liposomal formulation of γ-oryzanol.

Table 3.1.1. Physiochemical properties of γ-oryzanol-liposomes

Sample	Size (nm)	Polydispersity index	Zeta potential (mV)
EPC-lipo (control)	123.5 $\pm$ 7.2	0.16 $\pm$ 0.02	-7.4 $\pm$ 0.72
γ-Ory-lipo	116.1 $\pm$ 6.2	0.15 $\pm$ 0.03	-5.92 $\pm$ 0.67

Data are means $\pm$ SD of three samples prepared on different days.

3.3.2. Effects of liposome on the improvement of chemical stability of γ-oryzanol

After preparing γ-ory-lipo, I examined the chemical stability of γ-ory-lipo and compared it with the free drug γ-oryzanol. I found that the peak area of incubated γ-oryzanol (incubated with PBS at 37°C for 24h) was decreased by almost 75% compared to non-incubated γ-oryzanol (Fig-3.2), representing higher chemical instability of γ-oryzanol. Interestingly, γ-ory-lipo exhibited improved chemical stability compared to free γ-oryzanol. As shown in Fig-3.2, the peak area of incubated γ-ory-lipo was not changed even after incubation with PBS.

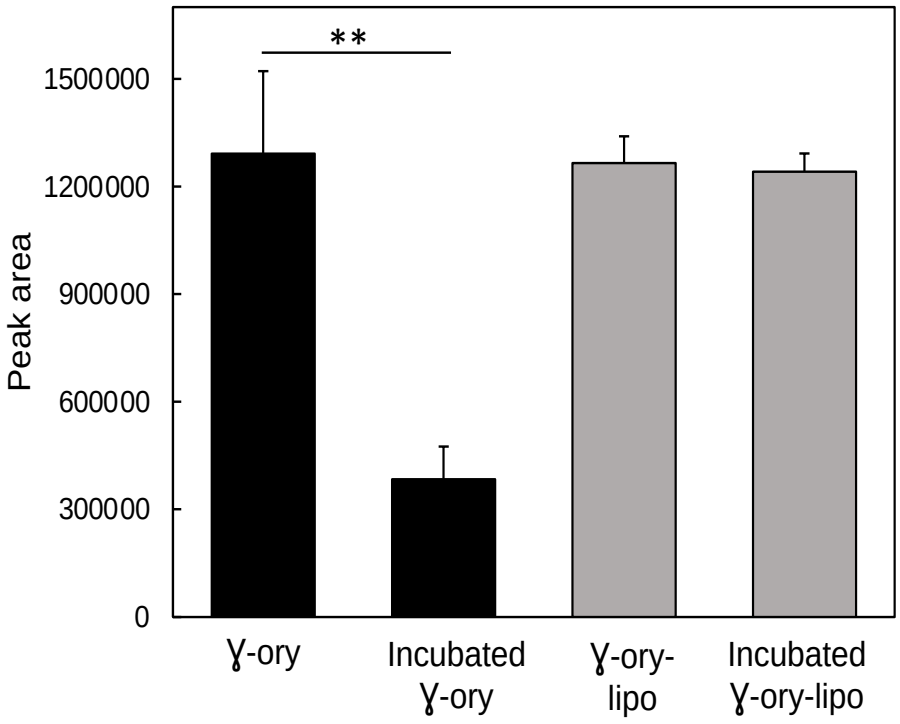

Figure 3.2. Assessment of chemical stability of γ-ory-lipo

(A) Chemical stability of γ-oryzanol (black column) and γ-ory-lipo (grey column) measured by HPLC. Incubated groups were treated with PBS at 37^0C for 24h. Data are means ± S.D. (n=3). *p< 0.05, **p<0.01.

3.3.3. Effects of γ-ory-lipo on hydroxyl radicals scavenging

To evaluate the antioxidative property of γ-ory-lipo, I examined the effects of γ-ory-lipo on hydroxyl radicals scavenging. Here, APF is used as an indicator of the generation of hydroxyl radicals. I found that γ-ory-lipo (20 μM) exhibited lower hydroxyl radical scavenging activity compared to ferulic-lipo (20 μM) based on fluorescence intensity measured during hydroxy radical generation (Fig-3.3). Besides, I observed that ferulic lipo could react with hydroxy radicals and be oxidized before reaching the target side. Additionally, there was no significant difference between γ-ory-lipo and EPC-lipo in preventing hydroxyl radical generation.

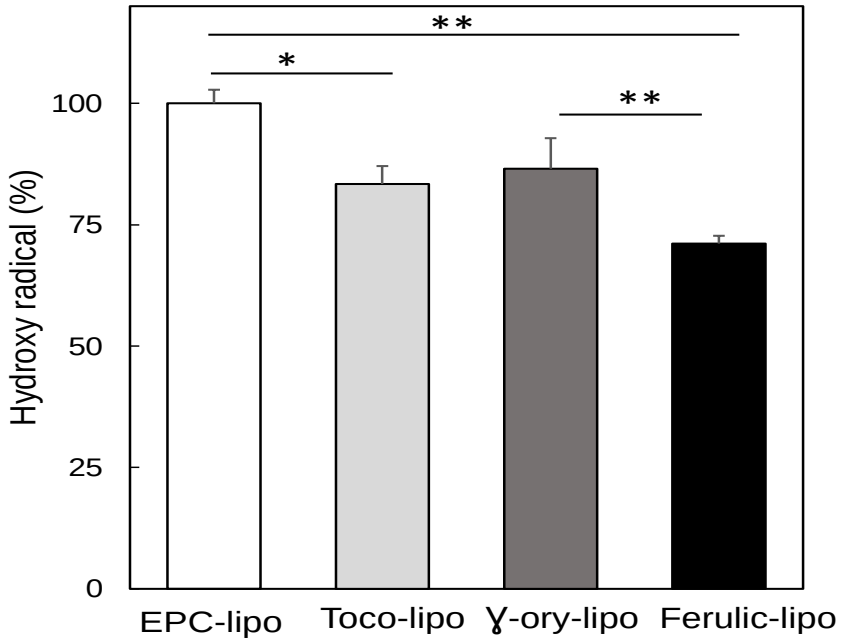

Figure 3.3. Effects of γ-ory-lipo on hydroxyl radicals scavenging

Hydroxyl radicals generated via Fenton reaction in the presence of liposomes encapsulating γ-oryzanol (dark grey column) or Ferulic acid (black column) were evaluated by the fluorescence intensity of APF. Data are means ± S.D. (n=3). *p< 0.05, **p<0.01.

3.3.4. Effect of γ-ory-lipo on cell viability

I also checked whether γ-ory-lipo had any effects on the viability of HepG2 cells. Here HepG2 cells were treated with γ-ory-lipo and cell viability were measured in a time-dependent manner. I found that the addition of γ-ory-lipo (100μM) on HepG2 cells had no impact on cell viability (Fig-3.4) measured at different time points (1h, 3h, 6h). I also observed no significant changes in the viability of HepG2 cells among the control, γ-ory-lipo, and Ferulic-lipo groups at three different time points.

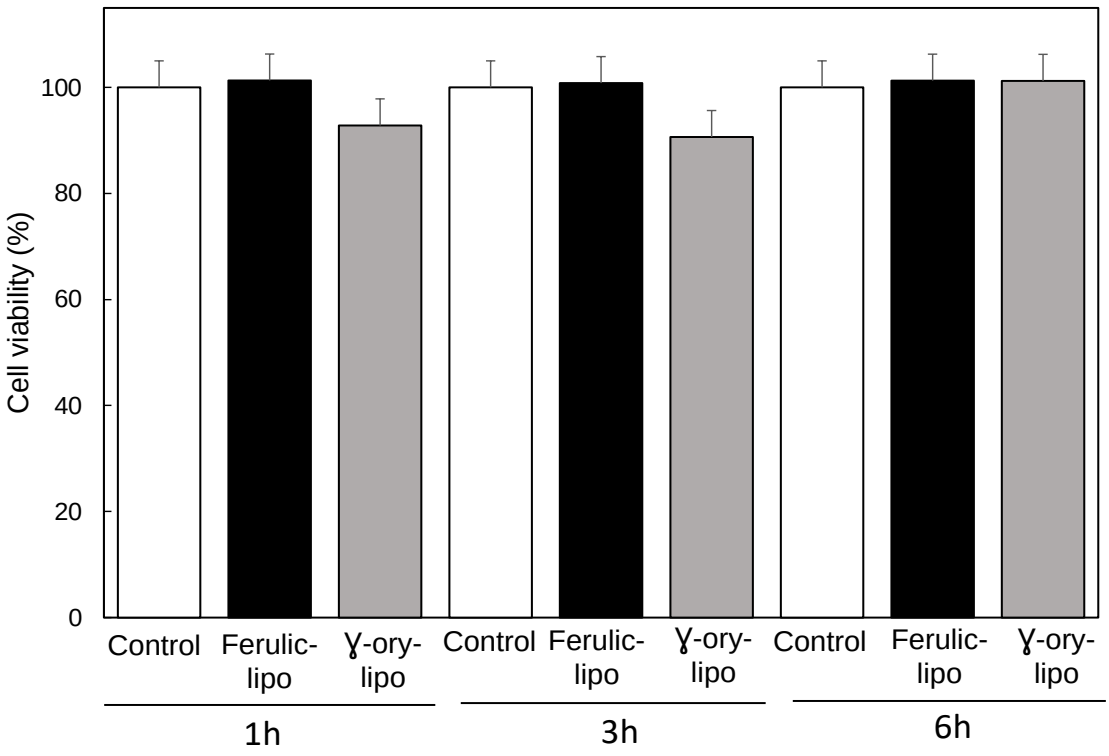

Figure 3.4. Effect of γ-ory-lipo on the viability of HepG2 cells

Cells were treated with 100 μM of liposomes containing γ-oryzanol and ferulic acid, respectively. Cell viability was determined after 1h, 3h, and 6h. Control (white column), γ-ory-lipo (grey column), and Ferulic-lipo (black column). Data are means ± S.D. *p<0.01, **p<0.001.

3.3.5. Effects of γ-ory-lipo on CCl_4-induced cytotoxicity in HepG2 cells

Next, I investigated the protective effects of γ-ory-lipo against CCl_4-induced toxicity in HepG2 cells. For measuring cytotoxicity, cells were pre-treated with CCl_4 (30 min), then γ-ory-lipo was administrated. Here, I found that the CCl_4 treatment significantly reduced the cell viability (~55%) compared to the control group (Fig-3.5). However, treatment with γ-ory-lipo (100 μM) significantly improved the cell viability (~ 25%). But the effect was not so potent. Besides, I observed that the inhibitory effects of γ-ory-lipo were lower than ferulic-lipo in preventing CCl_4-induced toxicity of HepG2 cells. Furthermore, free-oryzanol did not improve cell viability compared to the CCl_4-treated group.

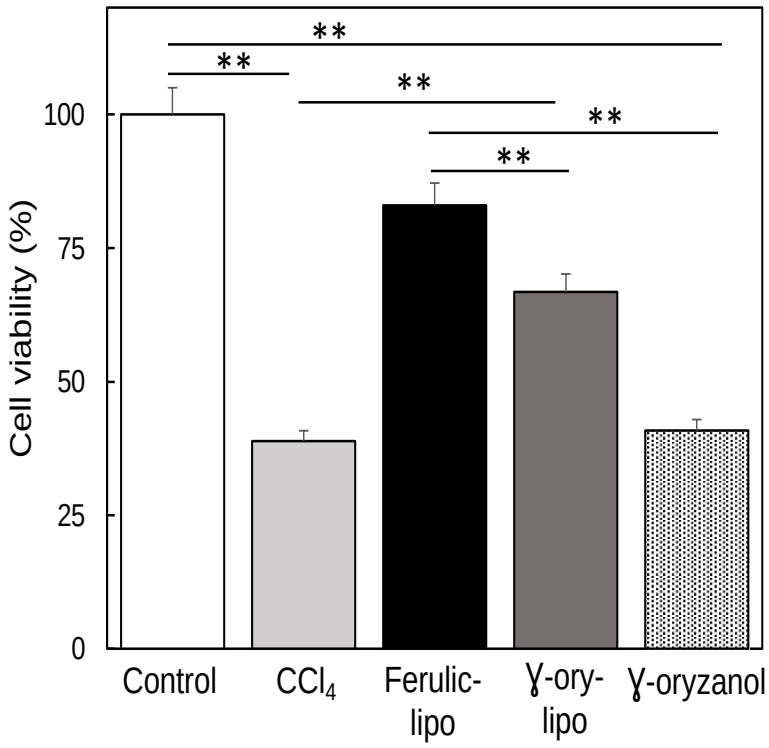

Figure. 3.5. Protective effects of γ-ory-lipo on CCl_4-induced toxicity of HepG2 cells

Cells were treated with 75 µM of CCl_4; liposomes((γ-ory-lipo & Ferulic-lipo) and free drugs were added 30 min after CCl_4 treatment. Cell viability was determined after 12h using the trypan blue staining method. Data are means ± S.D. *p<0.01, **p<0.001.

3.3.6. Assessment of the conversion of γ-oryzanol to ferulic acid (*in vitro & in vivo*)

As γ-oryzanol tends to rapidly metabolize to ferulic acid inside the body, I examined the conversion of γ-oryzanol to ferulic acid in both *in vitro and in vivo* conditions (Fig-3.6). First, I checked the conversion of γ-oryzanol in the cultured cells (HepG2 cells). After adding γ-ory-lipo in HepG2 cells, I incubated it for 1h. After 1h, I checked the conversion of γ-oryzanol by HPLC. Interestingly, I found the peak of ferulic acid (Fig-3.6C). Based on *in vitro* conversion, I also examined hydrolysis of γ-oryzanol *in vivo* conditions. For this, Wistar rats were treated with γ-ory-lipo, and 1h later rat was sacrificed, and the liver was collected. Liver tissue was homogenized, and the supernatant was collected to assess the conversion of γ-oryzanol in the live (Fig-3.6D). Here, I also found the peak of ferulic acid. Taking together, it can be said that γ-oryzanol was successfully and immediately converted into ferulic acid in both cultured cells and the liver.

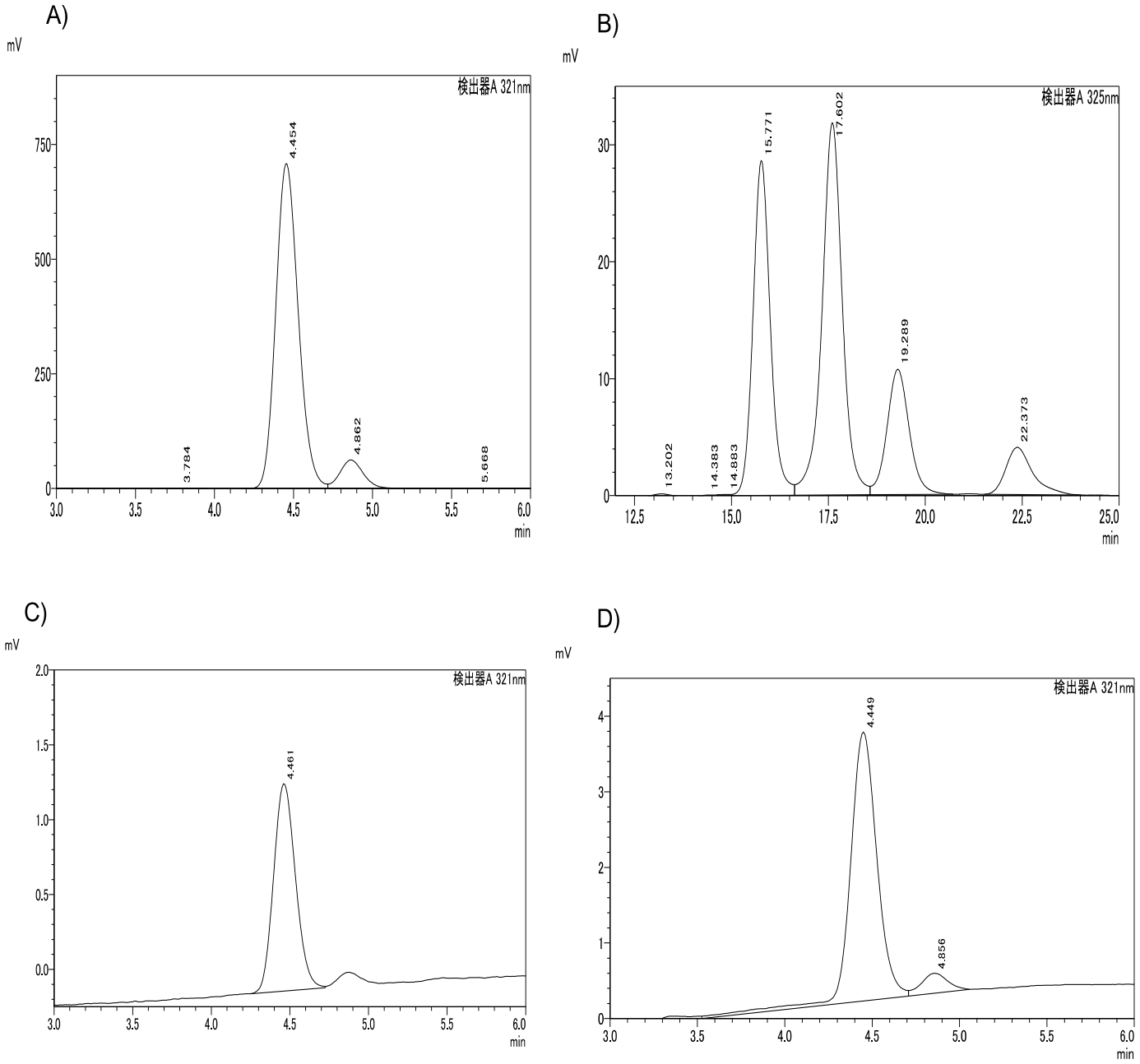

Figure 3.6. Evaluation of hydrolysis of γ-oryzanol into Ferulic acid (*in vitro* and *in vivo*)

Representative chromatograms- (A) Typical chromatogram of Ferulic acid (Standard); (B) Typical chromatogram of γ-oryzanol (Standard); (C) Chromatogram of Ferulic acid (*in vitro* conversion of γ-oryzanol into ferulic acid); (D) Chromatogram of Ferulic acid (*in vivo* conversion of γ-oryzanol into ferulic acid) determined by HPLC.

3.3.7. γ-Ory-lipo prevents cytotoxicity mediated by CCl_4 in HepG2 cells

As the conversion of γ-oryzanol to ferulic acid required some time based on *in vitro* and *in vivo* conversion of γ-oryzanol, here I incubated cells after adding γ-ory-lipo for 1h for assessment of protective effects of γ-ory-lipo on CCl_4-mediated toxicity of HepG2 cells. HepG2 cells were pretreated with γ-ory-lipo for 1h, then treated with CCl_4 and incubated for 12h. I found that γ-ory-lipo showed similar protective effects as Ferulic-lipo in improving the viability of HepG2 cells (Fig-3.7). Although 30 min after the addition of γ-ory-lipo did not show such potent effects. It may be due to the conversion of γ-oryzanol into ferulic acid and showed similar effects for preventing CCl_4-induced toxicity in HepG2 cells.

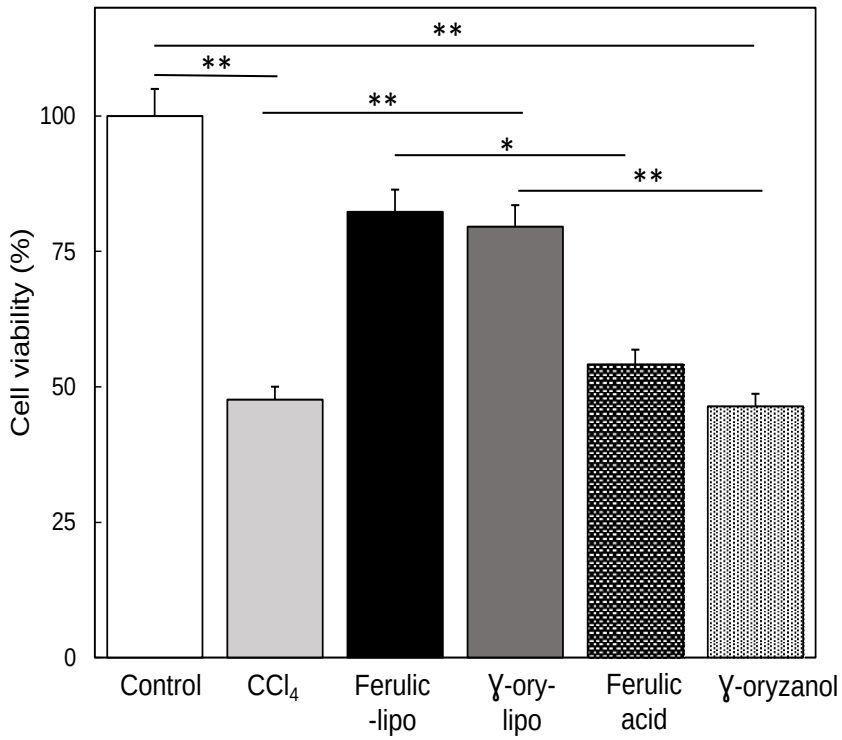

Figure 3.7. Cell viability of HepG2 cells treated with CCl_4 in the presence of γ-ory-lipo

Cells were pretreated with liposomes((γ-ory-lipo & Ferulic-lipo) and free drugs; 75 μM of CCl_4 were added 1h after liposomes and free drug treatment. Cell viability was determined after 12h using the trypan blue staining method. Data are means ± S.D. *p<0.01, **p<0.001.

3.3.8. Hepatoprotective effect γ-ory-lipo against CCl_4-induced liver injury model

Based on the protective effects of γ-ory-lipo against CCl_4-induced cytotoxicity in HepG2 cells, I examined the impact of γ-ory-lipo *in vivo* in a CCl_4-induced liver injury model of rats. Since serum AST and ALT level act as a marker of the severity of liver damage, I checked the effect of γ-ory-lipo in lowering serum enzymes level in a liver-injured rat model. Here, I found that in the CCl_4-treated group, serum ALT and AST levels were significantly high, approximately 400 IU/L and 180 IU/L, respectively, indicating severe liver damage (Fig-3.8). Intravenous administration of γ-ory-lipo (2.5 μM/kg) results in a significant reduction in serum enzyme level that was elevated with CCl_4 treatment. The conversion of γ-oryzanol into ferulic acid may be responsible for similar protective effects of γ-ory-lipo and Ferulic-lipo against CCl4-induced hepatotoxicity.

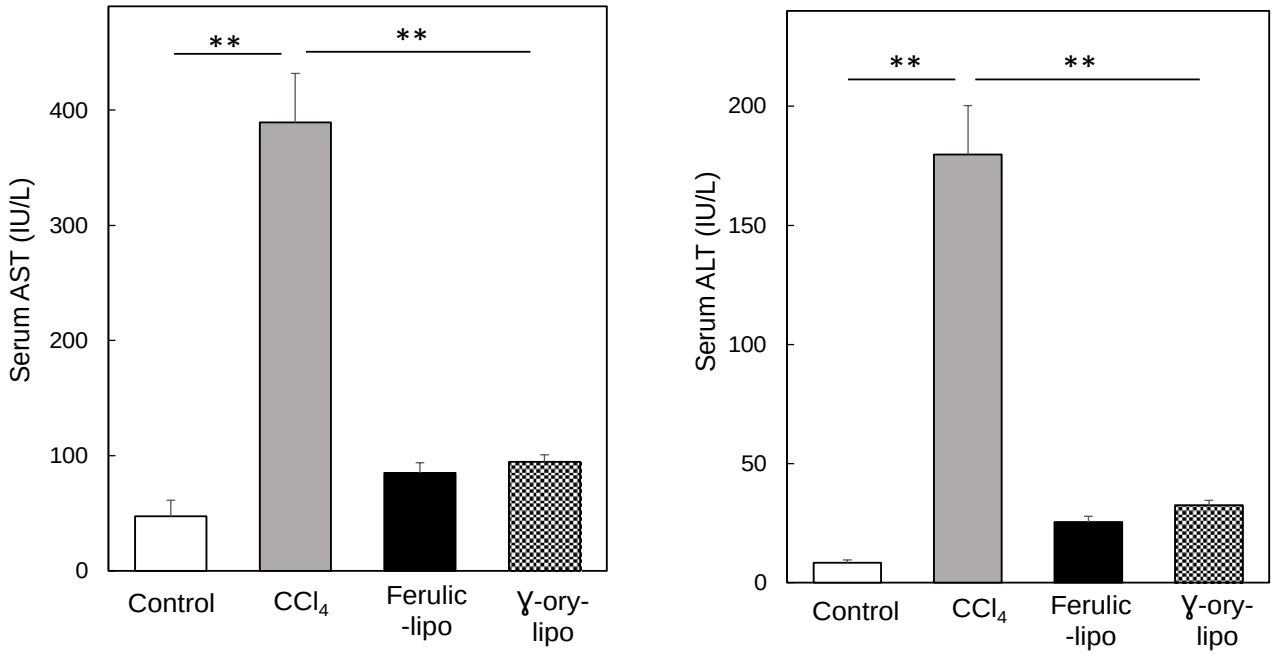

Figure 3.8. Protective effects of γ-ory-lipo on CCl_4-induced liver injury in rats

CCl_4 was used for the induction of liver injury. γ-ory-lipo and Ferulic-lipo were intravenously administrated. 1h after liposomes treatment, CCl_4 was administrated intraperitoneally. Serum was collected after 24h, and the enzymatic activities of ALT and AST were determined. Data are means ± S.D. *p<0.01, **p<0.001.

3.4. Discussion

Despite having numerous biological activities, the instability of ferulic acid is a major drawback to its large-scale application. In this study, I attempted to improve the stability of ferulic acid. It has been reported that γ-oryzanol hydrolyzes to its active metabolite ferulic acid inside the body [97]. Therefore, I focused on γ-oryzanol as a prodrug of ferulic acid. Here, I prepared a liposomal formulation of γ-oryzanol (γ-ory-lipo) and assessed its preventive effects against CCl_4-induced liver injury. Previous studies showed that γ-oryzanol is susceptible to oxidation in the presence of oxygen from air, light, and heat [98]. Thus, after preparing γ-ory-lipo (Table-3.1.1), I checked its chemical stability by HPLC and compared it with free γ-oryzanol to assess the effect of liposome on the stability of γ-oryzanol (Fig-3.2). I found that incubation of γ-oryzanol with PBS (37^{o}C, 24h) results in a significant reduction of peak area (~ 75%) compared to γ-oryzanol only. These results demonstrated the high chemical instability of γ-oryzanol. It is probably due to the increased reactivity of γ-oryzanol with reactive oxygenase species. Contrarily, encapsulation of γ-oryzanol with liposomes significantly improved its stability compared to free γ-oryzanol. It was assumed that liposomal formulation might reduce its possibility of reacting with reactive oxygenase species. After confirming the chemical stability of γ-ory-lipo, I checked its antioxidative effect by measuring hydroxyl radical scavenging capacity and compared it with Ferulic-lipo (Fig-3.3). Here, I found that γ-ory-lipo exhibited lower hydroxyl radical scavenging capability than ferulic-lipo. Based on chemical structure, γ-oryzanol comprises one ferulic moiety and one sterol moiety (Fig-3.1). It was supposed that the bulky structure of γ-oryzanol in liposomes might affect its mobility, reducing its possibility of interacting with hydroxyl radicals compared to ferulic-lipo.

Next, I examined the effects of γ-ory-lipo on the viability of HepG2 cells (Fig-3.4). γ-Ory-lipo did not show any effect on cell viability. After that, I assessed the protective effects of γ-ory-lipo against CCl_4-mediated toxicity of HepG2 cells (Fig-3.5). Here, I observed that γ-ory-lipo significantly improved cell viability (~25%) compared to the CCl_4-treated group. However, the effect was not so potent and even lower than the ferulic-lipo-treated group. It evidenced that ferulic moiety's presence in the structure of γ-oryzanol is mainly responsible for its antioxidative properties [99]. Therefore, the conversion of γ-oryzanol to ferulic acid is required for its potent antioxidative effects. For this, I checked the conversion of γ-oryzanol to ferulic acid in both *in vitro and in vivo* conditions by HPLC (Fig-3.6). Interestingly I found that treatment of HepG2 cells with γ-ory-lipo for 1h results in the successful conversion of γ-oryzanol into ferulic acid (Fig-3.6C). Similarly, I examined the conversion of γ-oryzanol into ferulic acid in the rat liver by treating Wistar rats with γ-ory-lipo for 1h. Here, I also observed the successful conversion of γ-oryzanol into ferulic acid (Fig-3.6D). As the conversion of γ-oryzanol into ferulic acid required some time (*in vitro & in vivo* study); subsequently, I incubated HepG2 cells with γ-ory-lipo for 1h, then treated them with CCl_4 for assessment of the protective effects of γ-ory-lipo on CCl_4-mediated cytotoxicity of HepG2 cells. Interestingly I found that γ-ory-lipo showed similar protective effects as ferulic lipo for improving the viability of HepG2 cells (Fig- 3.7). Although 30 min later addition of γ-ory-lipo didn't show such potent effects. It can be said that the conversion of γ-oryzanol to ferulic acid might be responsible for its similar protective effects. Based on *in vitro* study results, I checked *in vivo* hepatoprotective effects of γ-ory-lipo in a CCl_4-induced liver-injured rat model. Here I found that intravenous administration of γ-ory-lipo results in almost similar effects as ferulic-lipo in preventing liver damage by reducing serum ALT and AST levels (Fig-3.8). Previous literature showed that the treatment of SD male rats with equimolar concentrations of ferulic acid and γ-

oryzanol exhibited similar effects in reducing high-fat and high-fructose diet-induced obesity and dyslipidemia [100]. Thus, the findings of this study are consistent with the prior study. Based on these results, it can be said that the conversion of γ-oryzanol into ferulic acid may be responsible for similar protective effects of γ-ory-lipo and ferulic-lipo against CCl_4-induced hepatotoxicity.

3.5. Conclusion

Liposomal formulation of γ-oryzanol exhibited improved stability compared to free γ-oryzanol and would be a good prodrug formulation of ferulic acid for solving its instability problem.

Chapter IV

Conclusion and Future perspectives

Chapter IV: Conclusion and Future perspectives

Conclusion

The overall findings of this thesis can be summarized as follow-

Chapter II

- Ferulic acid in liposomal formulations showed higher antioxidant activity than α-tocopherol liposomes.
- Intravenous administration of ferulic-lipo significantly attenuated liver injury induced by CCl_4 (*in vivo & in vitro*).
- Ferulic-lipo could be a promising therapeutic for the treatment of oxidative liver damage.

Chapter III

- γ-Oryzanol successfully converted to its active metabolite ferulic acid (*in vivo & in vitro*).
- γ-Ory-lipo showed similar protective effects as ferulic-lipo in preventing CCl_4-induced liver injury.
- γ-Ory-lipo could be a good prodrug formulation of ferulic acid for improving its stability.

Future perspectives

Although the liposomal formulation of ferulic acid showed significant protective effects, it is still vulnerable to oxidization; therefore, a more stable formulation is required. Further studies needed to be carried out for the development highly stable formulation of ferulic acid with enhanced therapeutic efficiency.

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I would like to dedicate this thesis to my late father and mother, who are the origin of my success. I wish they were alive, and I could share this precious moment with them. I miss both of you in every sphere of my life.

TABASSUM ARA

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