Regular Article

Nanoparticles Encapsulated γ-Oryzanol as a Natural Prodrug of Ferulic Acid for the Treatment of Oxidative Liver Damage

Tabassum Ara[a] and Kentaro Kogure*[,b]

[a] *Department of Pharmaceutical Health Chemistry, Graduate School of Pharmaceutical Sciences, Tokushima University, 1 Shomachi, Tokushima 770–8505, Japan: and* [b] *Department of Pharmaceutical Health Chemistry, Graduate School of Biomedical Sciences, Tokushima University, 1 Shomachi, Tokushima 770–8505, Japan.*
Received March 19, 2023; accepted August 9, 2023

Antioxidants are promising therapeutics for treating oxidative stress-mediated liver diseases. Previously, we studied a potent natural antioxidant, ferulic acid, and developed a liposomal formulation of ferulic acid (ferulic-lipo) to improve its solubility. Ferulic-lipo significantly attenuated oxidative damage in the liver by inhibiting reactive oxygenase species (ROS). However, antioxidative liposomes must be less reactive with ROS prior to reaching the target sites to effectively neutralize existing ROS. But ferulic-lipo tends to be oxidized before reaching the liver. Besides, γ-oryzanol has been reported to decompose into ferulic acid *in vivo*; accordingly, we hypothesized that γ-oryzanol could be employed as a natural prodrug of ferulic acid to improve stability and antioxidative effectiveness. Therefore, in this study, we prepared a liposomal formulation of γ-oryzanol (γ-ory-lipo) and investigated its therapeutic effects in a CCl_4-induced rat model of liver injury. We found that γ-ory-lipo has a higher chemical stability than does free γ-oryzanol. Although the antioxidative effect of γ-ory-lipo was lower than that of ferulic-lipo, pretreatment of the HepG2 cells with γ-ory-lipo improved the viability of CCl_4-treated cells to a similar level as treatment with ferulic-lipo. γ-Oryzanol was shown to be converted into ferulic acid *in vitro* and *in vivo*. Furthermore, intravenous administration of γ-ory-lipo exhibited a similar effectiveness as ferulic-lipo against CCl_4-induced hepatotoxicity, which should be the due to the conversion of γ-oryzanol into ferulic acid. These findings demonstrated that γ-ory-lipo could be a good natural prodrug of ferulic acid for eradicating its stability problem.

Key words γ-oryzanol, prodrug, stability, liposome; oxidative-stress, antioxidant

INTRODUCTION

Liver disease is a major public health problem that affects approximately 844 million people globally, with a yearly fatality rate of 2 million.[1)] As a key metabolic center, the liver provides a front-line defense against potentially toxic chemicals through the phase-1 detoxification process.[2)] Disruption of metabolic homeostasis in the liver results in the excessive generation of reactive oxygenase species (ROS).[3)] Accordingly, the overproduction of ROS has been implicated as one of the common landmarks for the initiation and progression of several types of liver diseases.[4)] Because of their high reactivity, ROS readily interacts with DNA, proteins, and other cellular molecules altering their structure and function and resulting in oxidative stress-mediated liver injury.[5)] Increasing evidence has demonstrated that ROS plays specific roles in the induction of hepatocyte inflammation, apoptosis, and necrosis.[6,7)] Despite the considerable progress made in the development of pharmacological therapeutics, the identification of novel drug therapies for treating liver injury remains a challenge.[8)] Antioxidants gained much attention in clinical studies because of their beneficial role in fighting against oxidative stress and its mediated diseases. Antioxidants are stable molecules that can directly scavenge ROS, thus aiding in inhibiting ROS generation in living organisms.[9)] Plant-based dietary antioxidants such as alkaloids and phenolics are found to have a preventive role against a number of ROS-mediated diseases.[10)] It has been demonstrated that antioxidative compounds containing phenolic and aromatic rings donate a hydrogen atom to free radicals and stabilize them by the resonance delocalization of the electron in the aromatic ring.[11)] However, antioxidants have also been revealed as promising therapeutics for treating diseases mediated by oxidative stress.[12,13)] In fact multiple studies have reported that antioxidants can prevent oxidative liver damage and help regenerate liver tissues.[14–18)] Resveratrol, a phytoalexin, was found to have a potential role against oxidative stress-mediated liver disorders. Previous studies showed that resveratrol protects HepG2 cells from ethanol and hydrogen peroxide-mediated oxidative stress by its antioxidative potentiality.[19)] Besides, Preclinical research with silymarin has confirmed its potent hepatoprotective effects against oxidative stress-mediated liver damage.[20)]

In our previous study, we focused on the natural antioxidant ferulic acid and encapsulated it in liposomes to improve its solubility and stability and to ensure its delivery to the liver to prevent CCl_4-mediated oxidative liver damage.[21)] The phenolic hydroxyl moiety in ferulic acid has been postulated to be responsible for its potent antioxidative properties,[22)] and this chemical property allows ferulic acid to inhibit the formation of ROS and to neutralize free radicals.[23)] However, poor water solubility and low bioavailability of ferulic acid are major limitations to its successful drug delivery.[24)] Therefore, we prepared a liposomal formulation of ferulic acid (ferulic-lipo) in an attempt to improve its water solubility and to targeted delivery of ferulic acid to the liver. Ferulic-lipo showed significant preventive effects against CCl_4-induced liver injury in both *in vitro* and *in vivo* models.[21)]

However, we found that the strong reactivity of ferulic-lipo with ROS led to its oxidation prior to reaching its target site. Accordingly, novel liposomal formulations of antioxi-

* To whom correspondence should be addressed. e-mail: kogure@tokushima-u.ac.jp

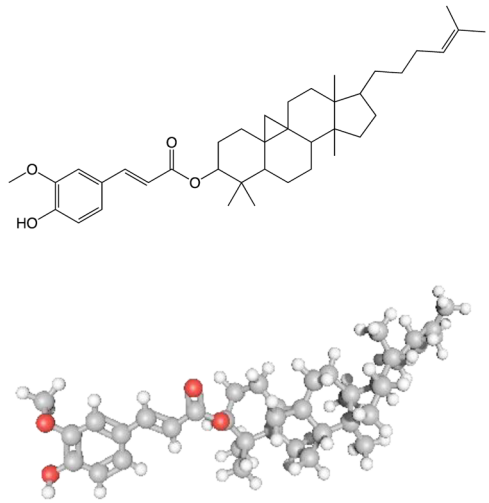

Fig. 1. Chemical Structure and 3D Stereostructure of γ-Oryzanol

In the 3D stereostructure, grey, white, and red spheres represent carbon atoms, hydrogen atoms, and oxygen atoms, respectively.

dants need to be less reactive with ROS prior to reaching the liver, where it could react with liver-specific ROS effectively. Therefore, we sought a more chemically stable formulation of ferulic acid as a potential tool to be used against oxidative stress. It has been reported that γ-oryzanol (Fig. 1) is rapidly hydrolyzed into ferulic acid inside the body.[25] Therefore, we hypothesized that γ-oryzanol would act as a natural prodrug of ferulic acid.

Prodrugs are pharmacologically inactive compounds that undergo biotransformation in the body and become converted to their active constituents through enzymatic or chemical cleavages.[26] Prodrug approaches are used when a pharmacologically active drug suffers from poor solubility, permeability and absorption, poor stability, or poor site specificity.[27] Multiple prodrugs have been designed to improve the physicochemical and biopharmaceutical properties of parent drugs, and prodrugs have frequently been found to improve stability and solubility problems. For example, curcumin diglutaric acid, a prodrug formulation of curcumin, was shown to exhibit enhanced water solubility and oral bioavailability relative to free curcumin in the treatment of neuropathy.[28] Similarly, capecitabine (Xeloda), a prodrug of 5-Fluorouracil, has been reported to show improved physiochemical and pharmacokinetics properties relative to 5-Fluorouracil in the treatment of solid malignant tumors.[29] In addition, resveratrol triacetate, a prodrug of resveratrol, exhibited significantly higher plasma concentrations than resveratrol in a rat model,[30] and quercetin pentabenzensulfonate, a prodrug of quercetin, demonstrated higher solubility relative to quercetin.[31] Thus, the prodrug strategy has been found to be effective in solving multiple problems, including instability, insolubility, and low bioavailability, in the development of biologically active drugs.

Therefore, in this study, we focused on γ-oryzanol as a ferulic acid prodrug to improve the chemical stability of antioxidative liposomal formulations. Specifically, we prepared a liposomal formulation of γ-oryzanol (γ-ory-lipo) and examined its chemical stability. Furthermore, we assessed the antioxidant activity of γ-ory-lipo and compared it with that of ferulic-lipo against CCl_4-induced oxidative liver damage in both *in vitro* and *in vivo* conditions.

MATERIALS AND METHODS

Materials and Animals γ-Oryzanol was 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 purchased from FUJIFILM Wako Pure Chemical Corporation. Amino phenyl fluorescein (APF) was purchased from Goryo Chemical (Sapporo, Japan). An octadecyl silyl (C_{18}) HPLC column (TSK gel ODS-80 TM) was purchased from Tosoh Corporation (Tokyo, Japan). HPLC-grade methanol and acetonitrile were used for HPLC analyses. All other reagents and solvents were of the highest grade, commercially available. Seven-weeks old male Wistar rats (weighing 180 to 200 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Ethical permission and approval for animal experiments were obtained from the Animal and Ethics Review Committee of Tokushima University.

Preparation of Liposomes Encapsulating γ-Oryzanol γ-Ory-lipo was prepared from EPC, and γ-oryzanol by the lipid hydration method.[32] Briefly, a chloroform solution containing 20 μM EPC and 0.2 μM γ-oryzanol was dried in an Erlenmeyer flask using a rotary evaporator (N-1000, EYELA, Tokyo, Japan) to form a lipid film. Then the lipid films were hydrated by the addition of 1 mL phosphate buffered saline (PBS). After subjection to a freeze-thaw cycle, the liposomal suspension was extruded through a poly-carbonate membrane filter with 100 nm pores (Nuclepore, Cambridge, MA, U.S.A.). A Zetasizer Nano ZS (Malvern Instruments, Worcestershire, U.K.) was used for the determination of the Size, polydispersity index, and zeta potential of liposomes. $DilC_{18}$ (1 mol% of total lipid) was incorporated into the liposomes to prepare fluorescently labeled γ-ory-lipo.

Measurement of Chemical Stability of γ-Ory-lipo by HPLC The HPLC analyses were performed using a C_{18} analytical column (4.6 mm × 15 cm; 5 μm particle size) at 25 °C. The analysis of ferulic acid involved an isocratic method with a mobile phase of methanol: water (48 : 52 (v/v)) adjusted to pH 3.0 using 0.1 N orthophosphoric acid and a flow rate of 1.0 mL/min.[33] The sample size was 20 μL, the detection wavelength was 321 nm, and the run time was 10 min. For γ-oryzanol, the mobile phase was acetonitrile: methanol (60 : 40 (v/v)), and the flow rate was 1.5 mL/min.[34] The wavelength used to detect γ-oryzanol was 325 nm, the with injection volume was 20 μL, and the run time was 25 min.

For analyses of chemical stability, a 0.35 μM/solution of γ-ory-lipo was prepared as described above. After removing free γ-oryzanol using a PD-10 column,[21] γ-ory-lipo (0.1 μM/mL) was incubated in PBS at 37 °C for 24h and then analyzed by HPLC (Shimadzu, Kyoto, Japan). The chemical stability of γ-ory-lipo was determined by measuring the area of the γ-oryzanol peak and comparing it to that of an equimolar suspension of free γ-oryzanol processed under identical conditions.

Evaluation of Hydroxyl Radical Scavenging Property of γ-Ory-lipo Hydroxyl radicals were produced by the Fenton reaction as described.[35] The generation of ROS was quantified with amino phenyl fluorescein (APF), which becomes fluorescent upon reaction with hydroxyl radicals. Briefly, hydroxyl radicals were generated in an aqueous solutions containing 10 μM APF, 1 mM H_2O_2 and 100 μM $FeSO_4$ with

1.5 mM γ-ory-lipo. The fluorescence intensity was measured using an Infinite M200 microplate reader (Tecan Group Ltd., Switzerland).

Assessment of Cell Viability HepG2 cells (2×10^5) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 atmosphere for 72 h. When the cells reached 80 to 90% confluency, the medium was exchanged with DMEM containing 100 μM of γ-ory-lipo or ferulic-lipo, and the plates were incubated at 37 °C for 1, 3, or 6 h. After incubation, the cells were washed with PBS and treated with trypsin. The viability of the cells was measured using the trypan blue exclusion method. The cell viability as a percentage was assessed by dividing the number of stained cells by the total number of cells.

Analysis of the Conversion of γ-Oryzanol to Ferulic Acid (*in Vitro* and *in Vivo*) For the study of γ-oryzanol conversion *in vitro*, HepG2 cells (2×10^5) were seeded in 35 mm petri dishes. After 72 h, the cells were treated with DMEM containing 100 μM of γ-ory-lipo and incubated at 37 °C for 1 h. The cells were washed with PBS and treated with lysis buffer (25 mM Tris–HCl, pH 6.5, 1% glycerol (v/v), 1% sodium dodecyl sulfate (SDS) (v/v), 5% 2-mercaptoethanol and a phosphatase inhibitor cocktail (PhosSTOP)). Protein was removed from the lysate by the addition of protein removal solution consisting of $HClO_4$ (60%): acetonitrile: methanol with volume (5 : 30 : 65), and incubation at room temperature for 30 min, followed by centrifugation at $1500 \times g$ for 15 min at 4 °C. The supernatant was collected and analyzed for γ-oryzanol and ferulic acid by HPLC as described in "Measurement of Chemical Stability of γ-Ory-lipo by HPLC."

To measure the conversion of γ-oryzanol to ferulic acid *in vivo*, γ-ory-lipo was administrated intravenously to Wistar rats at a dose of 2.5 μM/kg body weight. After incubation for 1 h, the rats were sacrificed, and the liver were collected. Next, 0.5 g of liver tissue was added to the 4.5 mL PBS, and the mixture was incubated for 30 min on ice. After incubation, the solution containing liver tissue was homogenized in the presence of ice. The liver tissue then homogenized on ice, and the lysate were centrifuged at $1000 \times g$ for 10 min at 4 °C. The supernatant was collected and incubated with the protein removal solution for 30 min. After centrifugation, the supernatant was collected and analyzed by HPLC.

Evaluation of CCl_4-Mediated Cytotoxicity *in Vitro* HepG2 cells (2×10^5) were seeded in 35 mm plates and cultured for 72 h. To pre-treat with γ-ory-lipo, the media was replaced with DMEM containing 100 μM γ-ory-lipo, and the cells were incubated for 1 h. Next, CCl_4 was added to a final concentration of 75 μM, and the cells were incubated at 37 °C for 12 h. To perform post-treatment with γ-ory-lipo, the cells were first treated with 75 μM of CCl_4 in DMEM for 30 min. After incubation, the cells were treated with 100 μM γ-ory-lipo and maintained at 37 °C for 12 h. The cells were then washed with PBS and treated with trypsin for detachment, and cell viability was determined using the trypan blue exclusion method.

Accumulation of γ-Ory-lipo in the Rat Liver To assess the accumulation of γ-ory-lipo in the liver, $DilC_{18}$-labeled γ-ory-lipo was administrated intravenously to the Wistar rats. After treatment, rats were sacrificed at multiple time points (1, 3, 6, 12, and 24 h). Their livers were excised and embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Tokyo, Japan). The tissues were then frozen in a dry ice/ethanol bath. Frozen sections (10 μm) were prepared using a cryostat (CM3050S, Leica Biosystems, Tokyo, Japan). PermaFluor Aqueous Mounting Medium (Thermo Fisher Scientific, Waltham, MA, U.S.A.) was used to mount the liver tissue sections, which were observed using a confocal laser scanning microscope (LSM700, Carl Zeiss, Jena, Germany). Quantitative estimation of the fluorescence intensity of liver tissue was performed using ImageJ analysis software.

Evaluation of the Hepatoprotective Effect of γ-Ory-lipo *in Vivo* To induce liver injury, Wistar rats were treated with CCl_4 according to a previously published reports with minor modifications.[36] Briefly, rats were injected with γ-ory-lipo intravenously at a dose of 2.5 μmol/kg bodyweight and incubated for 1 h. Then, the rats were injected with 1 mL/kg body weight of 10% CCl_4 dissolved in liquid parafilm and sacrificed 24 h later. Finally, blood was collected and incubated for 3 h. After incubation, serum was collected by centrifugation at $1000 \times g$ for 10 min at 4 °C. Serum ALT and AST levels were measured using transaminase CII-Test Wako.

Histopathological Observation of Liver Damage To assess the morphological changes to the liver tissue, hematoxylin–eosin (H&E) staining was performed. Frozen liver sections (10 μm) were prepared using a cryostat. A PAP pen was used to create a hydrophobic border around the sections. The sections were incubated for 10 min in 4% paraformaldehyde and washed with PBS prior to staining with Mayer's hematoxylin and 1% eosin solutions (FUJIFILM Wako Pure Chemical Corporation) for 10 and 1 min, respectively. The sections were then dehydrated using an ethanol gradient (80 to 100%) and mounted using a mounting agent (Entellan® New, Merck Millipore, Burlington, MA, U.S.A.). A fluorescence phase contrast microscope (BZ-9000, Keyence, Osaka, Japan) was used for observing stained sections.

Statistical Analysis Statical analyses were performed using one-way ANOVA with *post-hoc* Tukey test for more than two groups and Student's *t*-test for comparing two groups. A p-value of <0.05 was considered to indicate statistical significance.

RESULTS AND DISCUSSION

Physiochemical Properties of γ-Ory-lipo Liposomal formulations of γ-oryzanol (γ-ory-lipo) were employed to improve the stability of ferulic acid. Following preparation of the liposomes by the lipid hydration method, their physiochemical characteristics were assessed. The particle sizes of γ-ory-lipo and control liposomes were found to be between 110 and 120 nm (Table 1), which is approximately the same size as has been determined for ferulic-lipo (approximately 110 nm).[21] The zeta potentials of γ-ory-lipo and control liposomes were

Table 1. Physiochemical Properties of γ-Oryzanol Liposomes

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

Data are means ± standard deviation (S.D.) of three samples prepared on different days.

−5.9 and −7.4 mV, respectively (Table 1). The similarity in size and zeta potential to those previously determined for ferulic-lipo provide evidence that the liposomal formulation of γ-oryzanol was successfully prepared.

Effects of Liposomal Encapsulation on the Chemical Stability of γ-Oryzanol Wide-scale application of antioxidants in therapies has been limited due to their poor solubility, instability, and low bioaccessibility.[37] Nanoparticle-mediated delivery of antioxidants is being developed to address these problems.[38] The broad application of potential antioxidant quercetin for treating oxidative skin damage has been limited because of its light-induced degradative nature. However, incorporating quercetin into lipoparticle significantly improved its chemical instability compared to free quercetin.[39] Furthermore, the use of the potent antioxidant lycopene is also often limited because of its susceptibility to oxidation in the presence of light, heat, and oxygen.[40] It was demonstrated that encapsulation of lycopene into the liposome improved not only its stability but also improved therapeutic effects against the oxidative stress-mediated aging process.[41] As gamma-oryzanol is also susceptible to oxidation in the presence of oxygen from air, light, and heat,[42] we hypothesized that encapsulation of γ-oryzanol into a proper formulation might improve its chemical stability. Besides, liposomes can increase the therapeutic efficiency of encapsulated drugs compared to free drugs and protect them from enzymatic degradation and hydrolysis.[43] Regarding the encapsulating process, the encapsulation of compounds in liposome membranes is appropriate for hydrophobic antioxidants. Thus, liposome formulation is suitable for hydrophobic γ-ory-lipo. On this basis, we encapsulated γ-oryzanol into liposomes to improve its chemical stability and compared it with the free drug, γ-oryzanol only. Upon incubation of free γ-oryzanol for 24 h at 37 °C in aqueous solution (PBS), the amount of γ-oryzanol was decreased by approximately 75% relative to the amount present prior to incubation (Fig. 2), confirming the chemical instability of γ-oryzanol. In contrast, encapsulation of γ-oryzanol in liposomes significantly improved its stability compared to free γ-oryzanol. However, encapsulation of hydrophilic antioxidants such as ascorbic acid in the aqueous phase of liposomes should reduce antioxidative activity due to shielding. As γ-oryzanol is highly hydrophobic, it was assumed that almost all the γ-oryzanol was encapsulated into the hydrophobic lipid bilayer of the liposomal membrane that reduces its possibility of reacting with oxidative agents.

Antioxidative Effects of γ-Ory-lipo against Hydroxyl Radicals After confirming the chemical stability of γ-oryzanol in a liposomal formulation, the antioxidant effect of γ-ory-lipo was assessed by comparing its hydroxyl radical scavenging capacity to that of ferulic-lipo. It was found that γ-ory-lipo (20 μM) exhibited lower hydroxyl radical scavenging activity as compared to the same concentration of ferulic-lipo (Fig. 3). Based on chemical structure, γ-oryzanol comprises a ferulic moiety as well as a sterol moiety (Fig. 1). It was assumed that the sterol moiety in chemical structure of γ-oryzanol might led to an increased steric hindrance that affects its mobility in liposomes and reduces interactions between the ferulic moiety and surroundings hydroxyl radicals.

The γ-ory-lipo formulation also exhibited a lower hydroxyl radical scavenging potential compared to tocopherol liposomes, and there was no significant difference between the capacities of γ-ory-lipo and EPC-lipo to prevent hydroxyl radical generation. Importantly, it was observed that ferulic-lipo could react with hydroxyl radicals and be readily oxidized before reaching the target side, whereas γ-ory-lipo exhibited less propensity to be oxidized, considered to be stable to deliver its cargo to its target.

Effect of γ-Ory-lipo on Cell Viability The biocompatibility of γ-ory-lipo was assessed by determining its effects on the viability of HepG2 cells over time. As shown in Fig. 4, treatment of HepG2 cells with γ-ory-lipo (100 μM) caused a little decrease in cell viability that was not statistically different from those control groups when measured at 1, 3, and 6 h after application.

Effects of γ-Ory-lipo on the Cytotoxicity of CCl_4 in HepG2 Cells To determine the extent to which the γ-ory-lipo would improve hepatotoxicity, we assessed the protective effects of γ-ory-lipo on a commonly used *in vitro* model of liver damage, CCl_4-induced toxicity to HepG2 cells. In this model, the cells were pre-treated for 30 min with CCl_4. The cells were then treated with 100 μM γ-ory-lipo for 12 h, and cell viability was measured by the trypan blue exclusion

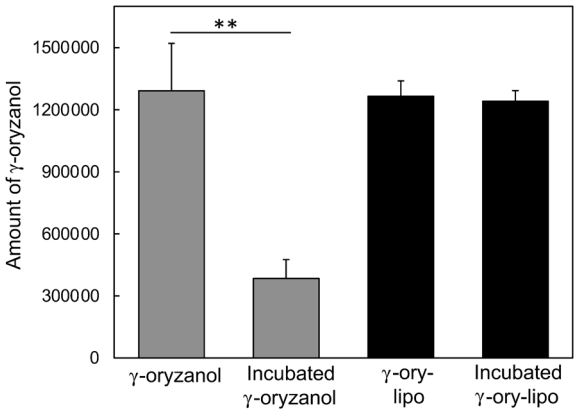

Fig. 2. The Chemical Stability of γ-Ory-lipo

The chemical stability of γ-oryzanol in its free form (grey column) and encapsulated in liposomes (black column) was measured by HPLC. The samples were incubated in PBS at 37 °C for 24 h prior to measurement. Data are displayed as mean ± standard deviation (S.D.) ($n = 3$). $^{**}p < 0.01$.

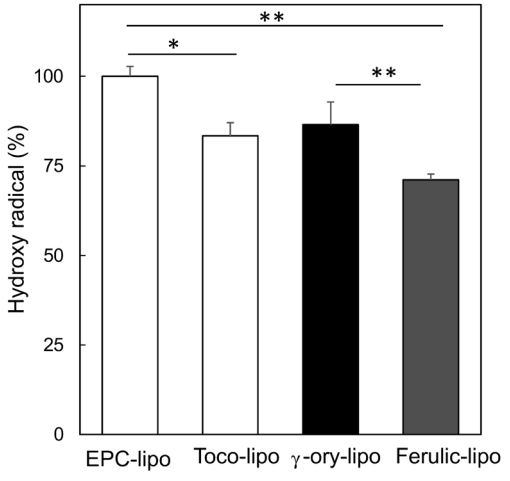

Fig. 3. Hydroxyl Radicals Scavenging Activity of γ-Ory-lipo

Hydroxyl radicals were generated through the Fenton reaction. Following the addition of liposomes encapsulating γ-oryzanol (dark grey column) or ferulic acid (black column), hydroxy radicals were quantified by measuring the fluorescence intensity of APF. Data are displayed as mean ± S.D. ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$.

method. It was found that CCl_4 treatment significantly reduced the viability of HepG2 cells by approximately 55% as compared to the control group (Fig. 5). Treatment with γ-ory-lipo (100 μM) significantly improved the cell viability by 25%, relative to cells treated with CCl_4 alone, while free γ-oryzanol did not improve cell viability as CCl_4-treated group. Notably, the preventive effect of γ-ory-lipo was lower than that of equal concentration of ferulic-lipo.

The Conversion of γ-Oryzanol to Ferulic Acid *in Vitro* and *in Vivo* The antioxidative properties of γ-oryzanol have been shown to be dependent on the presence of the ferulic moiety.[44] Therefore, the transformation of γ-oryzanol to ferulic acid is likely required for its potent antioxidative effects. Accordingly, we used HPLC analyses to investigate the conversion of γ-oryzanol to ferulic acid was checked in both *in vitro and in vivo* (Fig. 6). Here, Figs. 6A and B show standard

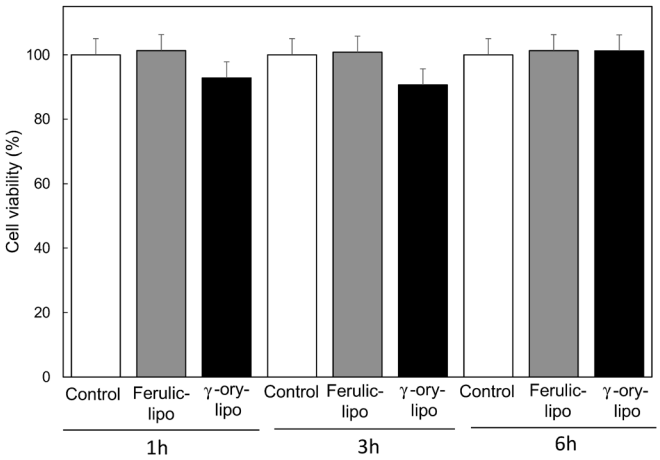

Fig. 4. Effect of γ-Ory-lipo on the Viability of HepG2 Cells

Cells were treated with 100 μM of liposomes containing γ-oryzanol (γ-ory-lipo) or ferulic acid (Ferulic-lipo). Cell viability was determined after 1, 3, and 6 h. Control (white column), γ-ory-lipo (grey column), and Ferulic-lipo (black column). Data are displayed as mean ± S.D. ($n = 3$).

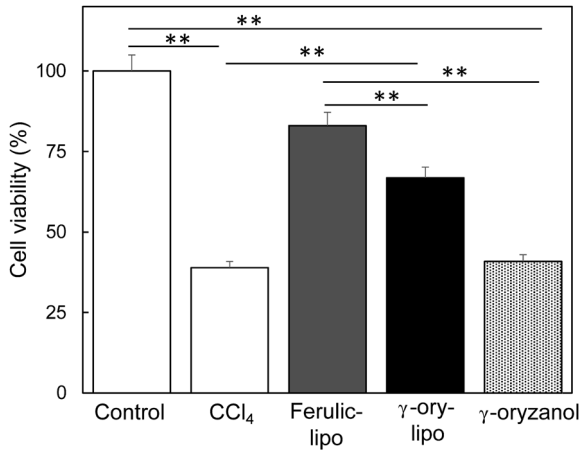

Fig. 5. Protective Effects of γ-Ory-lipo on CCl_4-Induced Toxicity of HepG2 Cells

HepG2 cells were treated with 75 μM of CCl_4 for 30 min, and then were treated with 100 μM free γ-oryzanol or with equivalent concentration of liposomes (γ-ory-lipo or Ferulic-lipo). Cell viability was determined after 12 h using the trypan blue staining method. Data are displayed as mean ± S.D. ($n = 3$). $^{**}p < 0.001$.

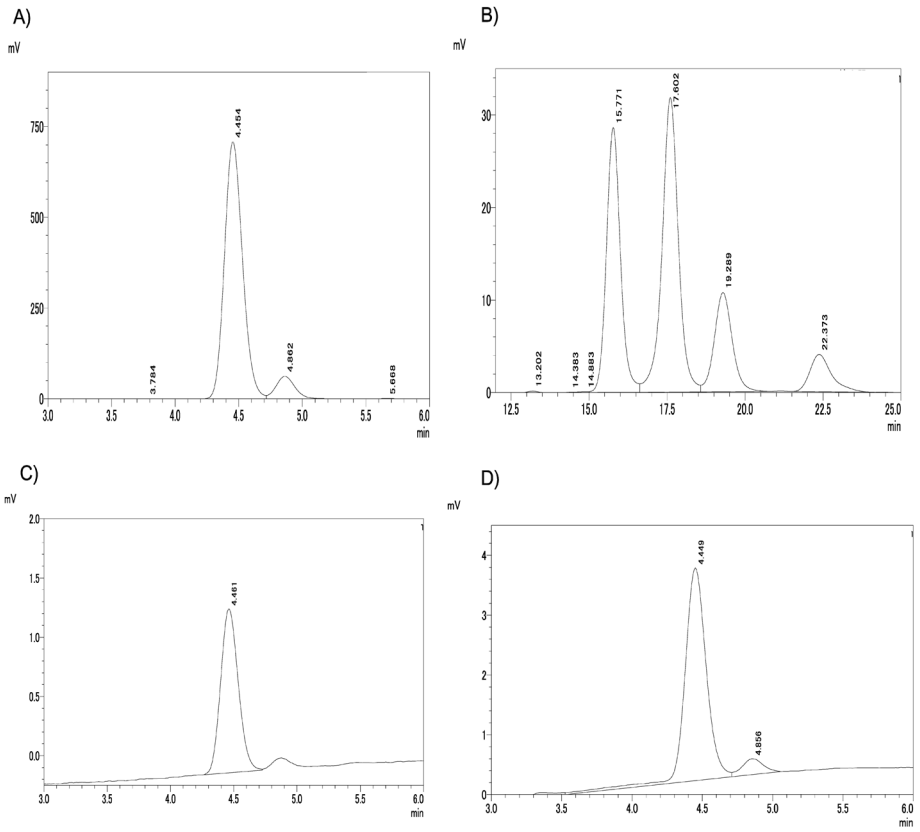

Fig. 6. The *in Vitro* and *in Vivo* Conversion of γ-Oryzanol into Ferulic Acid

γ-Oryzanol and ferulic acid were analyzed by HPLC. (A) Standard chromatogram of ferulic acid. (B) Standard chromatogram of γ-oryzanol. (C) HepG2 cells were treated with γ-ory-lipo for 1 h, and cellular lysate was analyzed by HPLC to investigate the *in vitro* conversion of γ-oryzanol to ferulic acid. (D) Wistar rats were treated intravenously with γ-ory-lipo for 1 h, and lysates from liver tissues were analyzed by HPLC to investigate the *in vivo* conversion of γ-oryzanol into ferulic acid.

chromatograms of ferulic acid and γ-oryzanol, respectively. It is reported that γ-oryzanols is chemically consist of four major phytesterol named cycloartenylferulate, 24-methylenecycloartenyl ferulate, camesterylferulate and sisterylferulate[45,46] which were clearly seen in Fig. 6B. First, the transformation of γ-oryzanol in the cultured HepG2 cells was determined. Here, HepG2 cells were incubated with γ-ory-lipo for 1 h and then lysed, and ferulic acid in the clarified lysates were analyzed by HPLC. Interestingly, a peak representing ferulic acid (Fig. 6A) was seen in the resulting chromatograms, suggesting that γ-oryzanol (Fig. 6B) was converted to ferulic acid (Fig. 6C) under these conditions.

Following confirmation of the conversion *in vitro*, we also examined the conversion of γ-oryzanol under *in vivo* conditions. For this assay, Wistar rats were intravenously treated with γ-ory-lipo, and 1 h later the conversion of γ-oryzanol (Fig. 6B) in the liver was assessed (Fig. 6D). Under these conditions, we again observed a peak representing ferulic acid. Taken together, these results suggest that γ-oryzanol was successfully converted into ferulic acid in both cultured cells and the liver.

γ-Ory-lipo Prevents the Cytotoxicity of CCl_4 in HepG2 Cells Because the *in vitro* and *in vivo* tests showed that time was required for the conversion of γ-oryzanol to ferulic acid, we checked whether pre-treatment of cells with γ-ory-lipo would enhance its protective effect against CCl_4-mediated toxicity. Therefore, cells were first incubated for 1 h with γ-ory-lipo prior to addition of CCl_4. Interestingly, when the viability of the cells treated for 12 h with CCl_4 was determined, it was found that γ-ory-lipo exhibited similar protective effects as ferulic-lipo (Fig. 7). As the addition of γ-ory-lipo 30 min after the addition of CCl_4 did not lead to such a potent protective effect (Fig. 5), we concluded that the conversion of the majority of the administrated γ-oryzanol into ferulic acid should be responsible for its similar protective effects for prevention of CCl_4-induced toxicity in HepG2 cells.

***In Vivo* Accumulation of γ-Ory-lipo in the Liver** The key target of CCl_4-induced toxicity *in vivo* is the liver, therefore the accumulation of γ-ory-lipo in the liver was investigated in a rat model. Here, $DiIC_{18}$-labeled γ-ory-lipo was intravenously administrated to Wistar rats, and the livers were harvested at multiple time points (1, 3, 6, 12, and 24 h

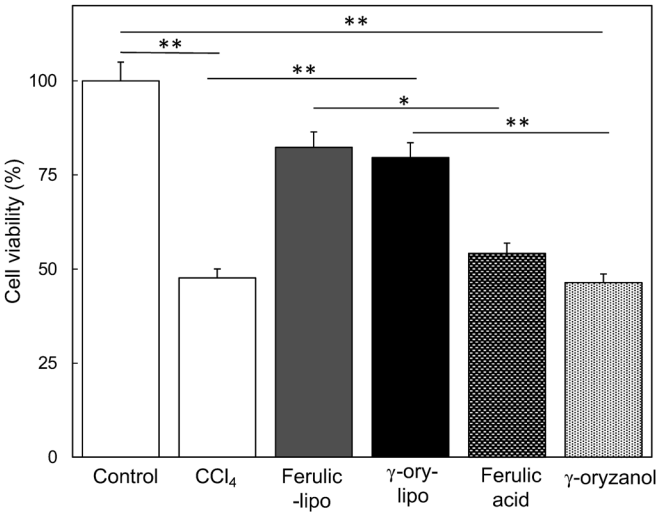

Fig. 7. Protection of HepG2 Cells against the Toxicity of CCl_4 by γ-Ory-lipo

Cells were pretreated with 100 μM free γ-oryzanol or ferulic acid or with an equivalent amount of encapsulating liposomes (γ-ory-lipo or Ferulic-lipo). After 1 h, the cells were treated with 75 μM of CCl_4. Cell viability was determined after 12 h using the trypan blue exclusion method. Data are displayed as mean ± S.D. ($n = 3$). *$p < 0.01$, **$p < 0.001$.

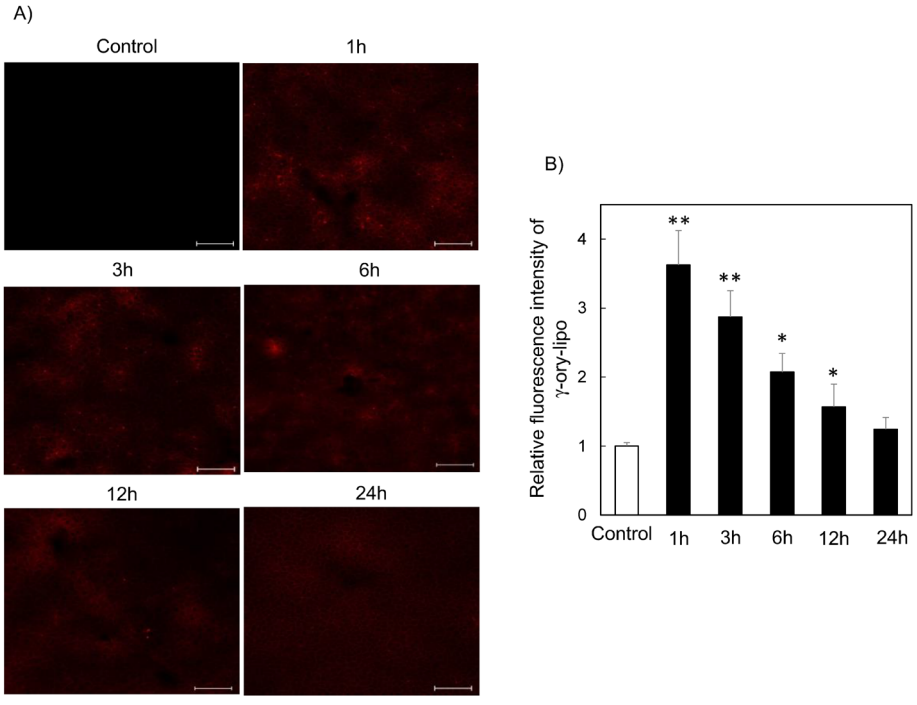

Fig. 8. Localization of γ-Ory-lipo in the Liver

$DiIC_{18}$-labeled γ-ory-lipo was administrated intravenously into Wistar rats. After the noted treatment time, the liver tissues were collected and sectioned using a cryostat. (A) Confocal laser scanning microscopic images of liver tissues analyzed at different time points (1, 3, 6, 12, and 24 h). (B) The relative fluorescence intensity of the liver tissue sections was quantified with ImageJ software. Data are displayed as mean ± S.D. ($n = 3$). *$p < 0.01$, **$p < 0.001$. Scale bar = 100 μm.

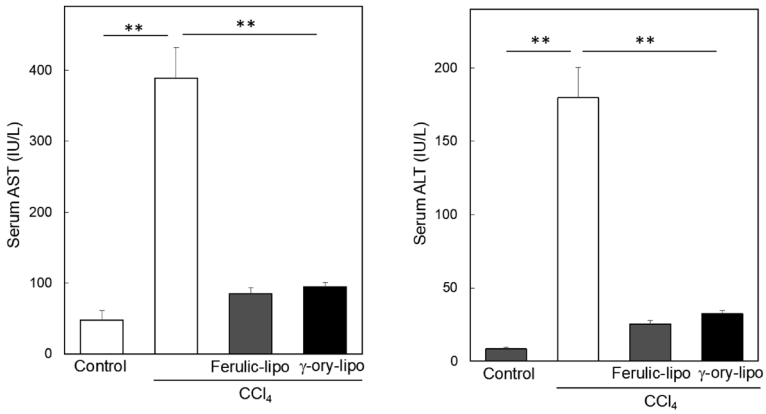

Fig. 9. Protective Effects of γ-Ory-lipo on CCl_4-Induced Liver Injury in Rats

Wistar rats were administrated equivalent concentrations of liposomes encapsulating γ-oryzanol (γ-ory-lipo) and ferulic acid (Ferulic-lipo) intravenously. After 1 h of treatment with liposomes, liver injury was induced by intraperitoneal administration of CCl_4. Serum was collected after 24 h, and the activities of ALT and AST were determined. Data are displayed as means ± S.D. ($n = 3$). $^{**}p < 0.001$.

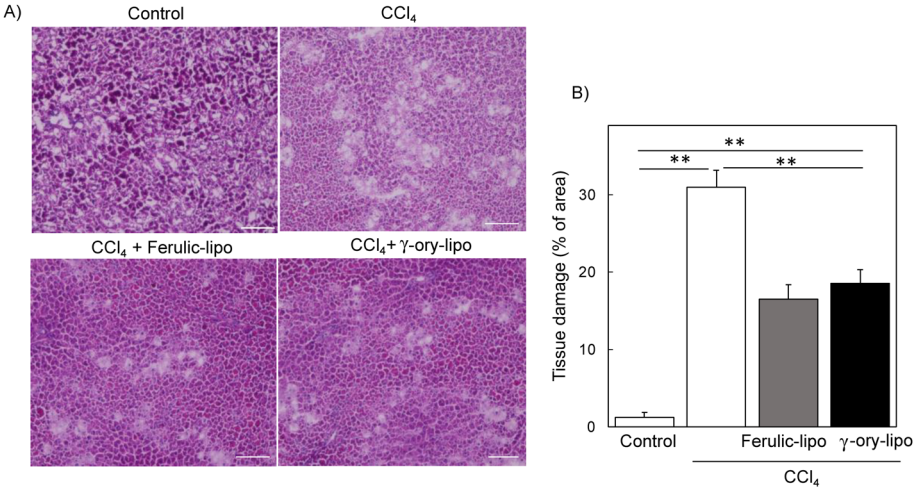

Fig. 10. Hepatoprotective Effects of γ-Ory-lipo against CCl_4-Induced Histopathological Damage

Liver tissues were collected 24 h after intravenous administration of γ-ory-lipo of Wistar rats, and sections were stained with hematoxylin and eosin to assess histological changes. (A) Microscopic image of liver tissues from control animals, CCl_4-treated animals, animals treated with CCl_4 and Ferulic-lipo, and animals treated with CCl_4 and γ-ory-lipo. (B) The relative area of tissue damage was quantified with ImageJ software. Data are displayed as means ± S.D. ($n = 3$). $^{**}p < 0.001$. Scale bar = 100 μm.

after administration). Resulting liver sections were subsequently observed by confocal laser scanning microscopy. As shown in Fig. 8A, the fluorescent intensity in tissue from γ-ory-lipo-treated rats was markedly increased relative to the control group, confirming the presence of γ-ory-lipo in the liver. Furthermore, quantitative estimation of relative fluorescence intensity (Fig. 8B) showed that fluorescence intensity in the liver was significantly higher at 1 h than at other time points. It was also found that the fluorescence of liposomes accumulated in liver decreased in a time-dependent manner (Fig. 8). Probably, the liposomes accumulated in the liver were gradually degraded, and the fluorescence dye labeled on the liposomes would be metabolized in the liver. As the result, the fluorescence intensity in the liver might have decreased. These results demonstrated the time-dependent *in vivo* distribution of γ-ory-lipo to the liver.

Hepatoprotective Effect of γ-Ory-lipo in a CCl_4-Induced Model of Liver Injury Because γ-ory-lipo exhibits protective effects *in vitro*, we tested the protective impact of γ-ory-lipo under *in vivo* conditions using a CCl_4-induced rat model of liver injury. Liver damage was quantified by the measurement of the activities of serum AST and ALT, which are serum markers of liver injury.[47] As shown in Fig. 9, CCl_4-treated rat had significantly higher serum ALT and AST levels, approximately 400 and 180 IU/L, respectively, as compared to control rats, indicating severe liver damage. However, intravenous administration of γ-ory-lipo significantly reduced serum ALT, and AST levels; the effect of γ-ory-lipo on reducing CCl_4-stimulated serum ALT and AST levels was similar to that of treatment with ferulic-lipo (Fig. 9). These results are consistent with a prior study in which treatment of SD male rats with equimolar concentrations of ferulic acid and γ-oryzanol led to similar effects in the obesity and dyslipidemia induced by a high-fat and high-fructose diet.[48] Based on these results, we conclude that the conversion of γ-oryzanol into ferulic acid is responsible for the similarity of the protective effects of γ-ory-lipo and ferulic-lipo against CCl_4-induced hepatotoxicity.

Protective Effects of γ-Ory-lipo against CCl_4-Induced Histopathological Damage Histopathological changes to liver tissues from rats of the CCl_4-induced model were observed in order to further investigate the protective effects of γ-ory-lipo. CCl_4-mediated liver tissue damage was clearly visible upon H&E staining of liver tissue from CCl_4-treated

rats (Fig. 10A), but intravenous administration of γ-ory-lipo led to a significant reduction of tissue damage (Fig. 10A), further demonstrating the hepatoprotective effects of γ-ory-lipo. Furthermore, quantitative estimation of the area of tissue damage (Fig. 10B) demonstrated that the protective effects of γ-ory-lipo and ferulic-lipo were comparable. These results provide additional evidence that the conversion of γ-oryzanol into ferulic acid is responsible for the effects of γ-ory-lipo on CCl_4-induced damage to liver tissue.

CONCLUSION

Based on our findings, we concluded that the γ-ory-lipo is a promising natural prodrug formulation of ferulic acid for the treatment of oxidative stress-mediated liver injury. In this formulation, γ-oryzanol is less reactive with ROS than ferulic acid, leading to an enhanced stability. The γ-ory-lipo formulation was also found to be effective at targeting the liver and in preventing CCl_4-induced damage to liver cells in culture and liver tissue *in vivo*.

Acknowledgments This study was supported by the Research Program for the Development of Intelligent Tokushima Artificial Exosome (iTEX) of Tokushima University, Japan.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Marcellin P, Kutala BK. Liver diseases: a major, neglected global public health problem requiring urgent actions and large-scale screening. *Liver Int*., **38** (Suppl. 1), 2–6 (2018).
2) Grant DM. Detoxification pathways in the liver. *J. Inherit. Metab. Dis*., **14**, 421–430 (1991).
3) Murie P, Gordillo KR. Role of oxidative stress in liver health and disease. *Oxid. Med. Cell. Longev.*, **2016**, 9037051 (2016).
4) Li S, Tan HY, Wang N, Zhang ZJ, Lao L, Wong CW, Feng Y. The role of oxidative stress and antioxidants in liver diseases. *Int. J. Mol. Sci*., **16**, 26087–26124 (2015).
5) Cichoż-Lach H, Michalak A. Oxidative stress as a crucial factor in liver diseases. *World J. Gastroenterol*., **20**, 8082–8091 (2014).
6) Bhogal RH, Curbishley SM, Weston CJ, Adams DH, Afford SC. Reactive oxygen species mediate human hepatocyte injury during hypoxia/reoxygenation. *Liver Transpl*., **16**, 1303–1313 (2010).
7) Tan LB, Norhaizan ME, Liew WPP, Rahman HS. Antioxidants and oxidative stress: a mutual interplay in age-related diseases. *Front. Pharmacol*., **9**, 1162 (2018).
8) Asrani SK, Devarbhavi H, Eaton J, Kamath PS. Burden of liver diseases in the world. *J. Hepatol*., **70**, 151–171 (2019).
9) Pooja V, Sunita M. Antioxidants and disease prevention. *International Journal of Advanced Scientific and Technical Research*, **2**, 903–911 (2014).
10) Ayoka TO, Ezema BO, Eze CN, Nnadi CO. Antioxidants for the prevention and treatment of non-communicable diseases. *J. Explor. Res. Pharmacol*., **7**, 178–188 (2022).
11) Wojtunik-Kulesza KA, Oniszczuk A, Oniszczuk T, Waksmundzka-Hajnos M. The influence of common free radicals and antioxidants on development of Alzheimer's Disease. *Biomed. Pharmacother.*, **78**, 39–49 (2016).
12) Kelly FJ. Use of antioxidants in the prevention and treatment of disease. *J. Int. Fed. Clin. Chem*., **10**, 21–23 (1998).
13) Wojtunik-Kulesza KA, Oniszczuk A, Oniszczuk T, Waksmundzka-Hajnos M. The influence of common free radicals and antioxidants on development of Alzheimer's Disease. *Biomed. Pharmacother.*, **78**, 39–49 (2016).
14) Casas-Grajales S, Muriel P. Antioxidants in liver health. *World J. Gastrointest. Pharmacol. Ther.*, **6**, 59–72 (2015).
15) Singal AK, Jampana SC, Weinman SA. Antioxidants as therapeutic agents for liver disease. *Liver Int*., **31**, 1432–1448 (2011).
16) Osawa T. Nephroprotective and hepatoprotective effects of curcuminoids. *Adv. Exp. Med. Biol*., **595**, 407–423 (2007).
17) Sumida Y, Naito Y, Tanaka S, Sakai K, Inada Y, Taketani H, Kanemasa K, Yasui K, Itoh Y, Okanoue T, Yoshikawa T. Long-term (> = 2 yr) efficacy of vitamin E for non-alcoholic steatohepatitis. *Hepatogastroenterology*, **60**, 1445–1450 (2013).
18) Li J, Guo C, Wu J. Astaxanthin in liver health and disease: A potential therapeutic agent. *Drug Des. Devel. Ther.*, **14**, 2275–2285 (2020).
19) Nah HY, Lee WS, Joo YE, Kim HS, Choi SK, Rew JS, Kim SJ, Jung Y, Ahn BW, Park JS, Shin BA. Resveratrol protects HepG2 and Chang liver cells from oxidative stress. *Chonnam Med. J*., **41**, 243–252 (2005).
20) Nnadi CO, Nwodo NJ, Kaiser M, Brun R, Schmidt TJ. Steroid alkaloids from *Holarrhena Africana* with strong activity against *Trypanosoma brucei rhodesiense. Molecules*, **22**, 1129 (2017).
21) Ara T, Ono S, Hasan M, Ozono M, Kogure K. Protective effects of liposomes encapsulating ferulic acid against CCl_4-induced oxidative liver damage *in vivo* rat model. *J. Clin. Biochem. Nutr.*, **72**, 46–53 (2023).
22) Bezerra GSN, Pereira MAV, Ostrosky EA, Barbosa EG, Moura MFV, Ferrari M, Aragao CFS, Gomes APB. Compatibility study between ferulic acid and excipients used in cosmetic formulations by TG/DTG, DSC, and FTIR. *J. Therm. Anal. Calorim*., **127**, 1683–1691 (2017).
23) Tee-ngam P, Nunant N, Rattanarat P, Siangproh W, Chailapakul O. Simple and rapid determination of ferulic acid levels in food and cosmetic samples using paper-based platforms. *Sensors*, **13**, 13039–13053 (2013).
24) Raj ND, Singh D. A critical appraisal on ferulic acid: biological profile, biopharmaceutical challenges, and nano formulations. *Health. Sci. Rev.*, **5**, 100063 (2022).
25) Sawada K, Rahmania H, Matsuki M, Hashimoto H, Ito J, Miyazawa T, Nakagawa K. Absorption, and metabolism of γ-Oryzanol, a characteristic functional ingredient in rice bran. *J. Nutr. Sci. Vitaminol*., **65** (Supplement), S180–S184 (2019).
26) Lohar V, Singhal S, Arora V. Prodrug: approach to better drug delivery. *Int. J. Pharm. Res*, **4**, 15–21 (2012).
27) Gandhi PM, Chabukswar AR, Jagdale SC. Carrier for prodrug synthesis: a review. *Indian J. Pharm. Sci*., **81**, 406–414 (2019).
28) Limcharoen T, Dasuni Wasana PW, Hasriadi H, Muangnoi C, Vajragupta O, Rojsitthisak P, Towiwat P. Curcumin diglutaric acid, a prodrug of curcumin reduces pain hypersensitivity in chronic constriction injury of sciatic nerve induced-neuropathy in mice. *Pharmaceuticals*, **13**, 212 (2020).
29) Wagstaff AJ, Ibbotson T, Goa KL. Capecitabine: a review of its pharmacology and therapeutic efficacy in the management of advanced breast cancer. *Drugs*, **63**, 217–236 (2003).
30) Liang L, Liu X, Wang Q, Cheng S, Zhang S, Zhang M. Pharmacokinetics, tissue distribution and excretion study of resveratrol and its prodrug 3,5,4′-tri-O-acetylresveratrol in rats. *Phytomedicine*, **20**, 558–563 (2013).
31) Peng Y, Deng Z, Wang C. Preparation, and prodrug studies of quercetin pentabenzensulfonate. *Yakugaku Zasshi*, **128**, 1845–1849 (2008).
32) Ishikawa M, Hirai S, Yoshida T, Shibuya N, Hama S, Takahashi Y, Fukuta T, Tanaka T, Hosoi S, Kogure K. Carotenoid stereochemistry affects antioxidative activity of liposomes co-encapsulating astaxanthin and tocotrienol. *Chem. Pharm. Bull*., **66**, 714–720 (2018).

33) Nadal JS, Toledo MG, Pupo YM, Padilha de Paula J, Farago PV, Zanin SMW. A stability-indicating HPLC-DAD method for determination of ferulic acid into microparticles: development, validation, forced degradation and encapsulation efficiency. *J. Anal. Methods. Chem*., **2015**, 286812 (2015).
34) Sakunpak A, Suksaeree J, Pathompak P, Charoonratana T, Sermkaew N. Antioxidative individual γ-oryzanol screening incold-pressed rice bran oil of different Thai rice varieties by HPLC-DPPH methods. *Int. J. Pharm. Sci*., **6**, 592–597 (2014).
35) Price M, Reiners JJ, Santiago AM, Kessel D. Monitoring singlet oxygen and hydroxyl radical formation with fluorescent probes during photodynamic therapy. *Photochem. Photobiol*., **85**, 1177–1181 (2009).
36) Wang CY, Ma FL, Liu JT, Tian JW, Fu FH. Protective effects of salvianic acid A on acute liver injury induced by carbon tetrachloride in rats. *Biol. Pharm. Bull*., **30**, 44–47 (2007).
37) Carrasco-Sandoval J, Aranda-Bustos M, Henriquez-Aedo K, Lopez-Rubio A, Fabra MJ. Bioaccessibility of different types of phenolic compounds co-encapsulated in alginate/chitosan-coated zein nanoparticles. *Lebenson. Wiss. Technol*., **149**, 112024 (2021).
38) Li S, Li H, Xu X, Saw PE, Zhang L. Nanocarrier-mediated antioxidant delivery for liver diseases. *Theranostics*, **10**, 1262–1280 (2020).
39) Scalia S, Mezzena M. Incorporation of quercetin in lipid microparticles: effect on photo- and chemical-stability. *J. Pharm. Biomed. Anal*., **49**, 90–94 (2009).
40) Shi J, Le Maguer M. Lycopene in tomatoes: chemical and physical properties affected by food processing. *Crit. Rev. Biotechnol*., **20**, 293–334 (2000).
41) Luong HV. Promotion of the stability of lycopene by liposomal formulations for anti-aging therapy. *Aging Sci*., **2**, 3 (2014).
42) Moongngarm A, Amornpan J, Homduang A, Haoteng C, Jelekovac A. Rice starch as coating materials for microencapsulation of gamma oryzanol to improve its stability. *Am. J. Appl. Sci*., **13**, 900–906 (2016).
43) Zhang Y, Cui H, Zhang R, Zhang H, Huang W. Nanoparticulation of prodrug into medicines for cancer therapy. *Adv. Sci.* (Weinh), **8**, 2101454 (2021).
44) Juliano C, Cossu M, Alamanni MC, Piu L. Antioxidant activity of gamma-oryzanol: mechanism of action and its effect on oxidative stability of pharmaceutical oils. *Int. J. Pharm*., **299**, 146–154 (2005).
45) Singanusong R. Garba Umar. Chapter 5—Micronutrients in Rice Bran Oil. *Rice Bran and Rice Bran Oil*, AOCS Press, Elsevier, Amsterdam, pp. 124–158 (2019).
46) Saenjum C, Chaiyasut C, Chansakaow S, Suttajit M, Sirithunyalug B. Antioxidant and anti-inflammatory activities of gamma-oryzanol rich extracts from Thai purple rice bran. *J. Med. Plants Res*., **6**, 1070–1077 (2012).
47) Giannini EG, Testa R, Savarino V. Liver enzyme alternation: a guide for clinicians. *CMAJ*, **172**, 367–379 (2005).
48) Wang O, Liu J, Cheng Q, Guo X, Wang Y, Zhao L, Zhou F, Ji B. Effects of ferulic acid and γ-oryzanol on high-fat and high-fructose diet-induced metabolic syndrome in rats. *PLOS ONE*, **10**, E0118135 (2015).