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RESEARCH ARTICLE

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Examining the hepatotoxic potential of cannabidiol, cannabidiol-containing hemp extract, and cannabinol at consumer-relevant exposure concentrations in primary human hepatocytes

Anneliese Striz | Yang Zhao | Estatira Sepehr | Cory Vaught | Kirsten Eckstrum | Kyra Headrick | Jeffrey Yourick | Robert Sprando

Division of Toxicology, Office of Applied Research and Safety Assessment, Center for Food Safety and Applied Nutrition, US Food and Drug Administration, Laurel, Maryland, USA

Correspondence
Anneliese Striz, MOD1 Laboratories, US FDA, 8301 Muirkirk Road, Laurel, MD 20708, USA.
Email: anneliese.striz@fda.hhs.gov

Abstract

Hemp extracts and consumer products containing cannabidiol (CBD) and/or other phytocannabinoids derived from hemp have entered the marketplace in recent years. CBD is an approved drug in the United States for the treatment of certain seizure disorders. While effects of CBD in the liver have been well characterized, data on the effects of other cannabinoids and hemp extracts in the liver and methods for studying these effects in vitro are limited. This study examined the hepatotoxic potential of CBD, CBD concentration-matched hemp extract, and cannabinol (CBN), at consumer-relevant concentrations determined by in silico modeling, in vitro using primary human hepatocytes. Primary human hepatocytes exposed to between 10-nM and 25-μM CBD, CBN, or hemp extract for 24 and 48 h were evaluated by measuring lactate dehydrogenase release, apoptosis, albumin secretion, urea secretion, and mitochondrial membrane potential. Cell viability was not significantly affected by CBD, CBN, or the hemp extract at any of the concentrations tested. Exposure to hemp extract induced a modest but statistically significant decrease in albumin secretion, urea secretion, and mitochondrial membrane potential at the highest concentration tested whereas CBD only induced a modest but statistically significant decrease in albumin secretion compared with vehicle control. Although this study addresses data gaps in the understanding of cannabinoid hepatoxicity in vitro, additional studies will be needed to determine how these results correlate with relevant consumer exposure and the biological effects of cannabinoids in human liver.

KEYWORDS
cannabidiol, cannabinol, hemp extract, hepatotoxicity, primary human hepatocytes

1 | INTRODUCTION

Cannabidiol (CBD) is a phytocannabinoid derived from the cannabis plant. Depending on the concentration of delta-9-tetrahydrocannabinol (THC), the psychoactive cannabinoid, cannabis is classified as either marijuana (THC > 0.3%) or hemp (<0.3%) by dry weight. CBD is generally the most abundant cannabinoid in hemp; however, there are over 100 naturally occurring cannabinoids in the hemp plant in various quantities (Bonini et al., 2018; Elsohly & Slade, 2005). Although THC has been the focus of cannabinoid research for many years, CBD, CBD-containing hemp extracts, and other cannabinoids such as cannabinol (CBN) have come to the forefront due to the increased presence of hemp extracts and hemp-derived cannabinoid-containing products in the marketplace after the passage of the Agricultural Improvement Act of 2018.

The cytotoxic effects of CBD are well known due to its use in the clinical setting. CBD was shown to decrease seizures in individuals suffering from Dravet syndrome and Lennox–Gastaut syndrome and was approved as the drug Epidiolex® by the Food and Drug Administration in 2018. In vivo studies and clinical trials evaluating CBD efficacy and safety showed that CBD alone, and in conjunction with other drugs, sometimes induced liver injury as indicated by increased liver enzymes (reviewed in Huestis et al., 2019). According to the FDA Epidiolex (CBD) Oral Solution Prescribing Information "ALT (alanine transaminase) elevations greater than 3 times the ULN (Upper Limit Normal) were reported in 17% of patients taking 20 mg/kg/day compared with 1% in patients taking EPIDIOLEX 10 mg/kg/day." Studies from healthy individuals in a Phase I clinical trial examining the effects of repeated dose administration of CBD on cytochrome P450 1A2 yielded similar results, indicating that hepatotoxicity from the highest clinical CBD dose (1500 mg/day, ~20 mg/kg/day for a 70-kg person) is independent of epilepsy disorder diagnosis (Watkins et al., 2021). The CBD doses used to treat patients with mild hepatic impairment are between 5 and 20 mg/kg/day, with maintenance doses of 10 mg/kg/day to avoid potential adverse effects of the 20 mg/kg/day dose (FDA Epidiolex [CBD] oral solution Reference ID: 4282447, 2018). Lower doses as low as 1 mg/kg/day are recommended for individuals who experience severe hepatic impairment. In a randomized safety trial for Dravet syndrome, mean plasma C_{max} for patients exposed to 5, 10, and 20 mg/kg/day of CBD for 22 days were found to be 130, 288, and 380 ng/ml and AUCs found to be 241, 722, and 963 h x ng/ml, respectively on Day 22 at target CBD dose (Devinsky et al., 2018). The highest observed clinical plasma concentrations were found to be between 1 and 3 μM (Chan & Duncan, 2021; Contin et al., 2021; Ohlsson et al., 1986). In comparison, a survey of cannabinoids in hemp-derived products available in the consumer marketplace performed by Office of Regulatory Science (FDA) found that cannabinoid concentrations ranged between <LOD (Limit of Detection) and 143 mg/serving (Dubrow et al., 2021).

Data on the potential hepatotoxicity of CBD in vitro which could be used to inform potential toxicity of less well characterized cannabinoids and studies specifically examining other cannabinoids are limited. Studies in HepG2 cells found that CBD and cannabidivarin (CBDV) induced cytotoxicity at and over 54 μM after 24 h (Russo et al., 2019). Both CBD and CBDV at subtoxic concentrations of 2, 6, and 18 μM induced a dose-dependent and significant, although modest (~2%–8%) increase in DNA damage after 24 h as measured by single cell gel electrophoresis (Russo et al., 2019). Further studies by Russo et al. found that the LOAEC in HepG2 cells after 72 h of CBD and CBDV exposure ranged from 10 to 40 μM or 10–22 μM, respectively, depending on the endpoint assay used (Russo et al., 2021). Mitochondrial metabolism, plasma membrane integrity of HepG2, and DNA synthesis in cells were significantly reduced upon exposure to CBD at above 10, 20, and 40 μM, respectively, with similar results observed upon exposure to CBDV (Russo et al., 2021). Studies in HepaRG spheroids exposed to CBD for 24 h identified an EC50 of 86.27 μM by measuring cellular ATP and determined very few altered gene targets at or below 1-μM CBD exposure after 24 or 72 h (Li et al., 2023). To our knowledge, there are no in vitro studies examining ethanolic hemp extract in hepatocytes. An in vivo study which utilized a mouse model to evaluate the toxicity of a CBD-rich hemp extract tested mice gavaged with a 57.9% CBD-hemp extract at either acutely toxic doses for 24 h or sub-acute doses for 10 days, based on allometrically scaled mouse equivalent doses of the maximum recommended human dose of Epidiolex (20 mg/kg) (Ewing et al., 2019). The two highest acute toxicity doses (738 and 2460 mg/kg) led to a moderate and statistically significant increase in aspartate aminotransferase and alanine transaminase levels after 24 h. The lowest subacute dose of 61.5 mg/kg (MED of 5 mg/kg CBD) had no effect on the mice over 10 days; however, foci of cytoplasmic swelling were observed in the 3× dose (184.6 mg/kg), and 33% of mice in the 10× dose (615 mg/kg) developed a moribund condition after the second dose (Ewing et al., 2019). The difference between CBD-rich hemp extract and CBD alone was not directly examined. In vitro studies evaluating CBN in hepatocytes were primarily limited to identification of metabolites and the effects on CBN on CYP450 metabolism. One of these studies identified CBN as a potent Cyp1A2 and Cyp1B1 inhibitor (Yamaori et al., 2010). A mechanistic study examining the effects of cannabinoids on ERK signaling in a normal diploid WB rat liver epithelial oval cell line found that a subtoxic concentration of CBN (15 μM) activated the ERK1 and ERK2 pathways indicating that CBN exposure may affect cell proliferation pathways (Upham et al., 2003); however, this result may be specific to the cell line tested.

To address gaps in the literature examining other cannabinoids and ethanolic hemp extract in vitro, this study evaluated the hepatotoxic potential of CBD, CBN, and an ethanolic hemp extract obtained from the University of Mississippi, using in vitro primary human hepatocytes. Based on CBD and CBN concentrations reported in hemp-derived products, in silico predicted C_{max} levels, clinical CBD exposure and toxicity data, and the limits of solubility in hepatocyte culture media, we chose a final exposure concentration range for testing between 10 nM and 25 μM (Liu & Sprando, 2023). Primary human hepatocytes were exposed to CBD, CBD concentration-matched hemp extract ([CBD]-matched hemp extract), or CBN for 24 and 48 h and evaluated cytotoxic and adverse functional effects. CBD and (CBD) matched hemp extract exposure in primary hepatocytes at the

concentrations tested were not found to induce cell death; however, some adverse functional effects were observed at the highest concentration tested. More studies are needed to determine the full impact of hemp extract, CBD, and CBN-containing consumer products on the human liver.

2 | METHODS

2.1 | Reagents

CBD (purity≥98%) and CBN (purity≥98%) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Ethanolic hemp extract was provided by the National Center for Natural Products Research at the University of Mississippi. A model was developed to quantitate the analytes in the hemp extract and is described in detail in Zhao et al. (2023). Certified standards of isotopically labeled CBD-D3 and CBN-D_3 at 0.1 mg/ml in methanol, usnic acid (positive assay control), and dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO, USA). Milli-Q water (18.2 MΩ-cm) was used throughout (Millipore Sigma, Burlington, MA, USA).

2.2 | Primary human hepatocyte cell culture

Gibco plateable primary human hepatocytes (metabolism qualified, transporter qualified, or induction qualified) and media components were obtained from Thermo Fisher Scientific (Waltham, MA, USA). A different primary hepatocyte lot was used for each individual experiment within each cannabinoid or the hemp extract to account for donor/lot variability (1 lot/experiment × 4 experiments per cannabinoid tested = 4 lots/cannabinoid tested). Donors ranged between 20 and 60 years old. Cells were thawed following manufacturer's instructions using Gibco CHRM (CM7000) and plated using William's E Medium without phenol red (A1217601) supplemented with Gibco Primary Hepatocyte Thawing and Plating Supplements (CM3000). Cells were plated in two tissue-culture treated 24-well plates and incubated at 37°C and 5% CO_2. After 6 h, the cells were coated with cold Matrigel (Corning Life Sciences, Tewksbury, MA, USA) diluted in 4°C hepatocyte maintenance media on ice (William's E media supplemented with Gibco Primary Hepatocyte Maintenance Supplements CM4000). The next day, media was exchanged with fresh 37°C hepatocyte maintenance media and incubated for another 24 hours at 37°C and 5% CO_2.

2.3 | CBD and hemp extract preparation in hepatocyte media

2.3.1 | CBD and CBN

To prepare the CBD and CBN dosing solutions for cell treatment, pure CBD or CBN were dissolved in DMSO to a concentration of 51.30 mg/ml (163.13 mM). Intermediate stocks in DMSO were diluted from the main stock solution to 200x (5 mM, 2 mM, 200 μM, 20 μM and 2 μM) to maintain a final working concentration of 0.5% DMSO and then aliquoted and stored at −80°C. The intermediate stocks were thawed and diluted in 37°C hepatocyte maintenance media in amber glass vials to the final working concentrations (10 nM, 100 nM, 1 μM, 10 μM, and 25 μM). Cells were treated immediately after dosing solution preparation.

2.3.2 | (CBD)matched hemp extract

Ethanol was removed from a 2-ml aliquot of the hemp extract by using a Labconco CentriVap vacuum concentrator (Kansas City, MO, USA) with no heat for 70 min. The pellet was then reconstituted with 800-μl DMSO. During the process, samples were kept on ice every 30 min to minimize degradation of the cannabinoids during centrifugation. Intermediate stocks of the hemp extract in DMSO were prepared to match CBD only stock concentrations of 200× (5 mM, 2 mM, 200 μM, 20 μM, and 2 μM CBD concentration in hemp extract) to maintain a final working concentration of 0.5% DMSO, aliquoted, and stored at −80°C. The hemp extract intermediate stocks were thawed and diluted in 37°C hepatocyte maintenance media in amber glass vials to the final working CBD concentrations (10 nM, 100 nM, 1 μM, 10 μM, and 25 μM). Cells were treated immediately after dosing solution preparation.

2.4 | Cannabinoid exposure in primary human hepatocytes

Two 24-well plates were identically treated with the 0.5% DMSO vehicle control, either CBD, (CBD)matched hemp extract, or CBN (10 nM, 100 nM, 1 μM, 10 μM, and 25 μM CBD), and 50 μM usnic acid positive assay control (PAC) in triplicate per condition and incubated for 24 and 48 h. The final three wells were treated with 0.5% DMSO vehicle control to have two lysis controls for the lactate dehydrogenase (LDH) assay and one negative staining control. Wells were randomized for each concentration and experiment to minimize plate edge effects.

2.5 | Cannabinoid stability in media

Samples of each CBD, (CBD)matched hemp extract, or CBN solution in hepatocyte media for at least two experiments were extracted and analyzed immediately after hepatocyte treatment (roughly ∼30–40 min after solution preparation) to determine the stability of CBD, CBN, and the other cannabinoids within the hemp extract in warm hepatocyte media and to verify the concentration the cells received. The methods for sample preparations and mass spectrometry analysis can be found in detail in Zhao et al. (2023).

2.6 | Lactate dehydrogenase cytotoxicity assay

Cytotoxicity (lysis) was measured via LDH release using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA) following assay instructions. Media from cells exposed for 24 or 48 h was collected and diluted 1:10 in Milli-Q H_20. Two 0.5% DMSO vehicle control exposed wells were lysed using the provided lysis buffer as directed. Samples were plated in duplicate. Absorbance was measured at 490 nm using a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Absorbance values were divided by the average of the two lysis controls to acquire a percent lysis. Percent lysis was then inverted from cell death and plotted as percent viability standardized to the average of the 0.5% DMSO vehicle control at both 24 and 48 h.

2.7 | Human albumin ELISA

Albumin secretion was measured using the SimpleStep Albumin ELISA (Abcam, Cambridge, MA), per manufacturer's protocol. Collected media from CBD, (CBD)matched hemp extract, or CBN exposed cells were diluted 1:1000 for the 24-h exposed cells and between 1:1000 and 1:4000 for the 48-h exposed cells depending on the 24-h 0.5% DMSO control levels from each hepatocyte lot in order to remain within the provided standard curve. Samples were plated in triplicate. Absorbance was measured at 450 nm using a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). The results of each triplicate were averaged and used to calculate average albumin secretion in μg/day per sample. The data was standardized to the average of the 0.5% DMSO vehicle control at both 24 and 48 h.

2.8 | Urea assay

Urea secretion was measured using the BioAssay Systems, QuantiChrom Urea Assay Kit (BioAssay Systems, Hayward, CA, USA) following the provided protocol. Media collected from cells exposed for 24 h was collected and run undiluted. Media collected from cells exposed for 48 hours was collected and diluted 1:3 in Milli-Q H_20. The samples were plated in duplicate. Absorbance was measured at 430 nm using a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). The resulting data were averaged and used to calculated urea secretion (mg/day). The data were standardized to the average of the 0.5% DMSO vehicle control at both 24 and 48 hours.

2.9 | Live staining procedure for mitochondrial membrane potential, apoptosis, and nuclei

Both the 24 and 48-h exposed cell plates were stained with tetramethylrhodamine methyl ester perchlorate (TMRM) for detecting mitochondrial membrane potential, caspase 3/7 for measuring apoptosis, and NucBlue for visualizing cell nuclei (Thermo Fisher Scientific, Waltham, MA, USA). Cells were washed with warm hepatocyte maintenance media to remove debris and waste products and then incubated at 37°C with 1-μM TMRM and 2-μM Caspase3/7 in media for 30 min. After staining, the cells were washed three times with hepatocyte maintenance media, and then nuclei were stained with NucBlue in hepatocyte maintenance media (2 drops/ml) for 20 min at 37°C. The cells were then washed three times with warm hepatocyte maintenance media and immediately imaged.

2.10 | Fluorescent imaging

Imaging and counting were performed on a BZ-X700 All-in-one Fluorescence Microscope (Keyence, Itasca, IL, USA) as described in detail in Eckstrum and Sprando (2021). Briefly, for wells, nine image sections were acquired with 6-μm z-stacks through the depth of the hepatocytes. For each well, the best image in the z-stack for each image was chosen automatically by the BZ-X Analyzer software and used for quantification.

2.11 | NucBlue (nuclei), TMRM (mitochondrial membrane potential), and Caspase 3/7 (apoptosis) quantification

The BZ-X Analyzer software was used to optimize and quantify NucBlue, TMRM, and Caspase3/7 as described in Eckstrum and Sprando (2021). Briefly, for nuclei counts, the total average nuclei/image and total count/nine images were found using the optimized nuclei count algorithm. Using Microsoft Excel©, the total nuclei count was then divided by the area quantified to determine cell density (cells/mm^2). This density was multiplied by the surface area of the well (200 mm^2) to obtain the total nuclei number for the well. The data were standardized to the average of the 0.5% DMSO vehicle control for each experiment.

To quantify TMRM and Caspase3/7 staining, an algorithm was created in the BZ-X analyzer. A control well without TMRM or Caspase3/7 was used to quantify background fluorescence and subtracted from the counts/totals of each well. The average area of TMRM or Caspase3/7 fluorescence was compared with the total area to yield percent coverage. TMRM staining was standardized to the average of the 0.5% DMSO vehicle control for each experiment. Because there were often cases of no Caspase3/7 detection, data were inverted to show increased apoptosis as a decrease in cell viability and standardized to average 0.5% DMSO vehicle control for each experiment.

2.12 | Statistical analysis

SigmaPlot® v. 14.5 (Systat Software, Inc., Chicago, IL, USA) was used to perform all statistical analyses. In general, a one-way ANOVA was run on the data obtained from each endpoint assay. CBD, (CBD)

matched hemp extract, and CBN data were all analyzed separately as experiments for each cannabinoid or hemp extract were performed on different days. The data were standardized to vehicle control from only the experiments performed within each cannabinoid or hemp extract experiment (i.e., CBD data were standardized to vehicle control for that CBD experiment). If the results of the ANOVA indicated significance among groups ($p < 0.05$), a post hoc test (either Holm–Sidak or Dunn's method) was run as recommended by SigmaPlot® based on normality or variance test results. The post hoc test used for each set of CBD, (CBD)matched hemp extract, or CBN data at each timepoint is indicated in the figure legends. Only significance relative to vehicle control is indicated in the graphs. Statistical analysis of the usnic acid PAC compared with 0.5% DMSO was performed using a t-test recommended by SigmaPlot® (Welch's, Student's, or Mann–Whitney) depending on normality and variance test results.

3 | RESULTS

3.1 | Determination of CBD, CBN, and hemp extract exposure concentrations

An exposure relevant concentration curve for treating the hepatocytes was chosen based on in silico predicted liver C_{max} levels from exposure to CBD and CBN containing consumer products. A maximum concentration was determined by using the in silico prediction for a consumer relevant dose to extrapolate from known plasma C_{max} values from the literature evaluating clinical CBD for treatment of seizure disorders to predicted liver C_{max} values. In their silico predictions, Liu and Sprando (2023) found that 30-mg oral CBD exposure in a 75-kg Caucasian male would yield a C_{max} of approximately 86 nM in the liver. Interestingly, CBD liver C_{max} was found to be 14.3× higher than the predicted plasma C_{max} of 6 nM (Liu & Sprando, 2023). Similarly, CBN liver C_{max} was predicted to be 129.2 nM which was 14.2× higher than the predicted plasma C_{max} of 9.1 nM (Liu & Sprando, 2023). Assuming that the relationship between CBD plasma C_{max} and liver C_{max} is linear, the highest observed clinical plasma CBD concentrations of generally 1–3 μM would predict a liver C_{max} of approximately 14–43 μM. Given the limit of solubility for CBD in hepatocyte culture media, a final concentration range for testing of 10 nM (0.01 μM), 100 nM (0.1 μM), 1 μM, 10 μM, and 25 μM for pure CBD, pure CBN, and (CBD)matched hemp extract was chosen (Figure 1). Concentration matching the hemp extract to pure CBD allowed comparison to determine the possible effects of the lower abundance cannabinoids and other constituents in the hemp extract. The concentration of CBD in the hemp extract was significantly higher than the other cannabinoids (Table 1). The next 4 highest abundance cannabinoids in the hemp extract ranked from highest to lowest concentration were cannabichromene (CBC), cannabigerol (CBG), cannabidivarin (CBDV), and CBN (Table 1).

Based on the CBD concentration of the CBD concentration curve, the relative expected concentrations of the next four most abundant cannabinoids in (CBD)matched hemp extract were calculated (Table 2). Stability studies found that not all the cannabinoids are stable in 37°C hepatocyte maintenance media. Immediately after treating, CBD, CBN, or all five cannabinoids in the hemp extract were extracted from the media and quantified using mass spectrometry. The recovered concentration of each cannabinoid in the solution as an average of 2–4 experiments is shown in Table 2. For pure CBD in media, approximately 74.14 and 72.68% of CBD for the 10 and 25 μM concentrations were recovered, respectively. Pure CBN also showed a decrease in recovery of ~25% at 1, 10, and 25 μM. CBD was more stable as part of the hemp extract with an average of 99.5% recovery at the 25-μM concentration. CBC and CBG were also found to be relatively stable in warm media. However,

TABLE 1 Cannabinoids concentrations in University of Mississippi hemp extract after ethanol evaporation and resuspension in dimethylsulfoxide .

Analyte	Measured concentration (μg/ml, mean ± *SD*, [RSD%])	Concentration (mM)
CBD	112,610.56 ± 587.02 (0.52%)	358.096
CBC	4294.45 ± 53.89 (1.25%)	13.657
CBG	2972.59 ± 26.57 (0.89%)	9.393
CBDV	1233.54 ± 17.39 (1.41%)	4.307
CBN	627.53 ± 24.44 (3.89%)	2.022

Abbreviations: CBC, cannabichromene; CBD, cannabidiol; CBDV, cannabidivarin; CBG, cannabigerol; CBN, cannabinol.

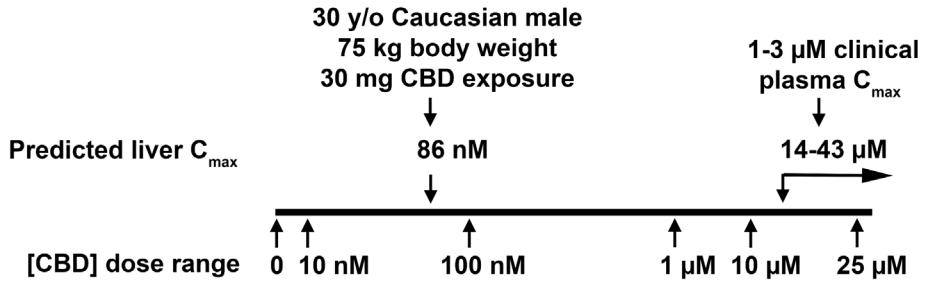

FIGURE 1 Determination of CBD and (CBD)matched hemp extract concentration curve. The in silico predicted liver C_{max} for a single individual and the predicted liver C_{max} for high dose clinical plasma C_{max} is shown above the line. The chosen CBD exposure concentrations used to encompass clinical and subclinical concentrations are shown below the line. These concentrations were also used for (CBD)matched hemp extract and CBN exposure. CBC, cannabichromene; CBD, cannabidiol; CBDV, cannabidivarin; CBG, cannabigerol; CBN, cannabinol.

TABLE 2 Cannabinoid recovery from dosing solutions immediately after dosing from either pure CBD, pure CBN, or (CBD)matched hemp extract in dosing media.

Recovery: pure CBD in dosing media			Recovery: pure CBN in dosing media		
CBD (μM)			CBN (μM)		
(conc)	Avg (R)	Avg %R	(conc)	Avg (R)	Avg %R
0.01	ND	ND	0.01	0.009	93.20
0.1	ND	ND	0.1	0.084	83.73
1	ND	ND	1	0.76	75.35
10	7.43	74.14	10	7.58	75.80
25	18.17	72.68	25	18.68	74.75

Recovery: (CBD)matched hemp extract cannabinoids in dosing media														
CBD (μM)			CBN (nM)			CBC (nM)			CBG (nM)			CBDV (nM)		
(conc)	Avg (R)	Avg %R	(conc)	Avg (R)	Avg %R	(conc)	Avg (R)	Avg %R	(conc)	Avg (R)	Avg %R	(conc)	Avg (R)	Avg %R
0.01	0.012	115	0.056	ND	ND	0.38	ND	ND	0.264	ND	ND	0.12	ND	ND
0.1	0.096	95.83	0.56	ND	ND	3.8	5	118.42	2.64	ND	ND	1.2	ND	ND
1	0.825	82.47	5.6	6	106.25	38	40	104.39	26.4	26	96.72	12	8	66.81
10	8.553	85.46	56	38	67.26	380	332	87.32	264	235	88.93	120	72	60.28
25	24.88	99.52	140	102	72.74	950	990	104.16	660	711	107.73	300	200	66.78

Note: (conc) = expected concentration; Avg R = average recovered concentrations from 2 to 4 experiments; Avg %R = average percentage recovery from 2 to 4 experiments. Pure CBD, pure CBN, and hemp extract CBD concentrations shown in μM, all other hemp extract cannabinoids show in (nM).
Abbreviations: CBC, cannabichromene; CBD, cannabidiol; CBDV, cannabidivarin; CBG, cannabigerol; CBN, cannabinol; ND, not detected.

CBN and CBDV within the hemp extract had an approximately 30%–40% decrease in recovery at the two highest concentrations for CBN and three highest concentrations for CBDV suggestive of decreased stability in pre-warmed hepatocyte media.

3.2 | Neither CBD, (CBD)matched hemp extract, nor CBN induced cell lysis or apoptosis

Hepatocyte media collected from cells treated with either CBD, (CBD) matched hemp extract, or CBN was tested for LDH after 24 h (Figure 2A) and 48 h (Figure 2B) to measure cell lysis. The results were calculated as a percent lysis, inverted, and plotted as viability as a percentage of 0.5% vehicle control. No decrease in cell viability was observed with exposure to either of the individual cannabinoids or the hemp extract at any of the concentrations tested after 24 or 48 h (Figure 2A,B). A very slight but statistically significant increase in viability was observed after exposure to (CBD)matched hemp extract for 24 h at the 10 μM ($p = 0.001$) and 25 μM ($p < 0.001$) concentration compared with 0.5% DMSO vehicle control (Figure 2A); however, it is likely this result is not biologically relevant as the difference was very small (1%–2%), the same result is not observed after 48 h, and there was no significant increase in nuclei count observed after 24 h (Figure 4C). The usnic acid assay PAC showed a significant time-dependent decrease as expected.

To determine if CBD, (CBD)matched hemp extract, or CBN induced programmed cell death, exposed hepatocytes were stained with Caspase3/7 as a marker for apoptosis. As described in the methods section, the results were calculated as the percent area of fluorescence and then inverted to show any increase in Caspase3/7 area as a decrease in cell viability. No change in viability was observed at any concentration for CBD, (CBD)matched hemp extract, or CBN after 24 or 48 hours of exposure (Figure 2C,D). These results indicate that at the chosen concentrations and within the time frame tested, neither CBD, (CBD)matched hemp extract, nor CBN induced cell lysis or apoptosis.

3.3 | Albumin and urea secretion were modestly but significantly affected by CBD and (CBD)matched hemp extract, but not CBN, at the highest concentration tested

Albumin and urea secretion were measured to determine if hepatocyte function was affected by CBD, (CBD)matched hemp extract, or CBN (Figure 3). Albumin secretion modestly but significantly decreased to 83.48% of control after 24 h of exposure to 25-μM CBD (Figure 3A) but appeared to recover after 48 h (Figure 3B). Exposure to 25-μM (CBD)matched hemp extract for 24 h significantly decreased albumin secretion more than CBD alone to 73.44% of control (Figure 3A). This difference may be due to the difference in stability between CBD alone and CBD in the (CBD)matched hemp extract or due to the presence of other cannabinoids and hemp constituents. When compared directly, the decrease induced by (CBD)matched hemp extract was significant compared with CBD alone ($p = 0.022$, significance not shown on graph). There was no significant decrease in albumin secretion observed from exposure to CBN (Figure 3A,B).

The same trend was observed in the results from examining urea secretion. Although CBD and CBN alone had no significant effect on urea secretion at any concentration or time tested, 25-μM (CBD)

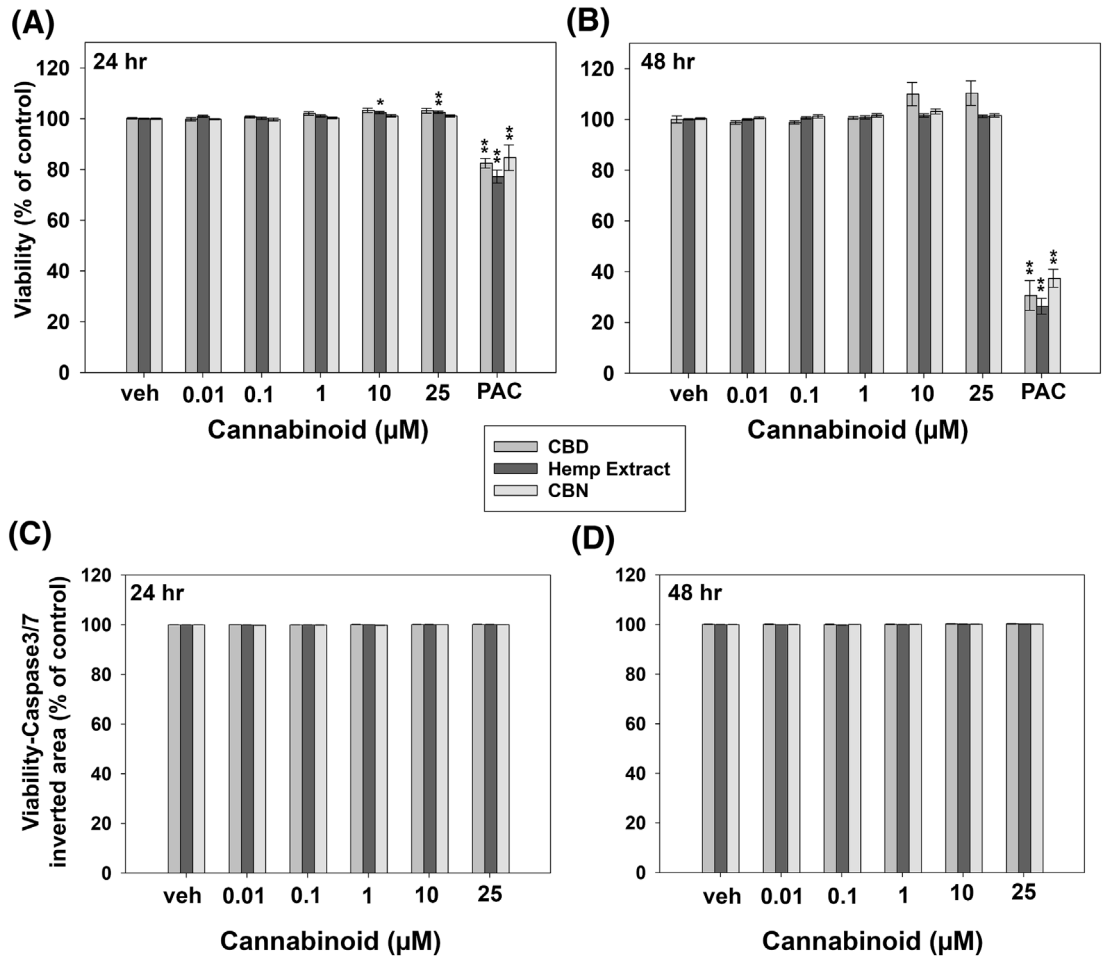

FIGURE 2 CBD, (CBD)matched hemp extract, and CBN did not induce cytotoxicity or apoptosis. (A, B) Primary hepatocytes were exposed to 0.01-, 0.1-, 1-, 10-, and 25-μM CBD, (CBD) matched hemp extract, or CBN for 24 h (A) or 48 h (B). Media was removed, and LDH was measured to determine cell lysis. The resulting data were divided by the average of the two lysis controls to obtain percent lysis. Percentage of lysis was inverted to show decreases in cell viability and is plotted as a percentage of control. A total 50-μM usnic acid was used as a positive assay control. Results represent the mean ± SEM for four independent experiments. $^{*}p = 0.001$ $^{**}p < 0.001$, statistical analyses: CBD, Dunn's method; (CBD)matched hemp extract, 24-h Holm–Sidak; CBN, Holm–Sidak. (C, D) Cells were stained with caspase3/7 to measure apoptosis after 24 h (C) or 48 h (D). The resulting staining area was divided by total area to obtain percentage coverage. This was inverted and plotted to show apoptosis as a decrease in cell viability. Results represent the mean ± SEM for four independent experiments. Statistical analyses: CBD, 24-h Dunn's method; (CBD)matched hemp extract, 48-h Holm–Sidak; CBN, 24-h Dunn's method. CBC, cannabichromene; CBD, cannabidiol; CBDV, cannabidivarin; CBG, cannabigerol; CBN, cannabinol; LDH, lactate dehydrogenase.

matched hemp extract very slightly reduced urea secretion after 24 h to 89.94% of control (Figure 3C); however, a similar decrease was not significant after 48 h of exposure due to variability among experiments (Figure 3D). These results suggest a modest but statistically significant decrease in hepatocyte function, which was slightly more pronounced upon exposure to the (CBD)matched hemp extract.

3.4 | Mitochondrial membrane potential varied with CBD and CBN exposure, but decreased with exposure to 25-μM (CBD)matched hemp extract

Mitochondrial membrane potential was measured to determine how CBD, (CBD)matched hemp extract, or CBN affect mitochondrial health. We found that mitochondrial membrane potential modestly but significantly decreased to 74.15% of the 0.5% DMSO vehicle control ($p = 0.007$) with exposure to (CBD)matched hemp extract at 25 μM after 24 h (Figure 4A). After 48 h of exposure the decrease was similar but no longer significant (Figure 4B). Interestingly, exposure to CBD and CBN appeared to show a trend for a dose-dependent increase after 48 h, particularly with exposure to CBD (Figure 4B). Despite the increase in TMRM staining observed compared with control, statistical analysis did not show significance due to variability among experiments as two CBD experiments showed no change and the other two showed a dose-dependent increase in TMRM staining. Similarly, two of the four lots of cells exposed to CBN showed a dose-dependent increase while the other two showed no change. Because a different cell lot was used for each individual

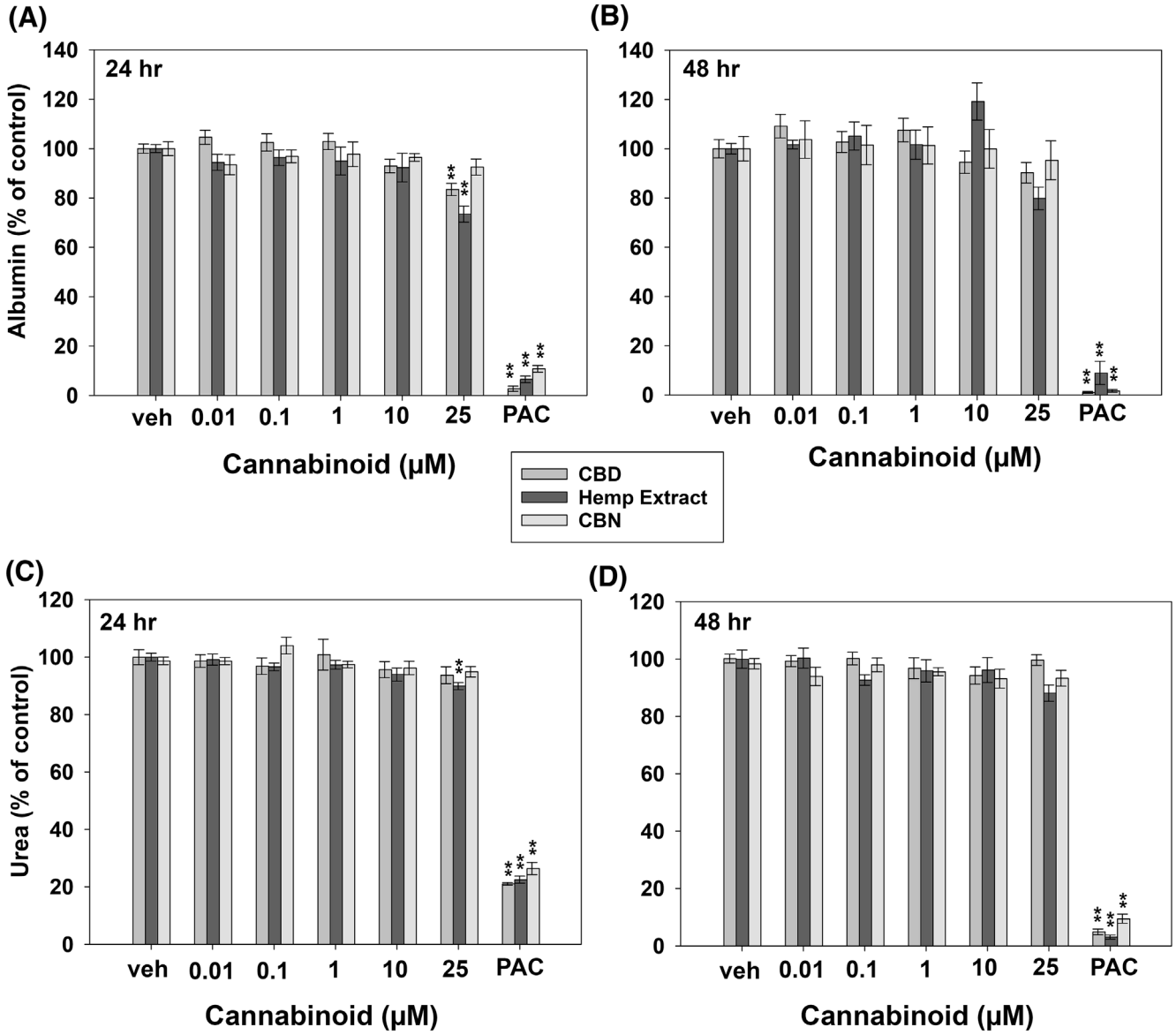

FIGURE 3 Albumin secretion decreased at the highest concentration tested with exposure to CBD and (CBD)matched hemp extract after 24 h whereas urea was only slightly decreased by (CBD)matched hemp extract. (A, B) Primary hepatocytes were exposed to 0.01-, 0.1-, 1-, 10-, and 25-μM CBD, (CBD)matched hemp extract, or CBN for 24 h (A) or 48 h (B). Media from exposed cells was collected and albumin secretion was measured using the Abcam SimpleStep Human Albumin ELISA. Albumin secretion was plotted as percent of vehicle control. A total of 50-μM usnic acid was used as a positive assay control. Results represent the mean ± SEM for four independent experiments. $^{**}p < 0.001$, statistical analyses: CBD, Holm–Sidak; (CBD)matched hemp extract, 24-h Holm–Sidak, 48-h Dunn's method. (C, D) Media was removed, and urea secretion was measured using the Bioassay Systems QuantiChrom Urea Assay kit after 24 h (C) and 48 h (B) of exposure. Urea secretion was plotted as percentage of vehicle control. A total of 50 μM usnic acid was used as a positive assay control. Results represent the mean ± SEM for four independent experiments. $^{**}p < 0.001$, statistical analyses: (CBD)matched hemp extract, 24-h Dunn's method. CBC, cannabichromene; CBD, cannabidiol; CBDV, cannabidivarin; CBG, cannabigerol; CBN, cannabinol.

experiment, nuclei counts were compared to determine if this variation was due to differences in cell number. However, nuclei count compared with 0.5% DMSO vehicle control was not significantly different across concentrations with exposure to either CBD, (CBD) matched hemp extract, or CBN after 24 or 48 h (Figure 4C,D). It is important to note that NucBlue is not a live/dead stain, so the nuclei count does not tend to decrease unless the nuclei are ruptured, DNA is degraded, or dead cells detach from the plate.

4 | DISCUSSION

This study was performed to determine if CBD, CBD concentration-matched hemp extract, or CBN induced toxicity in human primary hepatocytes in vitro. The concentration range was chosen based on in silico calculations of liver C_{max} modeled from exposure to a consumer-product relevant dose and extrapolated to serum C_{max} levels identified in clinical trials to determine the maximum concentration tested. The

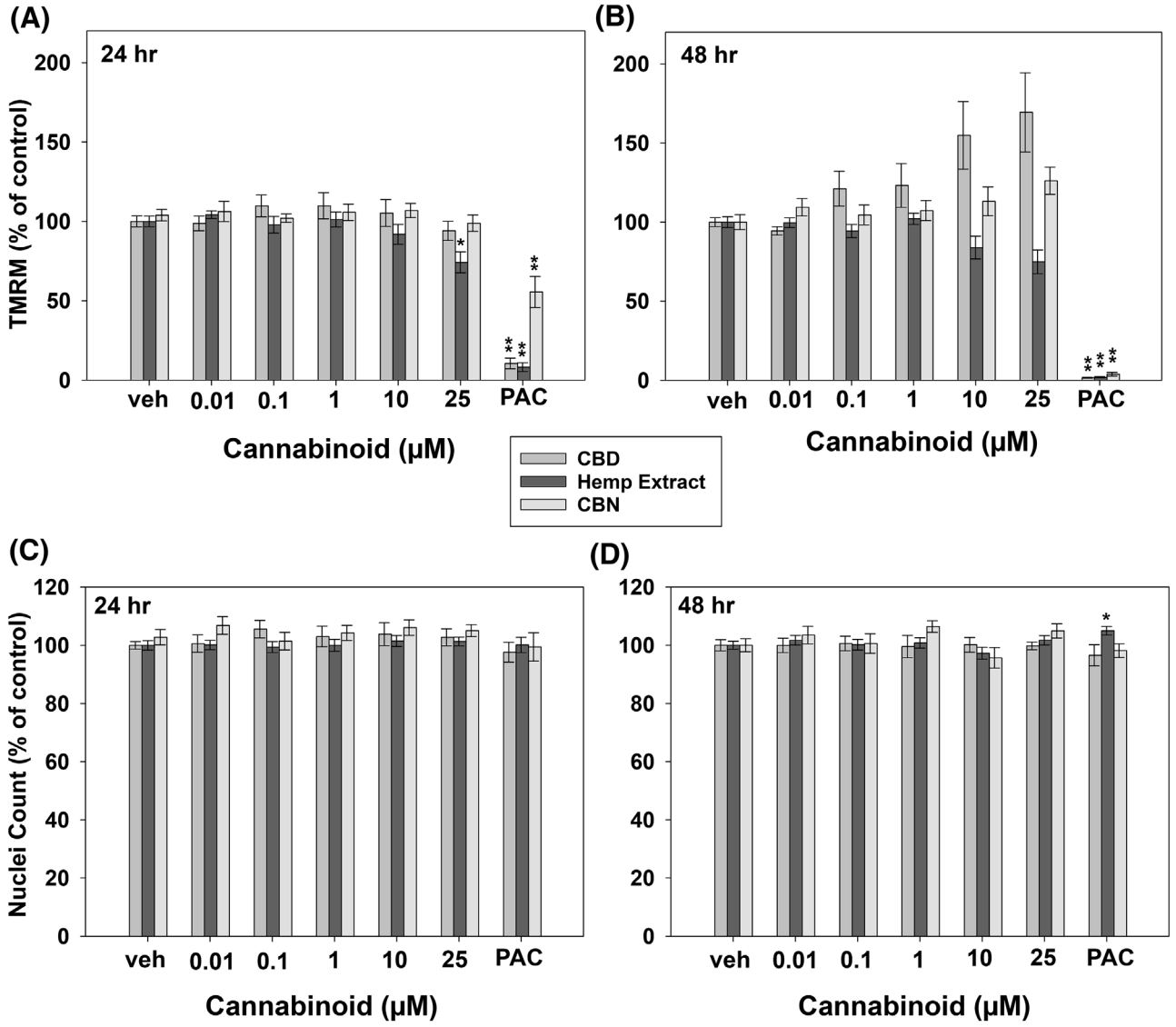

FIGURE 4 Mitochondrial membrane potential varied with CBD and CBN exposure but decreased with exposure to 25 μM (CBD)matched hemp extract after 48 h. (A, B) Primary hepatocytes were exposed to 0.01-, 0.1-, 1-, 10-, and 25-μM CBD, (CBD)matched hemp extract, or CBN for 24 h (A) or 48 h (B). Cells were stained with TMRM to measure mitochondrial membrane potential. The resulting staining area was divided by total area to obtain percentage coverage and then plotted as percentage of vehicle control. A total of 50-μM usnic acid was used as a positive assay control. Results represent the mean ± SEM for four independent experiments. $^{*}p = 0.007$, $^{**}p < 0.001$, statistical analyses: CBD, 48-h Dunn's method; (CBD)matched hemp extract, 24-h Holm–Sidak, 48-h Dunn's method. (C, D) Cells were stained with NucBlue to label and count nuclei after 24 h (C) and 48 h (D) of exposure. The nuclei were counted within the image area, and then total well area was used to determine total nuclei/well. Nuclei count was plotted as percentage of vehicle control. A total of 50-μM usnic acid was used as a positive assay control. Results represent the mean ± SEM for four independent experiments. $^{*}p = 0.021$. CBC, cannabichromene; CBD, cannabidiol; CBDV, cannabidivarin; CBG, cannabigerol; CBN, cannabinol.

results showed that CBD, (CBD)matched hemp extract, and CBN did not induce cell lysis or apoptosis at any of the concentrations or time-points investigated which correlates well with studies performed in HepG2 cells that showed cytotoxicity when exposed to CBD ≥ 54 μM for 24 h (Russo et al., 2019). However, CBD and (CBD) matched hemp extract did have some negative effects on albumin and urea secretion at the highest concentration tested (25 μM). If the assumption based on in silico modeling that the relationship between the plasma and liver CBD concentrations is correct (Figure 1), the data obtained from both CBD and (CBD)matched hemp extract showing an initiation of adverse effects at 25 μM match well with in vivo and clinical data showing adverse effects in the range of the highest clinical dose (Devinsky et al., 2018, 2019; Ewing et al., 2019; Gingrich et al., 2023; Huestis et al., 2019; Watkins et al., 2021). However, without clinical liver C_{max} data, this is only a prediction. Concentrations <10 μM showed no indications of toxicity at either the 24- or 48-h timepoint. In vitro studies examining gene expression in HepaRG spheroids also found very few changes below 10 μM (Li et al., 2023)

further suggesting that CBD concentrations below 10 μM have little to no effect in hepatocytes in vitro, although there may be differences in response between immortalized and primary hepatocytes.

The mechanism of CBD-induced hepatoxicity has not yet been determined. Although other drugs such as acetaminophen induce hepatotoxicity via glutathione depletion, in the mouse model, there were no significant differences in total glutathione levels observed at the highest sub-acute doses of CBD-rich hemp extract (Ewing et al., 2019). Several clinical and in vivo studies have also shown that CBD is most toxic in the presence of other drugs suggesting drug interaction as a possible mechanism (Devinsky et al., 2018). Furthermore, CBD is known to inhibit several cytochrome P450 enzymes including Cyp1A1, Cyp2B6, and Cyp3A4 while CBN has been identified as a potent inhibitor of Cyp1A2 and Cyp1B1 (Yamaori et al., 2010; Zendulka et al., 2016). mRNA analysis in the mouse model found significant changes in several hepatotoxicity pathways including oxidative stress response, lipid metabolism, and drug metabolizing enzymes at single doses as low as 246 mg/kg (Ewing et al., 2019). These data suggest that the mechanism may be linked to disruption in metabolism, but more studies investigating mechanism of action are needed.

The (CBD)matched hemp extract appeared to be slightly more toxic than CBD alone as the hemp extract induced a slight but significant decrease in urea secretion and a significant decrease in mitochondrial membrane potential not seen when hepatocytes were only treated with pure CBD. It is unclear whether the slight increase in toxicity was due to the increased stability of CBD as part of the extract, or due to the presence of the other cannabinoids and/or other constituents in the hemp extract. Similarly, the lack of effect observed in response to CBN exposure may be due to decreased stability of CBN in warm hepatocytes media. As drug–drug interaction and the inhibitory effects of CBD on CypP450 enzymes are a proposed mechanisms of CBD toxicity, the other constituents of the hemp extract, even at significantly lower concentrations, could influence hepatotoxic outcomes.

In experiments measuring changes in mitochondrial membrane potential, pure CBD- and CBN-treated hepatocytes showed much higher variability between experiment and hepatocyte lot compared with (CBD)matched hemp extract. In both cases, two out of the four lots tested showed a dose-dependent increase in TMRM staining. As a different hepatocyte lot was used for each individual CBD experiment to account for variability among donors and lots, the difference in response may be donor specific. There also may be quality control differences stemming from the hepatocyte perfusion and collection process that could affect overall cell health after thawing for each experiment. It is known that CBD's effect on mitochondria can be vastly different among cell types and exposure concentrations (Chan & Duncan, 2021), but it would also be interesting to determine how differences between hepatocyte lots affect the response to CBD, other cannabinoids, or whole hemp extracts. As only a fraction of patients receiving the highest clinical dose of CBD developed hepatotoxicity, the differences seen between cell lots from different donors may translate to differences in response seen among patients (Devinsky et al., 2018, 2019; Watkins et al., 2021). The variation among donors may not be an idiosyncratic effect, but due to the highest concentration being on the edge of a toxic response which is supported by the modest effects observed. If the concentration was just slightly increased, a toxic response may have been observed in all cell lots.

Cannabinoid stability was a notable limitation for future studies examining cannabinoids or other lipophilic compounds. In the hepatocyte maintenance media used for these studies, CBD and CBN were found to be stable at room temperature but not at the 37°C used for treating the hepatocytes. CBD was apparently more stable as a component of the hemp extract when diluted in warm hepatocyte media. Within the hemp extract, CBG and CBC also appeared to be relatively stable; however, CBN and CBDV showed some loss in the moments immediately following exposure. Studies examining the stability of cannabinoids in hepatocyte maintenance media and other types of media found that stability of the individual cannabinoids varies depending on media composition, temperature, incubation conditions, and possible absorption to plastic (Zhao et al., 2023).

Based on the data reported here, CBD and (CBD)matched hemp extract concentrations in the low micromolar and nanomolar range did not have a significant negative effect on the cellular functional biomarkers measured in vitro in the primary hepatocytes. CBN had no effect on any of the endpoints tested; however, this could also be due to decreased stability in warm hepatocyte media. As this was a short-term toxicity study looking at broad indicators of hepatotoxicity, this study did not address repeat exposure to CBD, hemp extract, or CBN over multiple days, or more sensitive indicators of cytotoxicity, which could be useful for drawing conclusions about the subclinical dose effects on human liver function. More studies are needed to fully evaluate the hepatic effects of CBD, hemp extract, or exposure to other cannabinoids from accessible consumer products.

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CONFLICT OF INTEREST STATEMENT

Mention of trade names or commercial products does not constitute endorsement or recommendation for use. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Food and Drug Administration. No competing financial interests exist.

DATA AVAILABILITY STATEMENT

Data available upon request from the author via a government FOI request.

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