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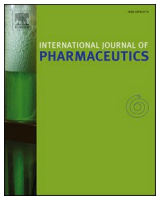

Rapeseed oleosomes facilitate intestinal lymphatic delivery and oral bioavailability of cannabidiol

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ABSTRACT

Due to high lipophilicity and extensive first-pass metabolic loss, cannabidiol (CBD) has low oral bioavailability. Co-administration of CBD and long-chain lipids facilitates the intestinal lymphatic delivery, resulting in higher systemic bioavailability, as well as high levels of the drug within the intestinal lymphatic system. However, despite previous attempts with various lipid-based formulations, the oral bioavailability of CBD is still limited. In this work, we have developed a novel formulation of CBD based on natural rapeseed oleosomes. *In vivo* studies in rats demonstrated that oral administration of CBD-loaded rapeseed oleosomes leads to substantially higher oral bioavailability and intestinal lymphatic targeting of CBD in comparison with rapeseed oil or artificial emulsion made of rapeseed oil and lecithin.

In vitro mechanistic assessments, including *in vitro* lipolysis and peroxide value determination suggest that the lower oxidative state of the oil in oleosomes in comparison to crude oil or artificial emulsion is likely to be the main factor responsible for the superior performance of the CBD-loaded rapeseed oleosomes *in vivo*. Although further investigation will be needed, the data suggest that natural seeds-derived oleosomes can be used as a promising lipid-based drug delivery platform promoting the bioavailability and lymphatic delivery of lipophilic drugs.

1. Introduction

Cannabidiol (CBD) is a highly lipophilic Biopharmaceutics Classification System (BCS) Class II phytocannabinoid originally isolated from the *Cannabis sativa* plant. According to previous reports, CBD has substantial immunomodulatory effects at high concentrations, suggesting its potential in treatment of autoimmune diseases (Hess et al., 2016; Hoffenberg et al., 2019; Nichols and Kaplan, 2019; Zgair et al., 2017). Efficient delivery of CBD to the intestinal lymphatic system can lead to sufficient exposure of the immune cells to the drug, potentially resulting in more efficient treatment of autoimmune diseases (Zgair et al., 2017). Moreover, enhanced systemic bioavailability of CBD could lead to benefits for patients affected by conditions such as epilepsy or cancer (Alice Brookes, 2024; Arzimanoglou et al., 2020; Chen et al., 2018; Seltzer et al., 2020). As reported previously, cannabidiol is metabolized by cytochrome P450 (CYP) enzymes (mainly by CYP3A4 and CYP2C19) and glucuronosyltransferases (minor contribution) in the liver (primarily) and intestinal wall (Court et al., 2024; Jiang et al., 2011; Perucca and Bialer, 2020). Due to the extensive first-pass metabolism and poor aqueous solubility, the oral bioavailability of CBD following oral administration is limited (Franco et al., 2020; Mechoulam et al., 2002; Perucca and Bialer, 2020).

It is reported that the oral bioavailability of CBD in fasting state in humans is as low as 6 % (Franco et al., 2020; Perucca and Bialer, 2020). Co-administration of highly lipophilic drugs and long-chain triglycerides to facilitate the intestinal lymphatic drug delivery is an

Abbreviations: AUC, Area Under Curve; BCS, Biopharmaceutical Classification System; BSU, Bio-Support Unit; CBD, Cannabidiol; DDT, Dichlorodiphenyltrichloroethane; HPLC, High-Performance Liquid Chromatography; MLN, Mesenteric Lymph Nodes; PC, Phosphatidylcholine; PDA, Photodiode Array Ultraviolet; SD, Standard Deviation; SDS, Sodium Dodecyl sulphate; TG, Triglycerides; CYP, Cytochrome P450.

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effective strategy to improve the oral bioavailability (Kalepu et al., 2013; Porter et al., 2007; Porter et al., 2013). Lipid-based formulations usually contain an oil phase mixed or not with various types of surfactants to ensure dispersion of the oil phase in the gastrointestinal tract contents (Porter et al., 2007). Both the initial components of lipid-based formulations and their lipolysis products are important for the intraluminal processing and absorption of the drug (Carrière, 2016). When long-chain triglycerides enter the gastrointestinal tract, they will be enzymatically hydrolysed to release fatty acids and monoglycerides. These lipid digestion products, together with bile salts and phospholipids form mixed micelles, facilitate the diffusion to the intestinal wall and eventually absorption into intestinal epithelial cells. In the enterocytes, long-chain fatty acids and monoglycerides are re-esterified to triglycerides and packed into chylomicrons. These large lipoproteins are then transported into the intestinal lymphatic system and from there to the thoracic lymph duct and systemic blood circulation, avoiding liver and hepatic first-pass metabolism (Kalepu et al., 2013; Porter et al., 2007). Therefore, if a highly lipophilic drug with appropriate physicochemical properties for affinity to chylomicrons is administered together with long-chain triglycerides, higher systemic bioavailability and efficient targeting into intestinal lymphatic system could be achieved, especially if the hepatic first-pass metabolism was a primary limiting factor of the oral bioavailability.

We have shown previously that sesame oil facilitates the delivery of CBD to the intestinal lymphatic system following oral administration in rats (Zgair et al., 2017). As a result, CBD concentrations in lymph fluid could reach ~ 250 times higher levels than in plasma, and systemic exposure was about 3-fold higher compared with lipid-free formulations (Zgair et al., 2017). In a later study it was found that olive oil formulation led to a similar extent of oral bioavailability and lymphatic delivery of CBD as sesame oil, but with somewhat lower variability (Feng et al., 2022). Interestingly, even with efficient intestinal lymphatic delivery of CBD associated with sesame or olive oil formulations, the oral bioavailability of the drug was only about 20 % (Feng et al., 2022; Zgair et al., 2016). In our previous attempts to improve further the bioavailability and lymphatic targeting of CBD, we have used pre-digested lipid formulations, medium-chain triglycerides or surfactants, resulting in higher micellar solubilisation of the drug *in vitro*, but natural vegetable oils still performed better than these formulations *in vivo* (Feng et al., 2021a; Feng et al., 2021b). This could potentially be due to existence of several minor constituents in natural oils, affecting their stability and digestion efficiency (Feng et al., 2021a). Therefore, in this work we have chosen to amend the approach of development of lipid-based formulations of CBD, and instead of pre-digesting, or increasing the emulsification or micellar solubilisation by various excipients, we have opted to use natural plant seeds-derived oleosomes as a drug delivery vehicle (Romero-Guzmán et al., 2020). Oleosomes are triglyceride (TG) storage organelles in plant seeds and consist of a triglyceride core surrounded by a monolayer of phospholipids and proteins (De Chirico et al., 2018; Romero-Guzmán et al., 2020). Although some *in vitro* studies assessed the potential use of oleosomes as drug delivery vehicles (Cho et al., 2018), it is unknown if they can indeed improve oral bioavailability and lymphatic delivery of CBD or other drugs.

Thus, the aim of this work is to test the hypothesis that natural rapeseed oleosomes could increase the oral bioavailability and intestinal lymphatic delivery of CBD in comparison to artificial oleosome-like protein-free rapeseed-based emulsion or pure rapeseed oil.

2. Materials and Methods

2.1. Materials

Rapeseed oil, sodium hydroxide solution (NaOH, 1 M), potassium bromide (KBr), L-α-phosphatidylcholine (~60 %, from egg yolk), Trizma® maleate, sodium taurocholate hydrate, pancreatin from porcine pancreas (8 × USP), 4,4-dichlorodiphenyltrichloroethane (DDT), acetic acid, isooctane, potassium iodide (KI, powder), sodium dodecyl sulphate (SDS), sodium thiosulphate and soluble starch were all purchased from Sigma-Aldrich (Dorset, UK). Synthetic CBD (Fig. 1) was kindly donated by CBDepot.eu (Teplice, Czech Republic). Pooled male Sprague Dawley rat plasma was purchased from Sera Laboratories International Ltd (West Sussex, UK). Rapeseed phosphatidylcholine (PC, > 98 %) was purchased from Stratech Scientific (Ely, UK). EnzyChrom™ Triglyceride Assay Kit was purchased from Generon Ltd (Slough, UK). All other solvents were of high-performance liquid chromatography (HPLC) grade or higher.

2.2. Preparation of oleosome-like protein-free artificial emulsion vehicle

The oleosome-like protein-free artificial emulsion preparation procedure was modified from previously reported methodology (Deleu et al., 2010). Rapeseed lecithin (40 mg) and rapeseed oil (760 mg) were first added into tubes, followed by addition of 1.2 mL water. The mixture was then homogenised using Bandelin Sonopuls HD 2070 ultrasonic homogenizer (Berlin, Germany) at 20 kHz, 15 s (full power, 60 % cycle, repeated 6 times with 25 s intervals). The emulsion was then cooled on ice for 1 min and filtered using syringe filter (pore size 5 μm). Triglyceride concentrations of the obtained artificial emulsion was measured by EnzyChrom™ triglyceride assay kit using the manufacturer's instructions (BioAssay Systems, Hayward, CA, US).

2.3. Preparation of rapeseed oleosomes vehicle

The procedure of extraction of rapeseed oleosomes from rapeseed seeds was based on a previous report (De Chirico et al., 2018). Briefly, rapeseed seeds were soaked in 0.1 M sodium bicarbonate buffer (pH 9.5) at 4°C for 16 h with a ratio of 1:4 (seeds: buffer, w / w). The liquid was then filtered off and seeds were crushed by a Kenwood BLX52 blender (Havant, UK) at full power for 90 s with the same buffer at a ratio of 1:7 (seeds: buffer, w / w). The mixture was filtered and centrifuged at 10,000 *g* for 30 min at 4°C using Beckman J2-21 centrifuge, JA-10 rotor (Brea, CA, US). The cream on the upper layer was collected and resuspended by gentle stirring in the sodium bicarbonate buffer 0.1 M (1: 4, cream: buffer, w: w). Subsequently, the emulsion was centrifuged again (10000 *g* for 30 min at 4°C). The cream was collected and resuspended in water by gentle stirring (1: 4, cream: water, w: w). The third centrifugation (10000 *g* for 30 min at 4°C) was then performed and the cream collected was the final rapeseed oleosomes batch. The triglyceride (TG) concentrations were measured by EnzyChrom™ triglyceride assay kit using the manufacturer's instructions (BioAssay Systems, Hayward, CA, US). The oleosomes were diluted to TG levels of 380 mg/mL with water to resemble TG levels in the artificial emulsion.

2.4. Preparation of CBD-loaded artificial emulsion, oleosomes and rapeseed oil

To prepare artificial emulsion loaded with CBD, the drug was pre-

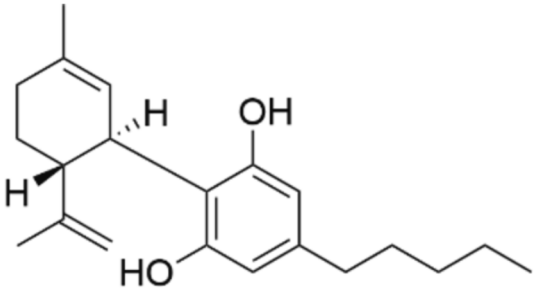

Fig. 1. 2D structure of Cannabidiol (Created by ChemDraw, Revvity Signals Software, Waltham, Ma, US).

dissolved in rapeseed oil at a concentration of 4 mg/mL, and the emulsion was prepared as described above in Section 2.2. For loading CBD into rapeseed oleosomes, 981 μL of rapeseed oleosome emulsion (380 mg/mL TG concentration) were mixed with 19 μL solution of 100 mg/mL CBD in ethanol. The mixture was then stirred using a magnetic stirrer (400 RPM) at 37°C for 1 h.

Density gradient centrifugation (1070 *g*, 22.5 h) was then applied by Eppendorf® 5810R Centrifuge with A-4–62 rotor (Hamburg, Germany) to separate the oleosomes or artificial emulsion from free CBD (Gershkovich and Hoffman, 2005; Muresan et al., 2023; Qin et al., 2021; Zgair et al., 2016). Subsequently, the upper layer containing the cream was collected and diluted in water to reach a TG concentration of 235 mg/mL. This resulted in CBD concentration of about 1 mg/mL in drug-loaded oleosomes and artificial emulsion.

CBD-loaded rapeseed oil formulation was prepared by solubilising CBD in rapeseed oil at a concentration of 4.44 mg/mL.

2.5. Particle size measurement of emulsions

The particle sizes of artificial emulsion and rapeseed oleosomes were measured by HORIBA LA960 laser diffraction particle size distribution analyser (Kyoto, Japan). Emulsion and oleosomes were diluted 5000-fold in water before measurement. All measurements settings were based on previous report (De Chirico et al., 2018). Median particle size and volume distribution were recorded.

2.6. Zeta potential measurement of emulsions

A series of 10000-times water-diluted artificial emulsions and rapeseed oleosomes were prepared in 1 mL Eppendorf tubes. The pH values were adjusted to be between 3 and 10 using 0.1 M NaOH and 0.1 M HCl. Samples were then analysed using Malvern Zetasizer Nano ZS (Malvern, UK). The measurement settings were set based on a previous report (De Chirico et al., 2018).

2.7. Triglyceride level determination

Triglycerides levels in artificial emulsion, rapeseed oleosomes and rapeseed oil were tested by colorimetric EnzyChrom^{TM} triglyceride assay kit using the manufacturer's instructions (BioAssay Systems, Hayward, CA, US).

2.8. In vitro lipolysis

The digestion of CBD-loaded rapeseed oil, rapeseed oleosomes and artificial emulsion was assessed using an *in vitro* lipolysis model, which mimics lipid digestion processes in the small intestine (Benito-Gallo et al., 2015; Benito-Gallo et al., 2016; Feng et al., 2022; Feng et al., 2021a; Feng et al., 2021b; Gershkovich et al., 2012). The complete digestion buffer (pH 6.8) simulating fasted state consisted of 50 mM Trizma® maleate, 150 mM NaCl, 5 mM $CaCl_2$, 5 mM sodium taurocholate hydrate and 1.25 mM L-α-lecithin. The incomplete digestion buffer (pH 6.8) that was used for lipase preparation consisted of 50 mM Trizma® maleate, 150 mM NaCl and 5 mM $CaCl_2$. After all components in buffers were solubilised, the pH values of buffers were adjusted to 6.8 at 37°C. To obtain lipase preparation from porcine pancreatin, 1 g of pancreatin was added into 5 mL incomplete digestion buffer and vortexed for 15 min at room temperature. The supernatant containing the lipase extract was collected after centrifugation at 5 °C, 1794 *g* for 15 min, and stored on ice. The CBD-loaded artificial emulsion and rapeseed oleosomes (0.9 mL, 1 mg/mL CBD concentration), or CBD-loaded rapeseed oil (202.5 μL, 4.44 mg/mL CBD concentration, followed by addition of 697.5 μL water), were premixed with complete digestion buffer (35.5 mL) at 37 °C for 15 min. Then, 3.5 mL of the lipase preparation was added to initiate the lipolysis process and the pH value was maintained at 6.8 by means of Mettler Toledo T50 pH-stat titrator (Greifensee, Switzerland) using 1 M NaOH solution. The termination of reaction was set to the rate of addition of 1 M NaOH dropping below the threshold of 3 μL/min. The lipolysis medium was then separated into three fractions (sediment, micellar and lipid layer) by ultracentrifugation (SORVALL Discovery 100SE ultracentrifuge (Waltham, MA, US), TH-641 rotor, 269,071 *g*, 37 °C, 90 min). All fractions were separately collected and stored at −80 °C until analysis for CBD content.

2.9. Peroxide value test

Oil contained in CBD-loaded rapeseed oleosomes and the artificial emulsion was released by freezing and thawing (di Bari et al., submitted manuscript). Briefly, the formulations were frozen in −20 °C for 24 h and then stored at ambient temperature for one hour to allow for complete thawing. The samples were centrifuged at 10,000 *g* for 15 min at ambient temperature using Biosan Microspin 12 centrifuge (Riga, Latvia) and released oil in the upper layer was collected. Subsequently, the peroxide values of these released oils and the CBD-loaded rapeseed oil were determined by sodium thiosulfate titration method (The American Oil Chemists' Society, 2011). Oils were weighed (~1 g) and dissolved in 30 mL of the 3:2 acetic acid-isooctane mixture. Saturated KI solution (0.5 mL) was added, and the medium was stirred for 1 min. Then, 30 mL of distilled water, 0.5 mL of 10 % sodium dodecyl sulphate and 0.5 mL of starch indicator solution were added. The medium was titrated with 0.01 M sodium thiosulphate until the disappearance of the blue colour.

Peroxide value (milliequivalents peroxide/ 1000 g of oil) was calculated using Equation (1):

$$\text{Peroxide value} = [(V_{oil} - V_{blank})^{*}M]/m \quad (1)$$

where V_{oil} is the titration volume of oil in mL, V_{blank} is the titration volume of blank in mL, M is the molarity of sodium thiosulphate solution in mmol/mL and m is the mass of oil in kg.

2.10. In vivo pharmacokinetics and biodistribution

All experimental protocols were authorised by the United Kingdom Home Office and University of Ethical Review Committee in accordance with the Animals [Scientific procedures] Act 1986. *In vivo* pharmacokinetics and biodistribution studies were carried out using male Sprague Dawley rats, 276 to 300 g body weight (Charles River UK, Margate, UK). The animals were kept in the University of Nottingham Bio-Support Unit (BSU) under regulated temperature and humidity, 12 h light–dark cycle.

In the pharmacokinetic study, the right jugular vein was cannulated under general anaesthesia and rats were allowed two nights of recovery. Animals were fasted overnight for up to 16 h before pharmacokinetic study with free access to water. Three lipid-based formulations of CBD: CBD-loaded rapeseed oil, artificial emulsion and rapeseed oleosomes were administered by oral gavage to animals. Water was administered by a second oral gavage in CBD-loaded rapeseed oil group to provide the same volume of water that is contained in CBD-loaded artificial emulsion and rapeseed oleosomes. The dose of CBD was 3 mg/kg in all groups. CBD-loaded rapeseed oleosomes and artificial emulsion contained 1 mg/mL CBD. CBD-loaded rapeseed oil contained 4.44 mg/mL of CBD and was administered to rats with water at a ratio of 22.5: 77.5 (oil / water, v / v) to achieve the same dose of CBD, oil and water across all formulations. During pharmacokinetic study, 0.3 mL blood samples were collected from jugular vein cannula at 1, 2, 3, 4, 5, 6, 8, 10 and 12 h following oral administration into EDTA-containing tubes. Collected blood samples were centrifuged at 3000 *g* for 10 mins at 4 °C using DLAB D1524R centrifuge (Beijing, China) for plasma separation. All samples were stored at −80 °C until analysis.

For biodistribution studies, the rats were fasted up to 16 h with free access to water. The same formulations and doses of CBD were administered by oral gavage to rats. Blood was collected under terminal anaesthesia from inferior vena cava at the plasma t_{max} and t_{max} − 1 h

(determined in pharmacokinetic studies). Rats were then sacrificed, and lymph fluid sample, mesenteric lymph nodes (MLN), brain, as well as spinal cord tissues were harvested. Collected blood samples were centrifuged at 3000 *g* for 10 mins at 4°C using DLAB D1524R centrifuge (Beijing, China) to separate plasma. Plasma, lymph, and tissues samples were stored at −80°C until analysis (Zgair et al., 2017; Zgair et al., 2015).

2.11. Bioanalytical conditions

The concentrations of CBD in all samples were analysed using a HPLC system consisting of Waters Alliance 2695 separations module (Milford, MA, US) and Waters photodiode array ultraviolet (PDA) 2996 detector (Milford, MA, US). The samples preparation procedure and chromatography conditions for determination of CBD were identical to a previously reported methodology (Feng et al., 2022; Feng et al., 2021a; Feng et al., 2021b; Zgair et al., 2015).

2.12. Data analysis

All data are presented as mean ± standard deviation (SD). Phoenix WinNonlin 6.3 Professional (Pharsight, Mountain View, CA, US) was used for non-compartmental analysis of plasma concentration–time profiles to obtain pharmacokinetic parameters. Power calculations (G*Power 3.1, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany) were used to determine the sample size of each group (Faul et al., 2007). GraphPad Prism 10.1.0 (GraphPad software, San Diego, CA, US) was used to perform statistical analysis. One-way analysis of variance (ANOVA), followed by Tukey's post-hoc comparison or unpaired *t* test where appropriate were used for analysis of differences between means. $P < 0.05$ was considered statistically significantly different.

3. Results

3.1. Physicochemical properties of CBD-loaded rapeseed oleosomes and artificial emulsion

An artificial emulsion was designed to have similar physicochemical properties as rapeseed oleosomes, including particle size of around 1 μm (Fig. 2A & 2C, Table 1). After CBD-loaded rapeseed oleosomes and artificial emulsion were prepared, various parameters were tested to ensure that they remain similar in their physicochemical characteristics. The particle volume distribution of CBD-loaded rapeseed oleosomes and artificial emulsion is presented in Fig. 2B showing overlapping particle size distributions. However, both CBD-loaded rapeseed oleosomes and artificial emulsion had a higher median particle size (around 2 μm) compared with unloaded lipid vehicles (Fig. 2B and Table 1). Zeta potential of these two formulations was also similar (Fig. 2D). In addition, Table 1 also shows that CBD-loaded rapeseed oleosomes and CBD-loaded artificial emulsion had practically identical CBD concentrations, while triglycerides content was already adjusted to be equivalent between these two formulations.

3.2. In vitro lipolysis of CBD-loaded rapeseed oil, rapeseed oleosomes and artificial emulsion

The distribution of CBD into the sediment, aqueous micellar and oil phases following *in vitro* lipolysis of CBD-loaded rapeseed oil, rapeseed oleosomes and artificial emulsion is shown in Fig. 3. The drug recovered in aqueous micellar phase is considered to be readily available for absorption. The CBD distributed more efficiently into micellar phase following lipolysis of CBD-loaded rapeseed oleosomes than following lipolysis of other formulations. The sodium hydroxide solution volumes used for titration during the lipolysis of three formulations were 0.49 ± 0.02 mL, 0.26 ± 0.04 mL and 0.12 ± 0.00 mL (mean ± SD, rapeseed oleosome, artificial emulsion, rapeseed oil, respectively).

3.3. Peroxide value of CBD-loaded rapeseed oil and released oils from CBD-loaded rapeseed oleosomes and artificial emulsion

The peroxide values of released oil from CBD-loaded rapeseed oleosomes and artificial emulsion, as well as of CBD-loaded rapeseed oil formulation were assessed (Fig. 4). The oil released from rapeseed oleosomes had a dramatically lower peroxide value than of other formulations.

3.4. Pharmacokinetics of CBD following oral administration in different formulations

The plasma concentration–time profiles of CBD following oral gavage administration of CBD-loaded rapeseed oil, artificial emulsion and rapeseed oleosomes are shown in Fig. 5. The pharmacokinetic parameters derived from plasma concentration–time profiles are shown in Table 2. The CBD-loaded rapeseed oleosomes led to the highest systemic exposure to CBD (AUC) and highest C_{max} in comparison to other formulations (2-fold increase versus rapeseed oil). C_{max} was also reached faster with CBD-loaded rapeseed oleosomes (t_{max} = 2h) than that of CBD-loaded rapeseed oil (t_{max} = 5h) and artificial emulsion (t_{max} = 2–3 h).

3.5. Biodistribution of CBD following oral administration

Plasma, lymph fluid, mesenteric lymph nodes (MLN), brain and spinal cord were obtained at plasma t_{max} and t_{max} − 1 h following oral administration of CBD-loaded rapeseed oil, artificial emulsion and rapeseed oleosomes. The results are shown in Fig. 6A and 6B. At plasma t_{max}, the CBD-loaded rapeseed oleosomes led to a significantly higher CBD concentration in MLN and lymph fluid compared with the CBD-loaded artificial emulsion and rapeseed oil (Fig. 6A). At t_{max}, the concentration of CBD in MLN following administration of CBD-loaded rapeseed oleosomes was 8.5-fold and 5.5-fold higher than following administration of CBD-loaded rapeseed oil or CBD-loaded artificial emulsion, respectively. In lymph fluid, CBD-loaded rapeseed oleosomes led to 26.5-fold and 9.2-fold higher CBD concentration than following CBD-loaded rapeseed oil and artificial emulsion administrations, respectively. At t_{max} − 1 h, the concentrations of CBD in MLN and lymph fluid following oral administration of CBD-loaded rapeseed oleosomes were also higher than for other groups (Fig. 6B). However, the differences at t_{max} − 1 h were less prominent than for plasma t_{max}. The distribution of CBD was also assessed in brain and spinal cord tissues and described in Supplementary Material Fig. S1.

4. Discussion

Lipid-based formulations are an effective strategy to increase the intestinal lymphatic delivery and oral bioavailability of highly lipophilic drugs (Feeney et al., 2016; Hauss, 2007; Porter et al., 2007). On the example of a highly lipophilic drug CBD, we have previously shown that natural vegetable oil vehicles led to higher intestinal lymphatic delivery and systemic bioavailability than pre-digested or self-emulsifying drug delivery systems (Feng et al., 2021a; Feng et al., 2021b). However, even the best performing vegetable oil vehicles (sesame and olive oils) resulted in systemic bioavailability of CBD of only about 20 % (Feng et al., 2022; Zgair et al., 2016). Therefore, inspired by the superior performance of natural vegetable oils in comparison to artificial formulations *in vivo* (Feng et al., 2021a; Feng et al., 2021b), in this work we have assessed the natural seed-derived oleosomes as an oral formulation vehicle for CBD. Oleosomes are triglyceride-rich particles surrounded by phospholipid monolayer with embedded proteins, the so called oleosins. The performance of CBD-loaded rapeseed oleosomes as a formulation for lymphatic delivery and systemic bioavailability following oral administration was compared with CBD-loaded rapeseed oil and rapeseed artificial emulsion controls.

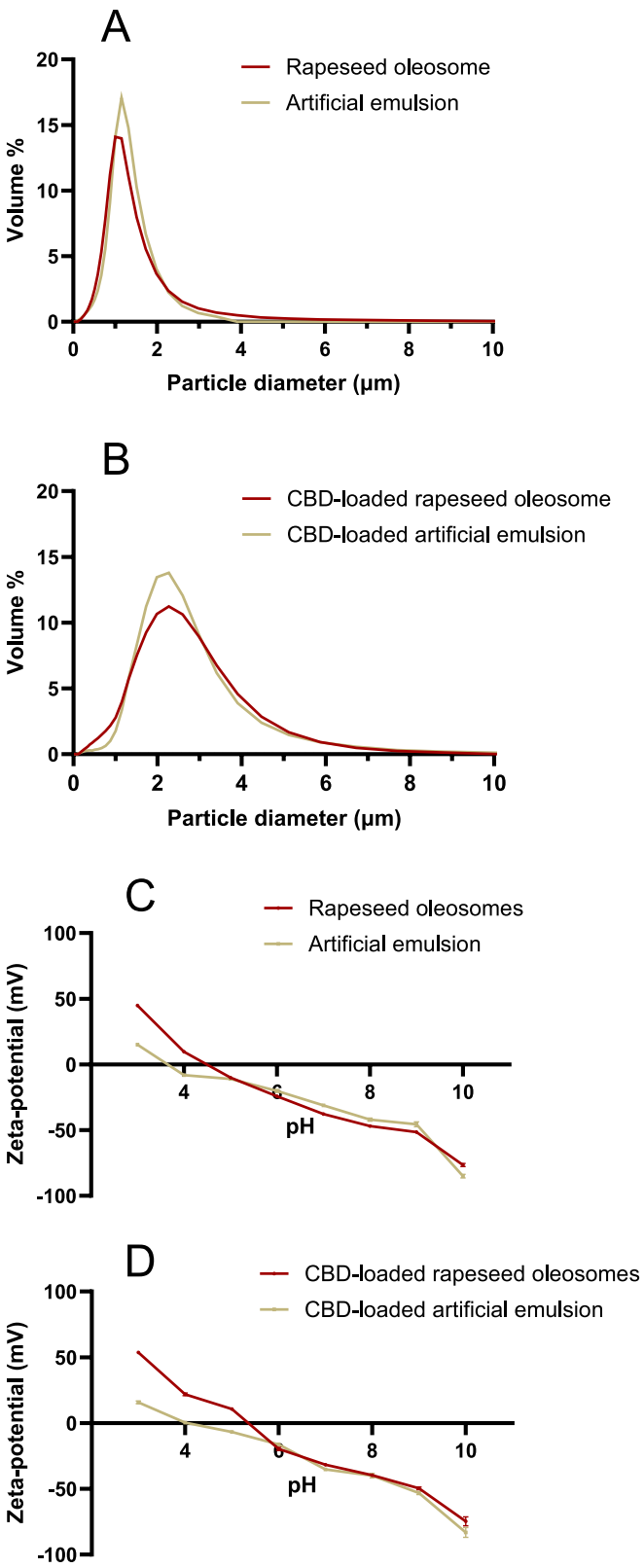

Fig. 2. Particle volume distribution and zeta-potential of rapeseed oleosomes and artificial emulsion before and after CBD loading (n = 3). A, volume distribution of rapeseed oleosomes and artificial emulsion; B, volume distribution of CBD-loaded rapeseed oleosomes and artificial emulsion; C, Zeta-potential of rapeseed oleosomes and artificial emulsion; D, Zeta-potential of CBD-loaded rapeseed oleosomes and artificial emulsion. Unpaired *t* test was used for analysis of differences in zeta potential values between artificial emulsion and rapeseed oleosomes at the same pH with or without CBD. It was found that almost all zeta potential values of artificial emulsion have significant differences compared with rapeseed oleosomes, except CBD-loaded artificial emulsion and CBD-loaded rapeseed oleosomes at pH 8. $P < 0.05$ was considered statistically significantly different.

Table 1
Properties of CBD-loaded rapeseed oleosomes and artificial emulsion (Mean ± SD, n = 3).

	TG level (mg/mL*)	Median particle size (μm)	CBD concentration (mg/mL)
Artificial emulsion	382.57 ± 24.50	1.09 ± 0.01[ns]	–
Rapeseed oleosomes	Adjusted to be identical to artificial emulsion	1.00 ± 0.06	–
CBD-loaded artificial emulsion	235.36 ± 11.44	2.03 ± 0.15[ns]	1.04 ± 0.03[ns]
CBD-loaded rapeseed oleosomes	Adjusted to be identical to CBD-loaded artificial emulsion	1.94 ± 0.04	1.03 ± 0.07

* Triglyceride levels are expressed as mg/mL triolein equivalents. The unit conversion formula is as follows: 1.0 mmol/L triglycerides = 0.885 mg/mL triolein equivalent triglycerides. The measured triglycerides concentration of pure rapeseed oil was 1043 ± 81 mg/mL.

ns not significantly different from corresponding oleosomes groups. Statistical analysis was performed using unpaired *t* test between artificial emulsion and rapeseed oleosomes groups with or without CBD. $P < 0.05$ was considered to represent a significant difference.

4.1. The physicochemical properties of CBD-loaded rapeseed oleosomes and artificial emulsion

After drug loading and density gradient centrifugation (to remove free CBD), around 1 mg/mL CBD content was found in the CBD-loaded oleosomes with a triglyceride concentration of approximately 235 mg/mL (Table 1). To assess the possible influence of various characteristics of rapeseed oleosomes on the absorption and lymphatic delivery of CBD, a protein-free CBD-loaded artificial emulsion was developed. The CBD-loaded artificial emulsion was designed to be similar to CBD-loaded rapeseed oleosomes in terms of rapeseed oil as a lipid core, rapeseed phospholipids as surface active agents, median particle size, triglyceride levels, and CBD content (Fig. 2B, Table 1). These two formulations also showed overall similar values for their zeta-potential (Fig. 2D). However, since there is no protein in the artificial emulsions, zeta potential of the CBD-loaded artificial emulsion was slightly different from CBD-loaded rapeseed oleosomes in the pH range. Considering the acidic pH environment of gastrointestinal tract (Fallingborg, 1999; McConnell et al., 2008), this difference may slightly affect formulations' behaviour in the stomach. Additionally, another noteworthy phenomenon is the change in particle size and zeta potential before and after CBD loading. Before CBD loading, both artificial emulsion and rapeseed oleosomes had a median particle size of approximately 1 μm (Fig. 2A, Table 1). After CBD loading, their median particle size increased to around 2 μm (Fig. 2B, Table 1). Although the drug loading could be responsible for this increase the particle size, the main reason is most likely due to density gradient centrifugation, which is used to separate the formulation from the unloaded free CBD (see Supplementary Material Fig. S2). Despite particle size increase, these two formulations showed only slight changes in zeta potential before and after CBD loading (Fig. 2C & D).

4.2. CBD-loaded rapeseed oleosomes improves the intestinal lymphatic delivery and systemic bioavailability of CBD compared with CBD-loaded rapeseed oil and rapeseed artificial emulsion

Oil-in-water emulsions can enhance the oral bioavailability of lipophilic drugs (McClements, 2018). However, in this work, the artificial emulsion enhanced the absorption rate and plasma C_{max} in comparison to CBD-loaded pure rapeseed oil, but did not change the AUC, which represents the overall systemic exposure to the drug and its bioavailability (Fig. 5, Table 2). The CBD-loaded artificial emulsion also increased the lymphatic delivery of CBD at plasma t_{max} and $t_{max} - 1$ h compared with rapeseed oil (Fig. 6A & 6B). Nevertheless, in comparison to rapeseed oleosomes, this improvement is not significant. The enhanced CBD absorption rate, plasma C_{max} and lymphatic delivery of CBD at plasma t_{max} and $t_{max} - 1$ h by artificial emulsion compared with rapeseed oil could be explained by the difference in their composition. The CBD-loaded rapeseed oil formulation consists only of CBD

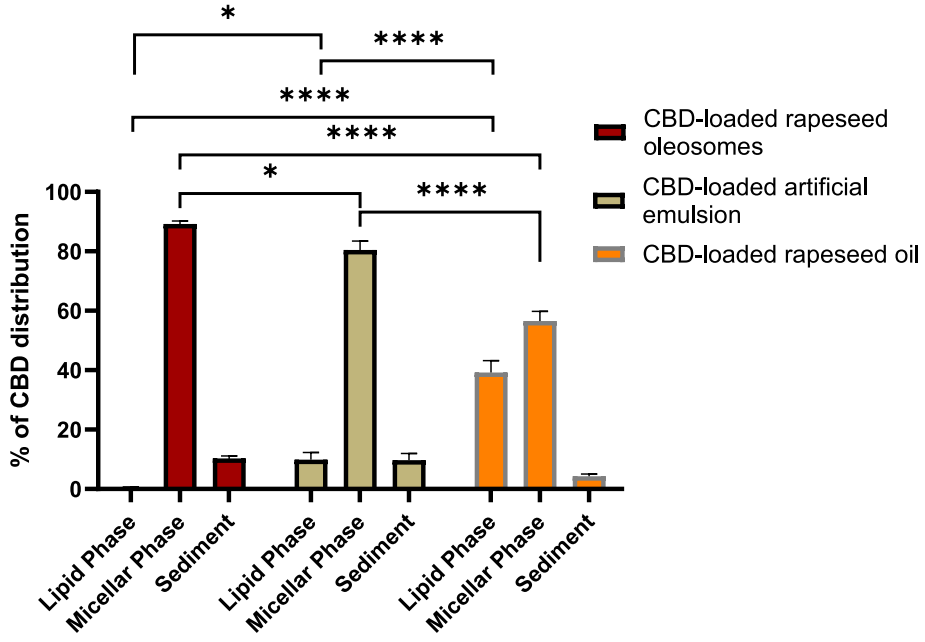

Fig. 3. CBD partitioning into lipid, micellar aqueous, and sediment phases after *in vitro* lipolysis of CBD-loaded rapeseed oil, rapeseed oleosomes and artificial emulsion (mean ± SD, n = 3). One-way ANOVA, followed by Tukey's multiple comparisons test was applied to evaluate the statistical significance of differences between means of CBD distribution into three phases. *, $p < 0.05$, ****, $p < 0.0001$.

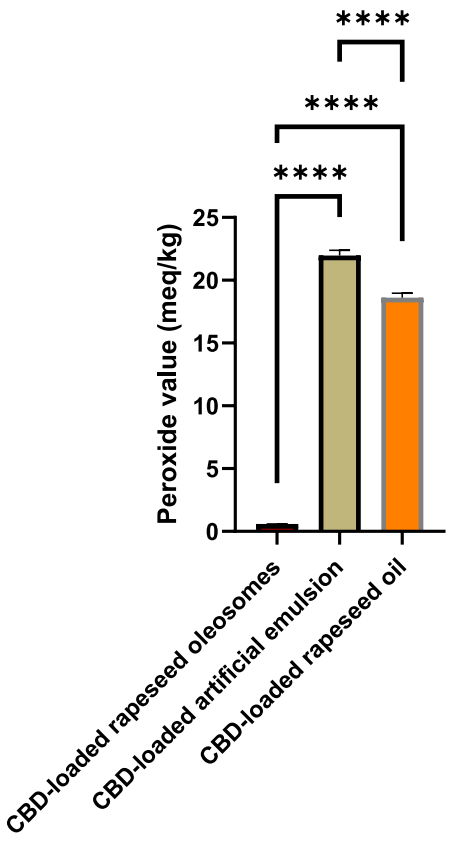

Fig. 4. Peroxide value for different rapeseed-based formulations (Mean ± SD, n = 3). ****, P < 0.0001. One-way ANOVA, followed by Tukey's multiple comparisons test was applied to evaluate the statistical significance of differences between means.

solubilised in pure oil and is not pre-emulsified. Therefore, it is likely to be digested and absorbed at a slower rate relative to the pre-emulsified formulations, which is indeed supported by the results of this study (Fig. 5). Moreover, *in vitro* lipolysis of the CBD-loaded rapeseed oil resulted in a substantially higher distribution of CBD into the undigested oil fraction compared with the other formulations (Fig. 3), further supporting the *in vivo* findings of slower absorption of CBD following administration of this formulation in comparison to pre-emulsified vehicles.

On the other hand, oral administration of CBD-loaded rapeseed oleosomes resulted in the fastest CBD absorption and highest plasma C_{max}, as well as substantially higher bioavailability in comparison to the other two lipid-based formulations. Importantly, CBD-loaded rapeseed oleosomes achieved significantly higher lymphatic delivery of CBD in comparison to CBD-loaded rapeseed oil or artificial emulsion (Fig. 6A & 6B). One possible explanation for the superior performance of the CBD-loaded oleosomes in comparison with the CBD-loaded artificial emulsion could be decreased coalescence of oleosomes during the early stages of digestion. As mentioned in Section 4.1, zeta potential of CBD-loaded artificial emulsion under acidic environment was lower than that of CBD-loaded rapeseed oleosomes (Fig. 2D). Since a lower absolute zeta potential value normally represent the lower stability of emulsion (Bhattacharjee, 2016; Kaszuba et al., 2010), this may lead to enhanced coalescence of the CBD-loaded artificial emulsion in the stomach. In addition, the presence of proteins on the surface of oleosomes could lead to lower coalescence (De Chirico et al., 2018; Deleu et al., 2010; Nikiforidis, 2019; Tzen and Huang, 1992; White et al., 2009). Thus, the eventual size of oleosomes' droplets within the intestinal tract *in vivo* might be smaller than that of the artificial emulsion and this could favour the lipolysis process by digestive lipases (Benzonana and Desnuelle, 1965). The physicochemical properties of lipid droplets appearing in the intestinal lumen can significantly affect the rate of lipid

Table 2
Plasma pharmacokinetic (PK) parameters of CBD following oral administration of CBD in different formulations (Mean ± SD).

Formulations	$t_{1/2}$ [a] (h)	t_{max} [b] (h)	C_{max} [c] (ng/mL)	$AUC_{0-\infty}$ [d] (h × ng/mL)	F [e] (%)	n
CBD-loaded rapeseed oil	3.5 ± 0.4	5	36 ± 8	273 ± 58	17.6 ± 3.8	5
CBD-loaded artificial emulsion	3.6 ± 0.8	2–3	50 ± 13	258 ± 53	16.7 ± 3.4	6
CBD-loaded rapeseed oleosomes	4.0 ± 0.7	2	79 ± 11	413 ± 25***	26.7 ± 1.6***	6

[a] half-life;
[b] time to maximum concentration in plasma;
[c] the maximum concentration in plasma;
[d] area under the curve (AUC) from 0 to infinity;
[e] bioavailability calculated based on (Zgair et al., 2015).
***, statistically significantly different from rapeseed oil and artificial emulsion groups (P < 0.001, one-way ANOVA followed by Tukey's multiple comparisons test within 3 lipid-based formulations).

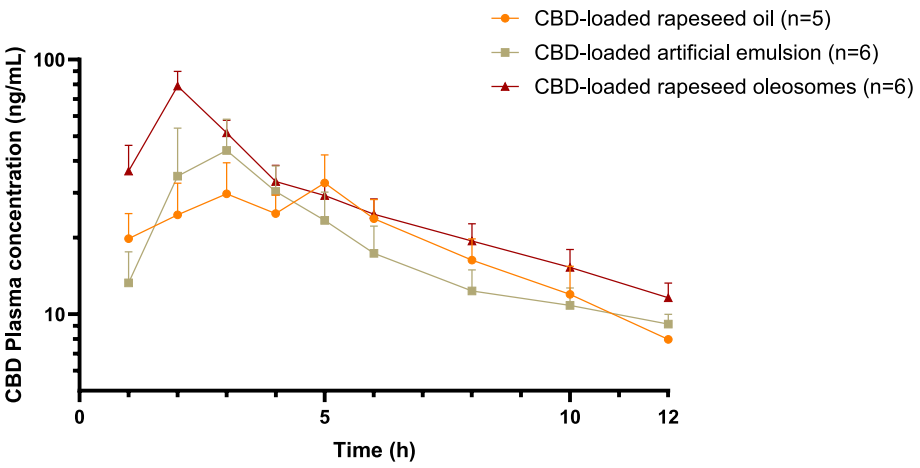

Fig. 5. Plasma CBD concentrations-time profile following oral administration of 3 mg/kg CBD in different formulations in Sprague Dawley rats (Mean ± SD, n = 5/6).

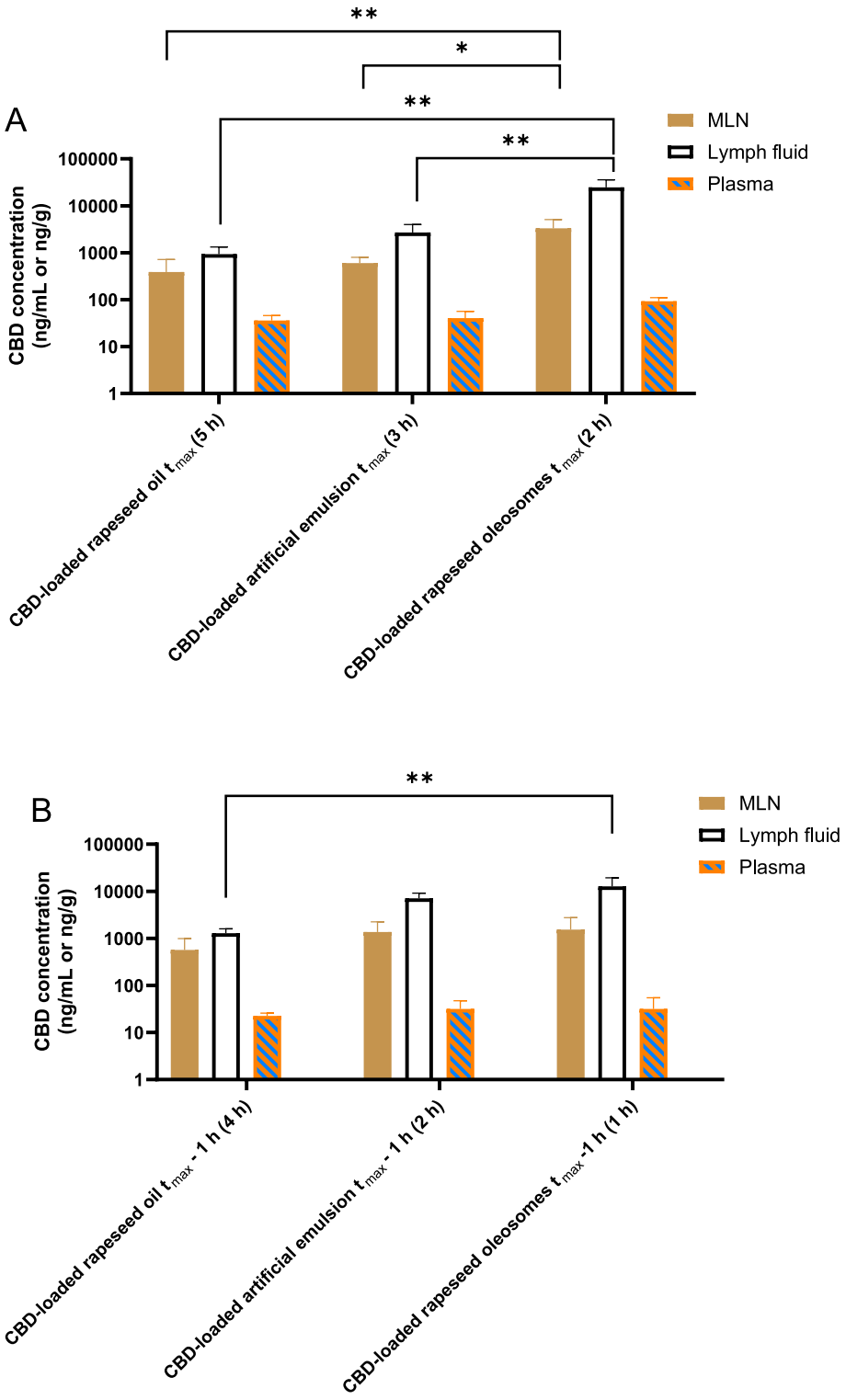

Fig. 6. Biodistribution of CBD at plasma t_{max} and t_{max} −1h following oral administration of 3 mg/kg CBD in different formulations in Sprague Dawley rats (Mean ± SD, n = 4). MLN, mesenteric lymph nodes. A, biodistribution of CBD at plasma t_{max}; B, biodistribution of CBD at plasma t_{max} −1h. One-way ANOVA, followed by Tukey's multiple comparisons test was applied to evaluate the statistical significance of differences between means. *, $p < 0.05$, **, $p < 0.01$.

digestion and affect the bioavailability of loaded drugs (McClements, 2018; Salvia-Trujillo et al., 2013; Salvia-Trujillo et al., 2017). Smaller droplet size of the emulsion droplets often leads to faster and more complete lipid digestion, efficient formation of mixed micelles, and eventually higher bioavailability of formulated drugs (Salvia-Trujillo et al., 2013; Yi et al., 2014). Therefore, although the particle size of the CBD-loaded artificial emulsion was designed to be similar to the particle size of CBD-loaded oleosomes before administration, oleosome formulation could lead to smaller size of droplets within the gastrointestinal tract, eventually resulting in higher bioavailability and intestinal lymphatic delivery of CBD. However, there is currently a lack of direct evidence regarding the changes in particle size of these two formulations during digestion to support this hypothesis.

Another potential explanation of the differences in oral

bioavailability and lymphatic delivery of CBD between CBD-loaded oleosomes and artificial emulsion is that the proteins on the surface of oleosomes could influence the intraluminal digestion or affect various stages of intra-cellular assembly and release of chylomicrons. The proteins present on the oleosome membrane are mainly oleosins (15——20 kDa), in addition to caleosins (25——35 kDa), and steroleosins (> 35 kDa) (Nikiforidis, 2019). The structures of these proteins have certain similarities with surfactants – the hydrophobic domains are anchored in the triacylglycerol core, while the hydrophilic domains are exposed to the aqueous phase (Jolivet et al., 2017; Nikiforidis, 2019). Some previous studies have demonstrated that the presence and type of proteins can affect the lipolysis of emulsions *in vitro* (Beisson et al., 2001; Ding et al., 2021; Ding et al., 2020). In addition, Couëdelo reported that the type of emulsifier could affect the expression of genes that are involved in absorption of fatty acids and chylomicron assembly and secretion in rats (Couëdelo et al., 2015). These results indicate the potential role of proteins in oleosomes on the absorption of CBD.

An *in vitro* model of lipid digestion was used to explore what might be occurring during the digestion of CBD-loaded lipid droplets in rats (Fig. 3). During lipolysis, substantially higher amount of sodium hydroxide solution was consumed during the digestion of the CBD-loaded rapeseed oleosomes in comparison with the CBD-loaded artificial emulsion and CBD-loaded rapeseed oil (0.49 ± 0.02 mL, 0.26 ± 0.04 mL and 0.12 ± 0.00 mL, mean ± SD,respectively). This leads to an estimated hydrolysis levels of these three formulations of 68.34 ± 2.79 %, 36.21 ± 5.57 % and 16.76 ± 0.56 % (mean ± SD, CBD-loaded rapeseed oleosomes, artificial emulsion and rapeseed oil, respectively). This means that the CBD-loaded oleosomes were hydrolysed most efficiently in comparison with the artificial emulsion and rapeseed oil systems. The hydrolysis level reached with rapeseed oleosomes corresponds to the release of two fatty acids out of the three esterified in rapeseed triglycerides, i.e. the complete conversion of triglycerides into free fatty acids and monoglycerides. This suggests that the lipids initially present in the oleosomes could be all transferred to the micellar phase where they can contribute to the formation of mixed micelles with bile salts and phospholipids. The process could increase the micellar solubilization of CBD and, indeed, the highest level of CBD in the micellar phase is observed with CBD-loaded oleosomes (Fig. 3). However, on this important parameter – the CBD distribution fraction, the performance of CBD-loaded rapeseed oleosomes did not differ significantly from CBD-loaded artificial emulsion. The lipolysis of the CBD-loaded oleosomes led to ~ 90 % of CBD distribution into micellar phase and very little CBD was left for the undigested oil phase (very little volume of oil left). For artificial emulsion, the CBD distribution into micellar phase and oil phase was ~ 80 % and ~ 10 %, respectively. Moreover, this *in vitro* lipolysis model only simulates the pre-enterocyte intestinal digestion processes. Since CBD is a highly lipophilic molecule, its oral bioavailability is greatly affected by the intestinal lymphatic transport, which is associated with affinity to chylomicrons in the enterocytes. It has been suggested previously that *in vitro* lipolysis models might be not predictive for oral bioavailability of drugs with substantial intestinal lymphatic transport component in their absorption following oral administration (Chu et al., 2023; Dahan and Hoffman, 2008). These considerations suggest that differences in intraluminal digestion of these two formulations could partially contribute to the differences in oral bioavailability and lymphatic delivery of CBD. It is, however, unlikely, that the observed differences in CBD distribution into micellar, oil and sediment phases following the *in vitro* lipolysis can fully explain the substantially higher lymphatic delivery and bioavailability of CBD following oral administration of CBD-loaded oleosomes *in vivo*. In addition, there is still insufficient evidence to explain how the hydrolysis rate of triglycerides could make an impact on the distribution of CBD.

Another hypothesis is that the oxidation level of lipids in the formulation could affect the oral bioavailability and lymphatic delivery of CBD. Polyunsaturated oils are prone to formation of peroxides during processing and storage and could potentially also undergo oxidation in the gastrointestinal tract following administration (Nieva-Echevarría et al., 2020). The initial oxidation of lipids may lead to reduced lipolysis (Martin-Rubio et al., 2019). This could explain at least partially the sodium hydroxide titration volume differences during lipolysis. On the other side, the oxidation level of lipids could substantially affect the digestion and absorption of lipids and the bioavailability of lipophilic drugs (Márquez-Ruiz et al., 2008; Yao et al., 2022). It was reported that the quantity and morphology of lipoprotein particles produced by intestinal epithelial cells depends on the oxidation state of the administered lipids, with oxidized lipids leading to smaller and more irregularly shaped lipoproteins (Yao et al., 2022). In addition, exposure to oxidized lipids might adversely affect the intestinal epithelial cells and subsequently influence the absorption of lipophilic substances (Yao et al., 2022). Furthermore, the oxidation of oil could also affect the minor constituents present in the natural oils, such as sterols and phenolic compounds (Márquez-Ruiz et al., 2008). The presence of these minor constituents could be one of the factors responsible for higher oral bioavailability and lymphatic delivery of CBD observed with natural vegetables oils in comparison to pre-digested or other artificial formulations (Feng et al., 2021a). To test this hypothesis, the lipids in the three formulations were extracted and the peroxide value was assessed (Fig. 4). The results show that the oil from CBD-loaded rapeseed oleosomes has dramatically lower peroxide value (~0.6 meq/kg) than that from the CBD-loaded artificial emulsion (~22.0 meq/kg). The peroxide value of CBD-loaded rapeseed oil is close to the oil from CBD-loaded artificial emulsion (~18.6 meq/kg). Given the dramatic difference in the peroxide values of the oil content of the CBD-loaded rapeseed oil, artificial emulsion and oleosomes, degree of oxidation of lipids could be an important factor for the differences observed in the lymphatic delivery and bioavailability of CBD. Further research will be needed to confirm or reject this hypothesis, as well as for mechanistic investigation of how the degree of oxidation affects the digestion of lipids, the bioavailability and lymphatic delivery of lipophilic drugs.

5. Conclusion

CBD has low oral bioavailability due to its high lipophilicity and extensive first-pass metabolic loss. Lipid-based formulations containing long-chain triglyceride or long-chain fatty acids are an effective strategy to facilitate the intestinal lymphatic delivery and enhance oral bioavailability of this lipophilic drug. Our previous work suggests that natural vegetable oils lead to higher lymphatic delivery and systemic bioavailability of CBD in comparison to pre-digested or self-emulsifying artificial formulations. In this work, we have developed a novel formulation of CBD based on natural rapeseed oleosomes and compared its CBD delivery performance to pure rapeseed oil and artificial oleosome-like rapeseed-based emulsion. The CBD-loaded rapeseed oleosomes led to substantially higher lymphatic delivery and systemic bioavailability of CBD in comparison with artificial rapeseed oil emulsion (stabilised with rapeseed phosphatidylcholine) or non-emulsified rapeseed oil. The lower oxidation level of the oil in oleosomes could be an important factor for the observed superior performance of natural oleosomes. Other potential explanations could include differences in intraluminal stability post-administration, influence of the surface proteins during the intraluminal digestion or the assembly and release of chylomicrons, or the lipolysis efficiency of the lipid vehicles. Further investigation is necessary to confirm or reject these hypothesises, as well as for mechanistic investigation on how the degree of oxidation, the presence of protein and other factors affect the digestion of lipids, the bioavailability and lymphatic delivery of CBD and other lipophilic drugs.

CRediT authorship contribution statement

Liuhang Ji: Writing – original draft, Methodology, Investigation. **Wanshan Feng:** Writing – review & editing, Methodology,

Investigation. **Haojie Chen:** Writing – review & editing, Methodology, Investigation. **YenJu Chu:** Writing – review & editing, Methodology, Investigation. **Abigail Wong:** Writing – review & editing, Methodology, Investigation. **Yufei Zhu:** Writing – review & editing, Methodology, Investigation. **Graziamarina Sinatra:** Writing – review & editing, Methodology, Investigation. **Filippo Bramante:** Writing – review & editing, Methodology, Investigation. **Frédéric Carrière:** Writing – review & editing, Validation. **Michael J. Stocks:** Writing – review & editing, Supervision, Methodology, Investigation. **Vincenzo di Bari:** Writing – review & editing. **David A. Gray:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Pavel Gershkovich:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2024.124947.

Data availability

Data will be made available on request.

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