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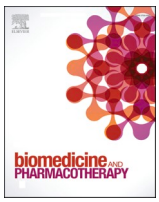

Exploring the safety of cannabidiol (CBD): A comprehensive *in vitro* evaluation of the genotoxic and mutagenic potential of a CBD isolate and extract from *Cannabis sativa* L

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ABSTRACT

Cannabidiol (CBD), a naturally occurring cyclic terpenoid found in *Cannabis sativa* L., is renowned for its diverse pharmacological benefits. Marketed as a remedy for various health issues, CBD products are utilized by patients as a supplementary therapy or post-treatment failure, as well as by healthy individuals seeking promised advantages. Despite its widespread use, information regarding potential adverse effects, especially genotoxic properties, is limited. The present study is focused on the mutagenic and genotoxic activity of a CBD isolate (99.4 % CBD content) and CBD-rich *Cannabis sativa* L extract (63.6 % CBD content) *in vitro*. Both CBD samples were non-mutagenic, as determined by the AMES test (OECD 471) but exhibited cytotoxicity for HepG2 cells (~$IC_{50\ (4\ h)}$ 26 μg/ml, ~$IC_{50\ (24\ h)}$ 6–8 μg/ml, MTT assay). Noncytotoxic concentrations induced upregulation of genes encoding metabolic enzymes involved in CBD metabolism, and CBD oxidative as well as glucuronide metabolites were found in cell culture media, demonstrating the ability of HepG2 cells to metabolize CBD. In this study, the CBD samples were found non-genotoxic. No DNA damage was observed with the comet assay, and no influence on genomic instability was observed with the cytokinesis block micronucleus and the γH2AX and p-H3 assays. Furthermore, no changes in the expression of genes involved in genotoxic stress response were detected in the toxicogenomic analysis, after 4 and 24 h of exposure. Our comprehensive study contributes valuable insights into CBD's safety profile, paving the way for further exploration of CBD's therapeutic applications and potential adverse effects.

1. Introduction

Cannabinoids are bioactive, oxygen-containing, aromatic hydrocarbon compounds [1,2] that are most commonly sourced from the plant *Cannabis sativa* L. This plant, unique in its components and physiological properties, produces well over 560 compounds (terpenes, alkaloids, phenols, flavonoids, amino acids, stilbenoids, fatty acids, carbohydrates, and hydrocarbons), among which over 120 cannabinoids have been identified. The exploration of cannabinoids has led to the discovery of the endocannabinoid system. A signalling system, widely distributed in the human body, particularly in immune-related cells and neurons, with the remarkable ability to regulate various physiological and pathophysiological processes, presenting potential targets for pharmacotherapy [3]. The most recognised cannabinoid in certain strains of *C. sativa L.* or *C. indica* is tetrahydrocannabinol (THC) due to its psychotropic effects in humans. However, in the last decade, many other cannabinoids such as cannabidivarin (CBDV), tetrahydrocannabivarin (THCV), cannabigerol (CBG), cannabinol (CBN), cannabichromene (CBC) and especially cannabidiol (CBD) have attracted interest, as they exert a variety of beneficial pharmacological effects and lack the psychotropic properties typical for THC.

CBD or 2-[(6 R)-6-isopropenyl-3-methyl-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol, interacts with specific cannabinoid receptors like CB1, CB2, and vanilloid receptors but in addition, it is known to non-specifically bind to more than five dozen macromolecular targets in humans, including multiple enzymes, receptors, ion channels, and

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transporters [4]. Although this potentially offers a myriad of potential therapeutic targets, off-target adverse effects can also be expected. There are numerous studies focused on the potential beneficial effects of CBD. Antiepileptic efficiency was demonstrated as early as 1980 [5], and the US Food and Drug Administration (FDA) approved Epidiolex® (a whole cannabis plant CBD-rich extract) for the treatment of refractory epilepsy in 2018 [6]. Numerous studies have delved into CBD's potential therapeutic impact on human anxiety, psychotic symptoms, and depression, consistently noting mild side effects in most cases [7–9]. CBD's anti-inflammatory and antioxidant properties present a novel avenue for neuroprotection [10]. A recent evaluation highlighted both preclinical and clinical evidence endorsing CBD-based treatments for various pain conditions [11]. Additionally, there's substantial enthusiasm regarding CBD's potential as an anticancer agent [12–14]. The literature is overflowing with studies addressing the therapeutic potential of CBD, while studies dedicated to identifying potential adverse outcomes are rare [15]. The surge in research on the beneficial effects of CBD and promising early results have prompted marketers, entrepreneurs, and investors to re-imagine the CBD brand. As a result, CBD is nowadays available to customers in a bewildering range of food supplements and food and cosmetic products. Preparations with CBD are commonly administered via the oral, sublingual, or topical exposure route and are available in a large variety of formulations containing wide-ranging concentrations of CBD, resulting in systemic exposure that exceeds the estimated historical norm (approximately 0.5 mg per day, mainly alongside THC dosing) by up to 150-fold [15].

The growing acceptance, accessibility, and use of CBD raise important public health concerns. Consequently, regulatory bodies and competent authorities in both Europe and the US are intensifying their scrutiny of the market concerning food and cosmetic items containing CBD. In the USA, it is unlawful to market CBD or THC products as, or in, dietary supplements with health claims (Federal Food, Drug, and Cosmetic (FD&C) Act) [16]. In Europe, extracts of *C. sativa* L. containing higher CBD and cannabinoid levels than the plant itself are categorized as novel food by the European Commission (EC). EC also classifies all (synthetic) cannabinoids as novel food. This means that all food products containing CBD or synthetic CBD in high concentrations require authorization under the Novel Food Regulation (EC No. 258/97) before entering the market. Due to several identified knowledge gaps and the inability to establish any "no-observed adverse effect levels"(NOAELs) on the basis of available animal and human studies, the Food Safety Authority (EFSA) panel was unable to assess the safety of CBD as a novel food, halted its use until more data are available, and suggested that unauthorized CBD products on the EU market should be labelled "unsafe" [17].

In the frame of the safety assessment of chemicals and products for human use, data on genotoxic activity are extremely important and are obligatory for all new chemicals as well as for products that are used as pharmaceuticals [18], food additives, supplements [19], cosmetics, etc. Despite the wide use of cannabis extracts and CBDs, the literature data on their potential genotoxic properties are extremely scarce [20,21]. The limited information available on the genotoxic potential of CBD is inconclusive and intricate owing to issues concerning the purity and potency of the test substances and constraints in study design and is therefore unable to provide clear information for human risk assessment.

In the present study, we aimed to explore the mutagenic and genotoxic activity of a CBD isolate (CBD crystal powder; CBD CP) containing 99.4 % CBD and a *Cannabis sativa* L. extract (CBD EX) containing 63.6 % CBD and to unravel the cellular responses to the exposure to these CBD samples that will enable better understanding of their possible health risks in humans. Potential mutagenicity was assessed using the Ames test (OECD 471), and potential genotoxic activity was assessed *in vitro* in human hepatocellular carcinoma (HepG2) cells. The obtained results will contribute substantially to the data needed for the evaluation of the safety of CBD-containing products and their authorization, as well as further development in the field of hemp exploitation for medical and nutritional purposes.

2. Materials and methods

2.1. CBD samples

Two samples derived from *C. sativa* L, containing different contents of CBD, were used in the study, obtained from PharmaHemp d.o.o. (Ljubljana, Slovenia). One of the samples was a CBD isolate (CBD crystalline powder; CBD CP, obtained by distillation and crystalization), and the other was a high-content CBD cannabis extract (CBD EX, prepared by extraction using supercritical CO2, cannabinoid concentration and removal of THC using preparative chromatography), with a CBD content of 99.4 % and 63.6 %, respectively. In addition to CBD, the CBD EX sample contained also other cannabinoids including CBDV (12.78 %), THCV (1.677 %), CBG (1.077 %), and others in small amounts (Table S1). For the experiments, the CBD samples were dissolved in ethanol (EtOH), and stock solutions with a concentration of 50 mg/ml for the Ames assay and 2.6 g/ml for the *in vitro* cell experiments were prepared.

2.2. Mutagenic activity – the AMES assay

Mutagenicity testing of the CBD samples was performed with the bacterial reverse mutation test - Ames test [22]. The plate incorporation method without and with external metabolic activation (10 % rat S9, Trinova Biochem GmbH) was performed according to the OECD guideline 471 [23], in accordance with Good Laboratory Practice (GLP) standards. To detect possible base pair substitution and frameshift mutations, five amino acid-requiring *Salmonella typhimurium* strains (TA97a, TA98, TA100, TA102, and TA1535) obtained from Trinova Biochem GmbH (Giessen, Germany) were used in the study. The CBD samples were tested at the concentration range of 0.0005–5 mg/plate. The plates were incubated for 48 h (TA97a, TA100, TA102, TA1535) and 72 h (TA98) at 37 °C. Afterwards, spontaneous and CBD CP or CBD EX-induced His+ revertants were counted. An inspection for potential toxic effects of CBD samples was carried out. Each experimental point was tested in three plates. A negative control (3.7 % MilliQ water), a solvent control (3.7 % EtOH) and strain-specific positive controls were included in the test. Direct mutagenicity positive controls included 4-nitroquinoline-N-oxide (NQO; 0.25 μg per plate) for TA97a and TA98; mitomycin C (0.05 μg/plate) for TA102; and sodium azide (NaN3; 0.25 and 0.125 μg/plate, respectively) for TA100 and TA1535. Benzo[*a*]pyrene (BaP) served as the positive control for S9-dependant mutagenicity for TA97a at 5 μg/plate and 2.5 μg/plate for TA98, TA100, TA102 and TA1535.

2.3. Assessment of toxic potential in vitro

The human hepatocellular carcinoma-derived cell line HepG2 (ATCC-HB-8065™, Manassas, VA, USA) was used in the study. The cell line expresses the wild-type TP53 tumour promotor protein and has retained the activity of several xenobiotic metabolizing enzymes [24,25], which makes it one of the widely used *in vitro* test systems for toxicological studies. Cells were grown in MEM medium (MEM-10370–047) supplemented with 10 % FBS, all from Gibco (Praisley, Scotland, UK) and 1 mM sodium pyruvate, 2 mM L-glutamine, and 100 IU/ml penicillin/streptomycin, all from Sigma-Aldrich (St. Louis, MO, USA), at 37 °C in a humidified atmosphere with 5 % CO2. Cell passages of between 3 and 10, with a confluency of approximately 70 %, were used for the experiments.

2.3.1. Cytotoxicity – the MTT assay

Cytotoxicity of the CBD samples was evaluated with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, a

colourimetric assay for assessing the metabolic activity of cells [26]. Cells were initially seeded onto 96-well plates (Corning Costar Corporation, New York, USA) at a density of 10,000 (4 h exposure) and 8000 (24 h exposure) cells/well, left overnight to adhere, and then exposed to graded concentrations (0.05 – 50 μg/ml) of CBD CP and CBD EX. Following 4 and 24 hours of exposure, MTT (Sigma, St. Louis, USA; 0.5 mg/ml) was added to each well, and the reaction was incubated for 3 hours. Post-incubation, the reaction solution was removed, DMSO (200 μl, (100 %) was added to dissolve the formazan crystals, and the absorbance was measured at 570 and 690 nm (Sinergy MX, BioTek, Winooski, VT, USA). The experiment was performed in five replicates per experimental point and repeated three times independently. Controls, including a negative (cell medium), solvent (0.2 % EtOH), and positive (3 % DMSO) control, were included in the experiments. The GraphPad Prism v9 software (GraphPad Software, San Diego, CA, USA) was used for the calculation of half-maximal inhibitory concentrations (IC_{50}) and statistical analysis. The IC_{50} values were determined using nonlinear regression (log(agonist) vs. normalized response—variable slope). Significant differences in cell viability between CBD sample-exposed cells and the solvent control group were analysed using a One-Way Analysis of Variance (ANOVA) and Dunnett's multiple comparison test.

2.3.2. Detection of CBD metabolism in HepG2 cells

The identification and the relative quantitation of CBD metabolites in HepG2 cell growth media was performed on an Agilent 1290 ultra-high performance liquid chromatograph coupled to a triple quadrupole tandem mass spectrometer 6460 (Agilent Technologies, Santa Clara, CA, USA). After the addition of internal standard (50 μl, haloperidol 224 ng/ml), the incubation media samples (100 μl) were extracted with ethyl acetate (900 μl) by 2 min vortex-mixing and subsequent brief centrifugation (15,000×*g*, 5 min); the supernatant was transferred to a separate vial, dried, and reconstituted in 100 μl of 50 % acetonitrile. The chromatographic and mass spectrometry parameters are described in Tables S9 and S10. For the identification of metabolites, specific mass transitions were used (Table S10). The CBD metabolites were determined semi-quantitatively due to the lack of analytical standards available at the time of experimental work. Two groups of metabolites were determined: phase I and phase II. In phase I, three different types of oxidative metabolites were monitored: the ring 1-hydroxylated CBD (CBD-OH I, Figure S1), the ring 2-hydroxylated CBD (CBD-OH II, Figure S2), and the 7-COOH-metabolite (Fig. 2). In phase II group, a direct CBD glucuronide metabolite was monitored, with the glucuronic moiety at ring 2 (Figure S3).

The method has been checked for accuracy for analyte CBD (88–110 %), precision (1.1–8.8 %), linear range (1–1000 ng/ml) and matrix effects (2–4 % RSD of calibration line slopes from different matrix donors). For CBD metabolites, only the linear response range and instrument precision could be tested due to the lack of analytical standard availability. Therefore, the CBD metabolites were determined semi-quantitatively, which was considered adequate for the aim of comparison of the metabolite formation from CBD CP and CBD EX.

2.3.3. The alkaline comet assay

DNA damage induction after exposure to the CBD samples was investigated using the alkaline comet assay. Cells were seeded onto 12-well plates at a density of 80,000 cells/well and allowed to adhere overnight. The subsequent day, the cell medium was replaced with graded concentrations (0.1, 0.5, 1, and 5 μg/ml) of either CBD CP or CBD EX. After 4 and 24 hours of exposure, the comet assay was conducted as previously described by Štampar et al. [27]. The slides were stained using the GelRed nucleic acid stain (Biotium, USA) as per the manufacturer's instructions. Subsequently, analysis and scoring were performed using a fluorescent microscope and Comet Assay IV software (Instem, Philadelphia, USA). A negative (cell culture medium), a solvent (0.2 % EtOH) and a positive (BaP, 30 μg/ml) controls were included. Each experiment was conducted independently three times, analysing fifty nuclei per experimental point. Statistical analysis employed the Kruskal–Wallis nonparametric test and Dunn's multiple comparison test in GraphPad Prism v9 (GraphPad Software, San Diego, CA, USA) to identify significant differences in the percentage of tail DNA among the tested cell populations.

2.3.4. The γH2AX focus assay and the determination of p-H3 positive events

Induction of γH2AX foci, a marker for DNA double-strand break (DSB) induction, and p-H3 positive events caused by the CBD samples were determined using flow cytometry. HepG2 were seeded in 6-well plates at a density of 300,000 cells/well and 500,000 cells/well, and the next day, the cell medium was replaced with graded concentrations (0.1, 0.5, 1 and 5 μg/ml) of CBD CP or CBD EX for 4 and 24 hours, respectively. Etoposide (3 and 1 μg/ml) and colchicine (0.5 μM) (both purchased from Sigma, St. Louis, MO, USA) were used as positive controls for γH2AX and p-H3, respectively, after 4 and 24 h treatment. Thereafter, all cells were collected, washed twice with 1x PBS and fixed overnight at 4°C in 75 % ethanol. For further analyses, cells were labelled with anti-γH2AX pS139 antibody (130–123–256, Lot:5230309463) for DNA DSB analysis and anti-histone H3 pS128 antibody (130–124–883, Lot:5230501378) for detection of p-H3 positive events as previously described [28,29]. REA-PE (130–118–347, Lot:5230301437) and REA-APC (130–120–709, Lot:5221007086) controls were used to exclude non-specific antibody binding. All antibodies and REA controls were purchased from Miltenyi Biotec, Bergisch Gladbach, Germany. Three biologically independent experiments were performed, and 10,000 events were acquired for each measured sample using a MACSQuant Analyzer 10 flow cytometer with MACSQuantify™ software (Miltenyi Biotech, Germany). To analyse the results, the raw data was exported from the MACSQuantify software and analysed using FlowJo V10 software (Becton Dickinson, New Jersey, USA). Statistically significant differences between CBD CP or CBD EX exposed and control samples in the number of pH3-positive cells were determined by one-way ANOVA with Dunnett's multiple comparison test, and the statistically significant difference in the intensity of APC fluorescence signal (γH2AX) was determined by two-way ANOVA with Uncorrected Fisher's LSD using GraphPad Software V9 (GraphPad Software, San Diego, CA, USA). Statistical significance was defined as $p \leq 0.05$.

2.3.5. The Cytokinesis-block micronucleus (CBMN) cytome assay

The potential impact of CBD CP and CBD EX on chromosomal instability was assessed by the Cytokinesis Block Micronucleus (CBMN) assay, evaluating DNA damage markers such as micronuclei (MNi), nuclear buds (NBUDs), and nucleoplasmic bridges (NPBs) in once divided, binucleated cells (BNCs). Cells were seeded onto T25 plates (Corning Costar Corporation, Corning, NY, USA) at a density of 700,000 cells/well and incubated overnight to adhere. Subsequently, they were exposed to graded concentrations (0.5, 1 and 5 μg/ml) of either CBD CP or CBD EX, followed by the CBMN assay after 24 hours, as described by Štraser et al. [30], with minor modifications. A negative (cell culture medium), solvent (0.2 % EtOH) and a positive (BaP, 30 μg/ml) controls were included. Automated image acquisition and analysis were performed using the Metafer system (Metasystems, Heidelberg, Germany). Slides were stained with Hoechst 33342 (Invitrogen, Waltham, MA, USA), and MNi, NBUD, and NPB were scored in 2000 BNCs per experimental point. Additionally, the nuclear division index (NDI) was determined by manually scoring cells with one, two, three or more nuclei in 500 cells through staining with acridine orange (20 μg/ml), using the fluorescent microscope Nikon Eclipse Ci (Nikon, Japan). Statistically significant differences between CBD sample-exposed cells and the solvent control group were analysed using ANOVA and Dunnett's multiple comparison test in GraphPad Prism V9 (GraphPad Software, San Diego, CA, USA).

2.3.6. The toxicogenomic analysis

The expression of selected genes after the exposure to the CBD samples was analysed at the transcriptional level using carefully selected qPCR primer assays (Applied Biosystems, USA) (SM2) and One 48.48 Dynamic Array IFC for Gene Expression (Fluidigm, USA). Cells were seeded on 6-well plates at the density of 300,000 cells/well and left to attach overnight. Cells were then exposed to CBD CP and CBD EX at concentrations 0.5 μg/ml and 5 μg/ml. After 4 and 24 h of exposure cells were harvested by trypsinization, and total RNA was isolated using the RNeasy mini kit from Qiagen (Qiagen, Germany), according to the manufacturer's instructions. The concentration and purity of the isolated RNA were evaluated using a NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific) and absorbance readings at 260/280 nm. The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, MA, USA) was used for the reverse transcription of 1 μg of total RNA per sample, as described by the manufacturer, using a PCR thermalcycler (BIO-RAD T100 Thermal Cycler), under reaction conditions described in Table S3.

For preamplification of the selected genes, 4 μl of each of the selected 24 Taqman assays to be analysed (SM2) was combined into the "primer poll", and the reaction mixture was prepared by mixing the TATAA PreAmp GrandMasterMix (Tataa Biocenter, Gothenburg, Sweden) and the prepared "primer pool" and nuclease-free water, according to the manufacturer's instructions. An NTC for the preamplification step and an NTCq for the qPCR reaction were included in the test. For the preamplification reaction, 8 μl of the prepared Reaction mix was mixed with 2 μl of the 5-times diluted cDNA samples in a 96-deep well plate, which was sealed with cover strips, vortexed, and centrifuged (1000 g, 1 min). The reaction was carried out on a PCR thermal cycler (BIO-RAD T100 Thermal Cycler), under the reaction conditions described in Table S4.

TaqMan Universal PCR Master Mix and Taqman Gene Expression Assays (Table 1) were used for the gene expression analysis. The preamplified samples were 10 times diluted by the addition of nuclease-free water into each sample well. Assays were prepared by mixing equal volumes (6 μl) of each specific assay with the Fluidigm Assay Loading Reagent Kit – 10IFCS. The reaction pre-mix was prepared by mixing the DNA Sample Loading Reagent and the Fast probe (Biotium) Master Mix (Roche), added to each preamplified diluted cDNA sample. The qPCR experiments were performed on 48.48 Dynamic Array™ IFC chips for gene expression on the Fluidigm BioMark™ HD System (BioMark, ID, USA), according to the manufacturer's instructions, with the conditions as described in Table S5. The generated data was analysed using the Fluidigm Gene expression Analysis Software and with a free-accessible web program quantGenious [31]. The difference in gene expression greater than 1.5-fold was considered a biologically important up/downregulation (relative expression >1.5 or <0.66, respectively). Statistically significant differences between CBD sample-exposed cells and the solvent control group were analysed using ANOVA and Dunnett's multiple comparison test in GraphPad Prism v9 (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

Despite the widespread use and numerous potential therapeutic properties of CBD, there is an overall lack of studies on the adverse effects and, in particular, on the genotoxicity of CBD and extracts of *C. sativa*. In animal toxicity assessments, CBD exhibited developmental toxicity, embryo-foetal mortality, central nervous system suppression, neurotoxicity, liver damage, decreased spermatogenesis, shifts in organ weights, changes in the male reproductive system, and lowered blood pressure [32]. However, these effects occurred at doses surpassing those typically recommended for human therapies. Human studies of CBD for the treatments of epilepsy and psychiatric conditions revealed CBD-triggered drug interactions, liver abnormalities, diarrhoea, fatigue, vomiting, and drowsiness [10]. However, these studies are not sufficient to evaluate the potential adverse effects of CBD upon chronic exposure, as the number of individuals in these clinical trials was small. In addition, as already mentioned, specific aspects of toxicological evaluations, including genotoxicity, are still missing. The limited data on the genotoxicity of CBD and CBD-containing extracts have been inconclusive and interactive due to challenges related to the purity and potency of the test material, and as limitations in the study design. In the present study, we have comprehensively investigated the potential *in vitro* mutagenic and genotoxic activity of a natural CBD isolate (CBD CP) and a well-defined CBD-rich *C. sativa* extract (CBD EX), to obtain critical information that will substantially contribute to the human health risk assessment of CBD.

3.1. Potential mutagenicity of the CBD isolate (CBD CP) and CBD extract (CBD EX)

As outlined in the EFSA guidance [33] on genotoxicity testing, the genotoxicity assessment should follow a tiered approach. It should commence with fundamental *in vitro* tests, typically starting with the bacterial reverse mutation assay (Ames test) to evaluate mutagenicity, followed by an *in vitro* micronucleus test to assess numerical and structural chromosomal changes [33]. The Ames test stands as a foundational tool in genetic toxicology for assessing the mutagenic potential of various compounds. It utilizes specific strains of bacteria, carrying mutations that disable them to synthesize a vital amino acid and to grow without it [22]. If a test substance induces mutations, it increases the frequency of reverse mutations, restoring the wild type that can grow on test plates, indicating the mutagenic potential of the test substance. Our results show that CBD CP and CBD EX did not induce mutations in *S. Typhimurium* strains TA97a, TA98, TA100, TA102 and TA1535, under the test conditions applied in this study (Table 1, Table S6). Induction factors (IF) for all strains, all tested concentrations and test variants are summarized in Table S6. Both CBD samples were toxic for the strain TA102 at concentrations $\geq$ 0.05 (CBD CP) and 0.0158 mg/plate (CBD EX) without external (S9) metabolic activation. CBD EX was toxic also in the test with S9, at concentrations $\geq$ 1.58 mg/plate for strain TA102 and strain 1535, both with and without S9, at the highest tested concentration (5 mg/plate).

The negative Ames test results are in line with previous published data. In a recent study, Handerson et al. reported that a hemp-derived CBD isolate (purity > 99.0) did not induce mutations at noncytotoxic concentrations up to 5 mg/plate in *Salmonella* tester strains TA98, TA100, TA1535, TA1537 and *E. coli* strain WP2 uvrA, with or without metabolic activation [34]. In addition, the non-clinical safety review of Epidiolex by EMA reported CBD to be negative for the strains TA98, TA100, TA1535, TA1537, and TA102, without or with external metabolic activation at concentrations up to 320 μg/plate [35]. Negative Ames assay data were also reported for hemp extracts with different CBD contents (approximately ranging from 7 % to 25 %) [36–38].

3.2. Cytotoxicity of the CBD isolate (CBD CP) and CBD extract (CBD EX) in HepG2 cells

Prior to genotoxicity testing, cytotoxicity was assessed in order to determine the toxicity profile of CBD CP and CBD EX in the chosen test system (HepG2 cells) and to select relevant non-cytotoxic concentrations for further testing, as cytotoxic concentrations may lead to false-positive results in genotoxicity tests. For this purpose, the MTT assay, a colourimetric assay that measures the reduction of MTT to insoluble formazan by dehydrogenases and other reducing agents present in metabolically active cells [39], was used. Assuming that all tested cell populations have equal metabolic activity, the amount of formazan produced is directly proportional to the number of viable cells. Our results show that both CBD samples exerted similar cytotoxicity in HepG2 cells (Fig. 1). The IC_{50} values for CBD CP and CBD EX were 26.9 ± 2.3 and 26.2 ± 5.3 μg/ml after 4 h of exposure and 6.7 ± 0.6 and 7.9 ±

Table 1
The mutagenic potential of CBD CP and CBD EX in the Ames assay, with and without metabolic activation (S9).

Sample/ concentration (mg/plate)		TA97a		TA98		TA100		TA102		TA1535	
		ø	S9	ø	S9	ø	S9	ø	S9	ø	S9
CBD CP	NC	-	-	-	-	-	-	-	-	-	-
	0.0005–0.0158	ND	ND	ND	ND	ND	ND	-	ND	ND	ND
	0.05–5	-	-	-	-	-	-	T	-	-	-
	PC	+	+	+	+	+	+	+	+	+	+
Sample/ concentration (mg/plate)		TA97a		TA98		TA100		TA102		TA1535	
		ø	S9	ø	S9	ø	S9	ø	S9	ø	S9
CBD EX	NC	-	-	-	-	-	-	-	-	-	-
	0.0005–0.005	ND	ND	ND	ND	ND	ND	-	ND	ND	ND
	0.0158	ND	ND	ND	ND	ND	ND	T	ND	ND	ND
	0.05–0.5	-	-	-	-	-	-	T	-	-	-
	1.58	-	-	-	-	-	-	T	T	-	-
	5	-	-	-	-	-	-	T	T	T	T
	PC	+	+	+	+	+	+	+	+	+	+

ND – not determined, T – toxic, + IF ≥ 2 or 3, - IF ≤ 2 or 3. PC – strain and test-specific positive control.

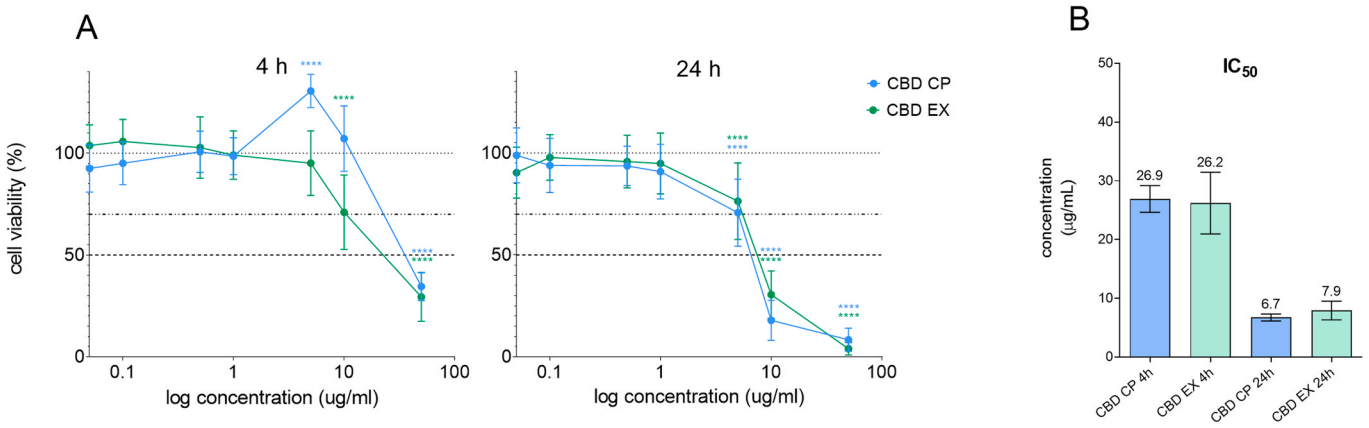

Fig. 1. Cytotoxicity of the CBD isolate (CBD CP) and CBD extract (CBD EX) in HepG2 cells. A) Effects of CBD CP (blue) and CBD EX (green) on cell viability (MTT assay) after 4 and 24 h of exposure are expressed as the percent of the solvent control (0; 0.2 % EtOH — upper dashed line). DMSO (3 %) was used as a positive control (data not shown). The middle-dashed line represents the threshold of 70 % viability and the lower dashed line represents 50 % viability. Asterisks (*) indicate statistically significant differences (ANOVA and Dunnet's multiple comparison test) between the solvent control and cells exposed to the test compound (**** $p \leq 0.0001$). B) IC_{50} values (right chart) were calculated using nonlinear regression (log(agonist) vs. normalized response—variable slope).

1.6 µg/ml after 24 h of exposure, respectively.

After 4 h of exposure, a significant increase in cell viability was observed at 5 µg/ml in cells exposed to CBD CP. At 10 µg/ml, the viability was only slightly increased, and at 50 µg/ml, cell viability decreased to 34.5 ± 6.9 %. The observed increase in cell viability (at 5 and 10 µg/ml) is most likely not due to an increased cell number but to an increased metabolic rate as a stress response. No increase in viability was observed after exposure to CBD EX, which induced a dose-dependent decrease in cell viability that was significant at 10 and 50 µg/ml, reducing cell viability to 71.0 ± 18.2 % and 29.4 ± 12.0 %, respectively. After 24 h of exposure, the dose-response curves were remarkably similar for both CBD samples. A significant decrease in viability was observed at 5, 10 and 50 µg/ml. Considering that the CBD samples exhibited similar cytotoxicity and the CBD content in the samples differed significantly, our results indicate that in the case of the CBD EX sample, other components present in the extract contributed to the observed cytotoxicity, directly or indirectly. Since the CBD content of cannabis extracts varies greatly, data are not comparable across studies. However, we can compare the results for CBD CP, which is a CBD isolate with 99.4 % purity. Although different methods for IC_{50} determination were used by different authors, the reported IC_{50} values are in a similar range. The IC_{50} values for CBD in HepG2 cells were recently reported as 61.6 ± 2.8 µM after 24 h of exposure [40] and in a previous study as 162 µM after 3 h and 54 µM after 24 h of exposure [41], which is about two times higher than the IC_{50} values for CBD CP in the present study (85.3 µM after 4 h and 21.2 µM after 24 h). Similar results were recently reported for several human hepatocellular carcinoma cells (42.98 µM in HepG2, 39.51 µM HUH7, 40.87 µM MHCC97H cells, and 53.8 µM in HCCLM3 cells) [42]. On the other hand, recently Acquavua et al., who used the same method (MTT) and a similar approach for IC_{50} determination (curve fitting using GraphPad Prism V6) as used in our study, reported lower IC_{50} values (16.82 ± 2.54 µM) in HepG2 cells after 24 h of exposure to CBD [43], closely comparable to our results.

The observed CBD cytotoxicity in HepG2 cells in the present study correlates with reports on CBD hepatotoxicity that were recently summarized in a review by Lo et al. [44], which concluded that elevated liver enzyme levels and drug-induced liver injury are common adverse drug reactions associated with CBD use at moderate-to-high doses, but the risk is low at doses below 300 mg CBD/day. After oral application, CBD has low bioavailability, due to its incomplete absorption from the gastrointestinal lumen and is mostly related to its extensive presystemic metabolism and poor aqueous solubility, especially at higher concentrations [45]. In addition, formulation and food effects may have a pronounced impact on CBD absorption and first pass metabolism [46]. Therefore, it is difficult to speculate about the CBD concentration that reaches the liver in relation to doses in humans. Data on the CBD blood plasma levels after consumption suggest that CBD concentrations in

blood after doses up to 800 mg would not reach the levels that were cytotoxic in our study, as the reported blood plasma levels are in the ng/ml range [47]. However, there are factors, which may increase CBD oral bioavailability by many folds, including the pronounced positive effects of milk, high-fat meals or advanced nano-drug delivery formulation strategies [48,49], and which underline the importance of toxicity testing in a wide-concentration range.

3.3. Metabolism of CBD in HepG2 cells

The metabolic transformation of CBD is an important aspect to be considered when studying CBD toxicity and potential genotoxicity. CBD serves as a favourable substrate for CYP450 mixed-function oxidases, undergoing substantial hydroxylation at various sites. Further oxidations lead to a multifaceted metabolic profile, yielding approximately 40 identified phase I oxidative metabolites in humans [50]. In general, the major metabolites of CBD are 7-OH-CBD, direct CBD glucuronide, and 7-COOH-CBD, which is thought to be formed from the 7-OH-CBD with further oxidation; apart from these major metabolites, several hydroxylated metabolites at the 4' side chain and their putative glucuronides have also been identified in human urine [50]. It was demonstrated that many CBD oxidative metabolites have potential pharmacologic activity as well. Russo et al.[9] demonstrated that CBD metabolites exert higher genotoxic potential than the parent molecules. Since the metabolites are believed to be the most important for the potential genotoxic effects of CBD, it is imperative to establish a relevant *in vitro* model that can correlate to the *in vivo* metabolite formation. In the present study, HepG2 cells were used, a cell line known to have preserved a substantial level of the expression of genes encoding enzymes involved in the metabolism of xenobiotic substances. To confirm that the exposure to the CBD samples induces up-regulation of the mRNA expression of known inducible metabolic enzymes, gene expression analysis of several phase I (*CYP1A1*, *CYP1A2*, *CYP3A4*, *CYP2D6* and *CYP2C19*), phase II (*UGT1A3* and *UGT2B7*) drug-metabolizing enzymes, and nuclear receptors (*AHR*, *PXR* and *CAR*) was performed.

The enzymes CYP3A4 and CYP2C19 are thought to be the major drug-metabolizing enzymes implicated in the oxidative metabolism of CBD, however, there is still some uncertainty regarding the relative contribution and the exact mechanism of each enzyme. *In vivo* metabolite pharmacokinetic data published by Taylor et al. [51] and further clarified by *in vitro* experiments by Beers et al. [52] propose a quite complex oxidative and phase II metabolism of CBD, consisting mainly of three major routes: the ring I oxidations, the ring II oxidations, and the glucuronidation at ring II (Fig. 2).

Our results show that both CBD samples induced the expression of the selected genes involved in the metabolism of xenobiotics and specifically in CBD metabolism in a dose and time-dependent manner (Fig. 3). The highest fold change was observed for *CYP1A1*, followed by *CYP3A4* and *UGT1A3*. Gene deregulation was much more pronounced after 24 h compared to 4 h of exposure. The sample CBD EX overall induced a slightly higher gene expression up-regulation than CBD CP. Of the selected genes, *CYP1A2* and *CYP2C19* were poorly expressed in HepG2 cells at baseline, so their potential differential expression resulting from exposure to the CBD samples could not be determined. The fold change data for each sample and assay is summarized in Table S7.

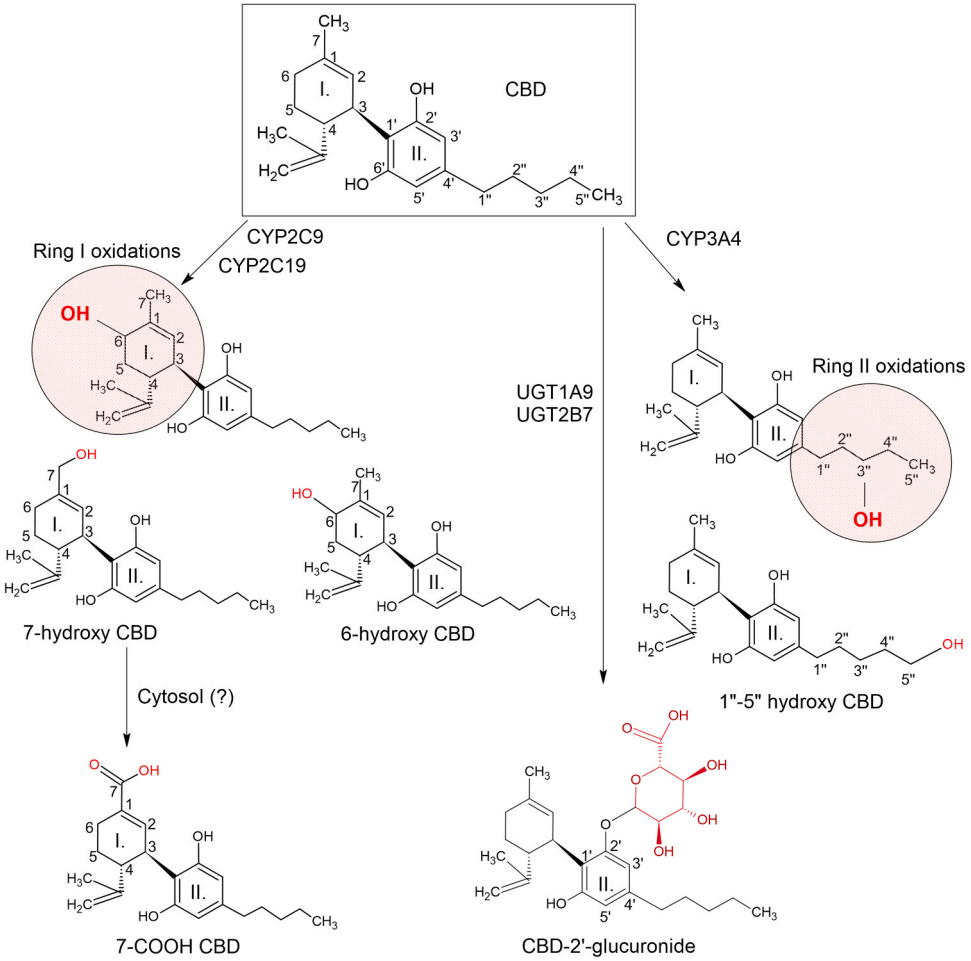

Fig. 2. The schematic presentation of CBD metabolism showing three main pathways: the ring I oxidations, the ring II oxidations, and the glucuronidation at ring II.

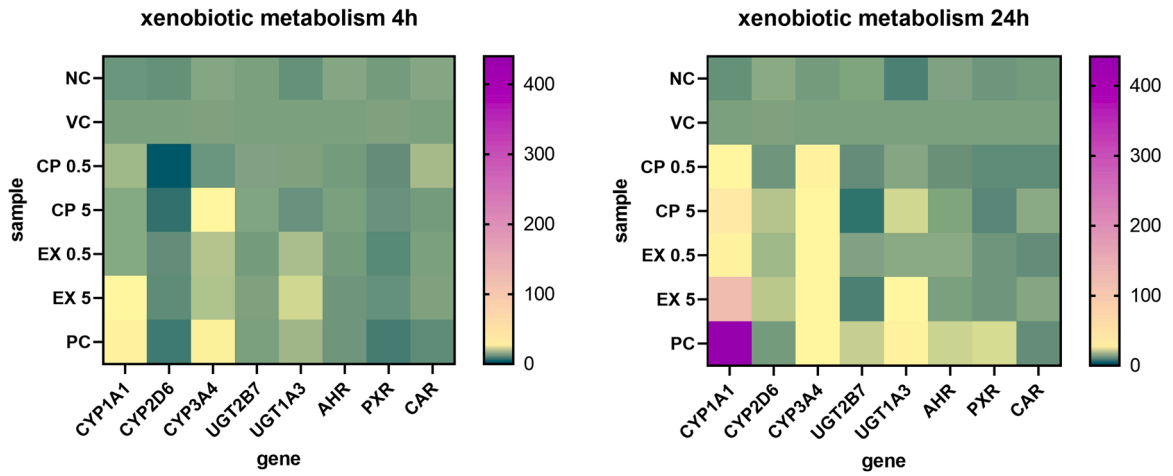

Fig. 3. Heatmap showing the induction of selected genes involved in CBD metabolism. Fold change deregulation of gene expression after exposure to CBD CP and CBD EX (0.5 and 5 μg/ml) for 4 (left) and 24 h (right) in comparison to the vehicle control (VC; 0.2 % EtOH) is presented in the form of heatmaps. VC fold-change value is 1 and is represented in light green colour. Down-regulation is indicated by a darker green colour, while up-regulation is indicated by a lighter green colour and changes to yellow and purple, as shown in the scale bar. NC is the negative control (growth medium), and PC is the positive control (30 μg/ml BaP).

To confirm that CBD is being metabolized and some of the major metabolites found in humans are also present in HepG2 cells, the analysis of metabolites in the cell culture media after exposure to the CBD samples was performed.

Based on the data obtained from semi-quantitative MS/MS analysis, significant formation of phase I and phase II CBD metabolites was observed in the HepG2 incubation media after 4 and 24 h of exposure. Among phase I biotransformations, two groups of hydroxylated CBD metabolites were distinctively observed based on MS/MS fragmentation data: the first group (CBD-OH I) consisted of at least three hydroxylated products at ring I, including the active major CBD metabolite, the 7-OH-CBD (Fig. 2 and S1). In the second group (CBD-OH II), the hydroxylation occurred at the second CBD ring (Figure S2).

The formation of CBD-OH I and II metabolites significantly positively correlated with the substrate concentration (Fig. 4A). Furthermore, the CBD-OH I metabolite formation rate was 2–5-fold higher in the presence of CBD EX as a substrate compared to the CBD CP (Fig. 4A), even though the content of CBD was significantly lower in CBD EX compared to that in CBD CP. This seemingly contradictory result points to specific metabolic enzymes involved in the formation of CBD-OH I, namely the CYP2C9/2C19. Indeed, as was already observed by Beers et al. [52], the formation of 7-OH CBD, the major active CBD OH I metabolite found *in vivo*, is catalysed mainly by CYP2C9 and 2C19. Furthermore, Beers et al., showed that enzyme kinetics for the formation of 7-OH CBD follows the substrate-inhibition model. The reaction velocity in human liver microsomes is peaking below 5 μg/ml of CBD. Therefore, the higher rate of CBD-OH I formation in the case of the CBD EX sample can be explained by the lower CBD content in CBD EX used as a substrate source, indicating that the same substrate inhibition may have been observed in our experiment, indirectly suggesting that CYP2C9/2C19 is responsible for the formation of CBD-OH I in our study despite the low CYP2C19 gene expression observed in HepG2 cells in the present study. Besides the CYP2C9/2C19, other cytosolic non-CYP oxidases may also be involved in the oxidative metabolism of CBD at the 7-position, as discussed by Beers et al. [52].

On the other hand, the formation of CBD-OH II (Fig. 4B) was shown to be primarily catalysed by CYP3A4 [52], the expression of which was confirmed and upregulated also in our HepG2 system. After 4 h of exposure, a significantly higher amount of CBD-OH II was formed from CBD CP compared to CBD EX (Fig. 4B, left panel), while after 24 h, the amount of CBD-OH II formed in the case of CBD EX was equal or higher (at 5 μg/ml) compared to CBD CP (Fig. 4B, right panel). A similar pattern was also observed in the case of CBD glucuronidation (Fig. 4C), where after 4 h, a higher metabolite formation rate was observed in the case of CBD CP, and after 24 h, the amounts of CBD-2'-glucuronide were similar in both samples, CP and EX. Higher phase I conversion rates have been observed for CBD ring I and ring II hydroxylation in the case of CBD EX compared to CBD CP, despite its lower substrate content, possibly indicating a greater HepG2 stimulation by other compounds present in the extract besides CBD. These results correlate with the higher induction of metabolic enzyme genes that was observed in the case of CBD EX, which was especially evident after 24 h of exposure (Figs. 3 and 4B).

Our results confirmed that although the gene encoding CYP2C19, one of the major enzymes involved in CBD metabolism, was poorly expressed in HepG2 cells, the cells were able to metabolize CBD into metabolites that are also encountered in humans, indicating their suitability as a model system for studying CBD metabolism-related adverse effects *in vitro*.

3.4. *Genotoxicity of CBD isolate (CBD CP) and CBD extract (CBD EX) in vitro*

For the genotoxicity assessment of CBD CP and CBD EX *in vitro*, DNA damage, DNA double strand break (DSB) induction, potential aneugenic activity, and chromosomal instability were evaluated using the comet assay, the γH2AX and p-H3 assays, and the CBMN assay, respectively. In addition, toxicogenomic analysis was performed to get insight into the cellular stress response after exposure to the tested CBD samples.

The comet assay is a sensitive technique that detects DNA damage at the level of individual cells. It detects mostly DNA single (SSBs) strand breaks, but in addition also DSBs, alkali labile sites such as apurinic/apyrimidinic sites, DNA-DNA and DNA-protein cross-links as well as SSBs associated with incomplete excision repair [53]. No significant increase in DNA damage was observed in HepG2 cells after 4 and 24 h of exposure to CBD CP and CBD EX at concentrations of up to 5 μg/ml (Fig. 5). Contrary to our results, Russo et al. observed DNA damage in HepG2 cells and buccal-derived cells (TR146) after exposure to CBD at concentrations equal to or higher than 6 μM (3 h) or 2 μM (24 h), using the comet assay [40]. The highest concentration used in our study was 5 μg/ml, which in the case of CBD CP corresponds to 15.9 μM CBD. Moreover, Russo et al. reported that the genotoxic activity of CBD depends on metabolic activation, as the addition of the S9 mixture of liver enzymes increased DNA damage, and they also observed induction of oxidative DNA damage by CBD in HepG2 and TR146 cells. The main difference between the studies was in the method of sample preparation (synthetic vs. plant isolate) and the solvent used for the preparation of

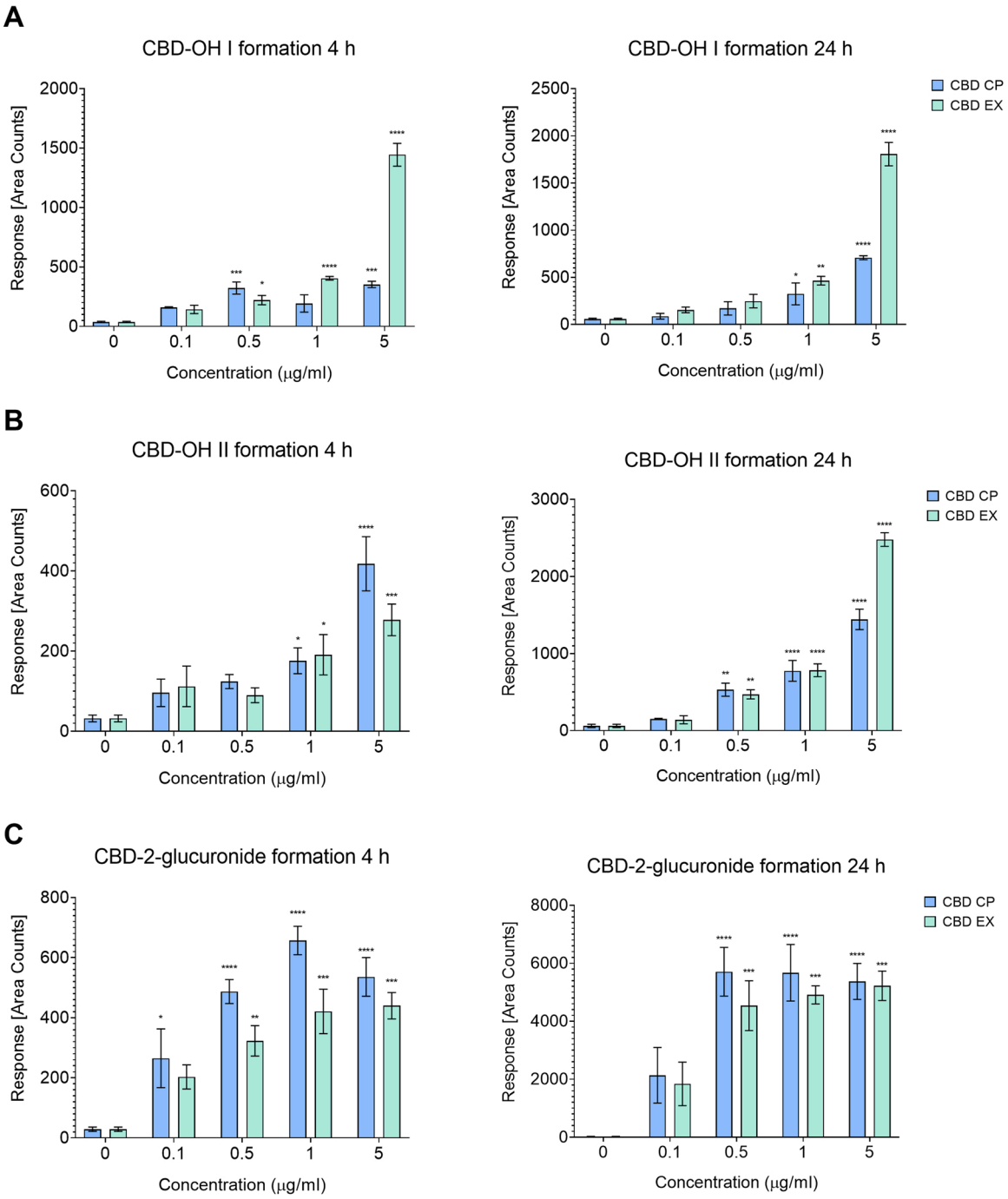

Fig. 4. CBD metabolite detection in HepG2 cell growth media after 4 (left panel) and 24 h (right panel) of cell exposure to CBD CP (blue bars), and CBD EX (green bars). A) the formation of the first group of oxidative metabolites (CBD OH I), B) the formation of the hydroxylated metabolites at ring 2 (CBD-OH II), C) the formation of CBD-2'-glucuronide. 0 is the solvent control (0.2 % EtOH). Asterisks (*) indicate a statistically significant difference (two-way ANOVA and Dunnet's multiple comparison test) between the solvent control and the exposed cells (* $p \leq 0.05$, ** $p \leq 0.01$, ** $p \leq 0.001$, **** $p \leq 0.0001$).

the CBD stock solution. Russo et al. used synthetic CBD dissolved in methanol, with a relatively high concentration of solvent in the final treatment solutions (1.70 % for 3 h and 0.52 % for 24 h treatment), while we used plant-based samples and ethanol as solvent, with a much lower final solvent concentration (0.2 %). However, the reasons for the diverging outcomes in a similar experimental system are not clear. In line with our results, another *in vitro* study applying the comet assay reported no induction of DNA damage in colon cancer cells Caco2 after exposure to 10 µM CBD after 24 h of exposure [54]. *In vivo* studies, on the one hand, reported no induction in DNA damage in rat liver after CBD administration at doses up to 500 mg/kg-bw/day [35], and on the other hand, reported notable DNA damage in sperm cells but not in leukocytes in male Swiss mice that received CBD at doses up to 30 mg/kg-bw for 34 consecutive days by gavage [55].

Furthermore, in the present study, CBD CP and CBD EX did not induce DNA DSBs in HepG2 cells after 4 and 24 h exposure to concentrations of up to 5 µg/ml (Fig. 6A). DNA DSB induction was measured indirectly by measuring the phosphorylated form of the histone H2AX, γH2AX. This histone is phosphorylated in response to DNA DSB induction and accumulates adjacent to DSB sites. Given its rapid and abundant occurrence and its correlation with the number of DSBs, phosphorylation of H2AX is a very sensitive marker for the induction of DNA DSBs

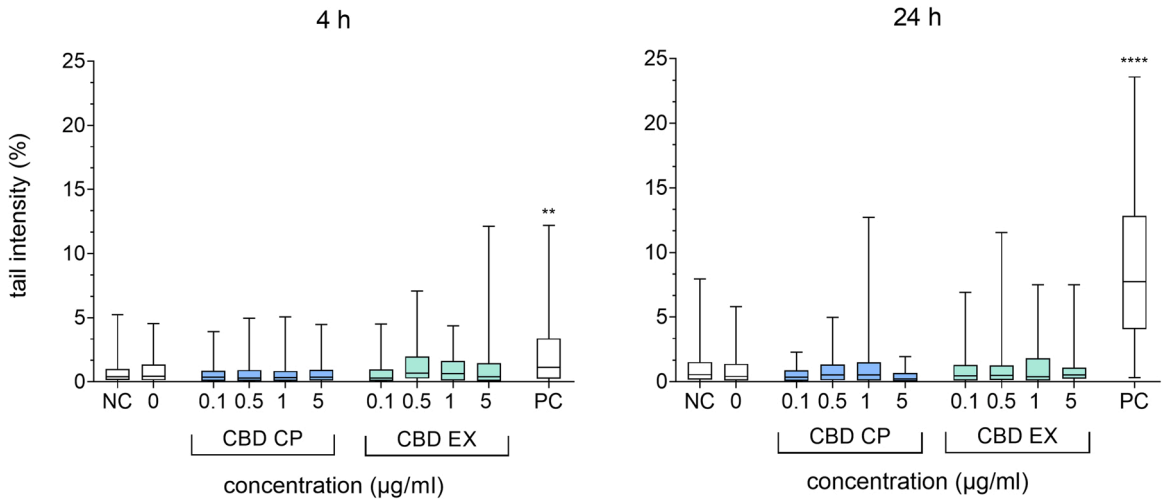

Fig. 5. Induction of DNA damage in HepG2 cells after exposure to CBD CP and CBD EX after 4 and 24 h. Data are expressed as % of DNA in the "comet tail" and presented as quantile box plots (95 % confidence interval). NC is the negative control (growth medium), 0 is the solvent control (0.2 % EtOH), and PC is the positive control (30 µg/ml BaP). Asterisks (*) indicate a statistically significant difference (Kruskal–Wallis nonparametric test and Dunn's multiple comparison test) between the solvent control and the exposed cells (** $p \leq 0.01$, **** $p \leq 0.0001$).

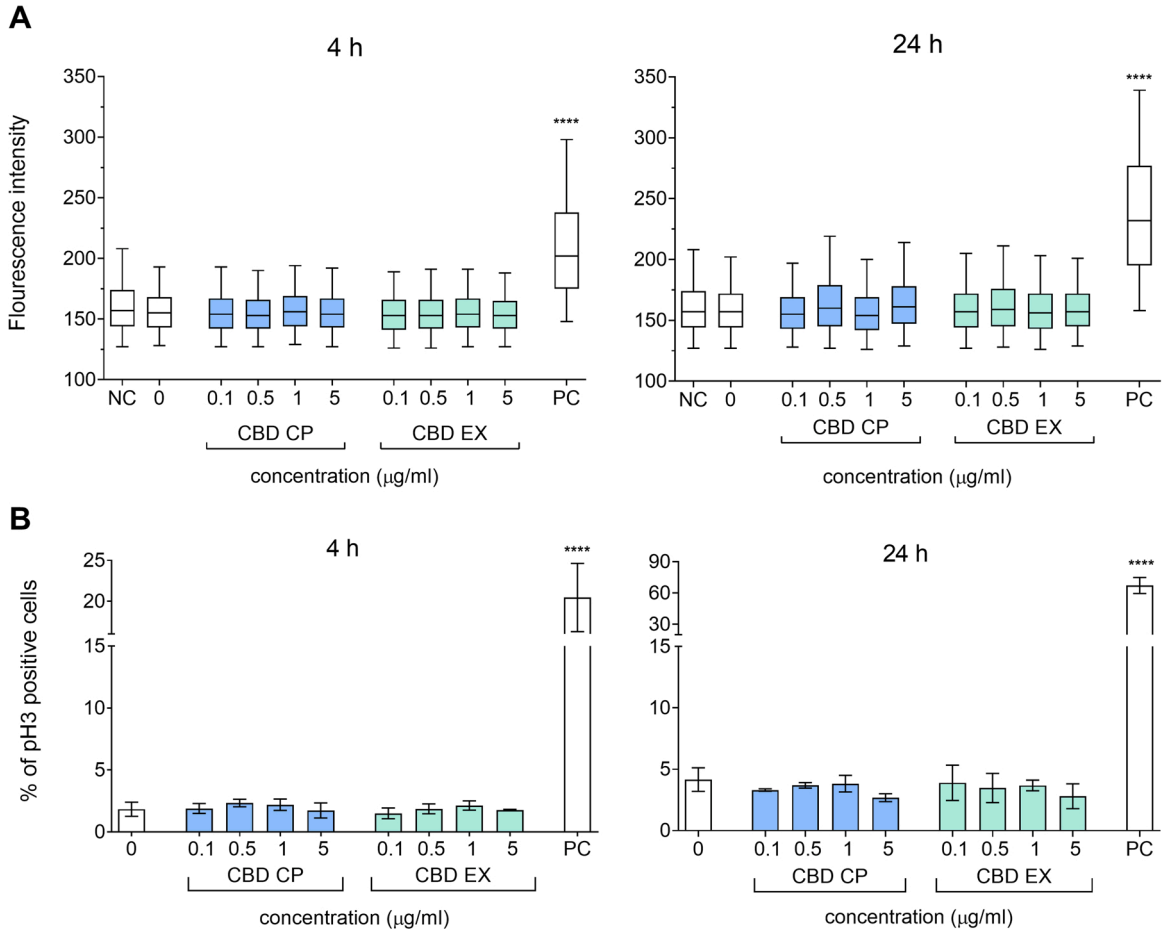

Fig. 6. DNA double strand break induction and potential aneugenic effects of CBD CP and CBD EX in HepG2 cells after 4 and 24 h of exposure. A) Expression of γH2AX a marker of DNA double-strand breaks was determined using flow cytometry. Etoposide (3 for 4 h and 1 µg/ml for 24 h) served as positive control (PC). The distribution of γH2AX APC fluorescence intensity is presented in boxplots (95 % confidence interval) and significant differences between treated samples and the solvent control (0; 0.2 % EtOH) were tested using two-way ANOVA with uncorrected Fisher's LSD. Statistically significant differences were determined using ANOVA and Dunnet's multiple comparison test, *** $p < 0.001$. B) Increase in the phosphorylation of the histone H3 (pH3) was determined using flow cytometry. Colchicine (0.5 µM) served as a positive control (PC). Results are presented as mean ± SD (N=3). Statistically significant differences in H3 phosphorylation (ANOVA and Dunnet's multiple comparison test) **** $p < 0.0001$.

[56], one of the most severe forms of DNA damage, that can lead to/cause clastogenic effects and, as a result, structural chromosomal aberrations. Clastogenic effects can manifest as visible changes in the structure of chromosomes, affecting genetic stability and potentially leading to mutations or other cellular dysfunctions. Aneugenic effects, on the other hand, result in alterations in the number of chromosomes within a cell, e.g. as a result of disruption of spindle fibres or the mitotic apparatus, preventing the accurate separation of chromosomes, potentially leading to genetic disorders or cellular dysfunction. The combined detection of γH2AX and mitotic cell (phospho-histone H3-positive event; p-H3) readings enables the distinction between clastogenic and aneugenic effects [57]. The aneugenicity of the tested CBD samples was assessed by measuring the increase in phosphorylation of histone H3 (specifically phosphorylation at serine 10, p-H3), a marker associated with mitotic chromatin condensation and chromosome segregation during cell division, and thus representing mitotic cells. Aneugenic agents or conditions that interfere with proper chromosome segregation during mitosis can affect the dynamics of histone H3 phosphorylation. Therefore, the measurement of p-H3-positive events can serve as an indicator of mitotic disturbances caused by aneugenic agents [29]. Our results show that neither CBD CP nor CBD EX affected phosphorylation of the H3 histone in HepG2 cells after 4 and 24 h exposure to concentrations of up to 5 μg/ml (Fig. 6B), indicating that the tested CBD samples have no aneugenic activity.

The results of the γH2AX and p-H3 tests were further confirmed by the cytokinesis-block micronucleus (CBMN) assay, as no changes in genomic instability were detected after 24 h of exposure to any of the tested CBD samples. The CBMN allows the detection of micronuclei (MNi), which result from chromosome breakage or loss of entire chromosomes, and other nuclear abnormalities including nucleoplasmic bridges (NPBs), which reflect dicentric chromosomes, and nuclear buds (NBUDs), which are the result of gene amplification. No significant increase in MNi, NBPs or NBUDs was observed after 24-h exposure to CBD CP and CBD EX at concentrations up to 5 μg/ml (or 15.9 μM of CBD in the CBD CP sample) (Fig. 7). Also, no impact on the nuclear division index (NDI) was observed, confirming that the tested concentrations were not cytotoxic and did not affect cell division.

Again, Russo et al. [41] reported different findings. The authors reported CBD-induced MNi in HepG2 cells at low concentrations (≥ 0.22 μM), which are in the range of levels found in the blood people using CBD products and in addition, a significant increase in NBUDs and NPBs was observed already after 3 h of exposure. On the other hand, the authors observed induction of cell death (necrosis and apoptosis), even at low concentrations, used for the genotoxicity assessment, which is contradictory to the cytotoxicity results, as the authors reported no changes in cell viability at concentrations of up to 54 μM. Similarly, an *in vivo* study reported positive CBMN assay results. Zimmerman and Raj [58] explored the induction of MNi and chromosomal aberrations in mouse bone marrow post-treatment with 10 mg/kg CBD. A 3–5-fold increase in MNi was observed compared to the untreated control. However, this study does not provide information on the source and purity of the compound tested, nor does it take into account possible

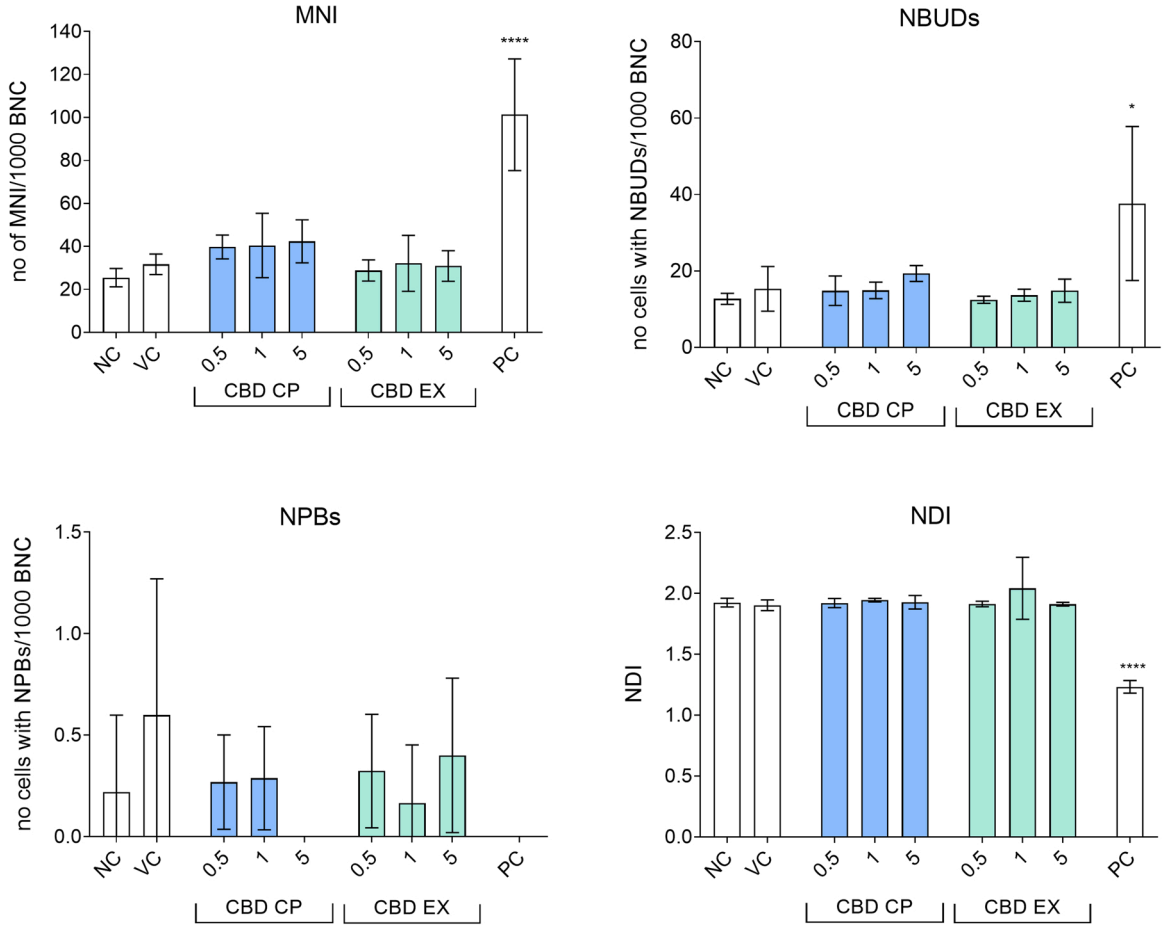

Fig. 7. Influence of CBD CP (blue) and CBD EX (green) on genomic instability of HepG2 cells. The frequency of micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) per 1000 binucleated cells (BNCs), and the nuclear division index (NDI) in HepG2 cells after 24 h of exposure to graded concentrations of CBD CP and CBD EX are shown. NC is the negative control (growth medium), 0 is the solvent control (0.2 % EtOH), and PC is the positive control (ET; 0.5 μg/ml). Asterisks (*) indicate statistically significant differences (ANOVA and Dunnet's multiple comparison test) between the solvent control and cells exposed to the test compound (* $p \leq 0.05$; **** $p \leq 0.0001$).

effects on erythropoiesis, which could contribute to false positive results.

In line with our results, several *in vitro* and *in vivo* studies reported negative results in the CBMN assay. No MNi induction was found in TK6 cells after 4 h of exposure with and without metabolic activation as well as after 27 h of exposure, and no increase in MNi was found in polychromatic erythrocyte from male or female Sprague Dawley rats for any CBD dose level tested, up to 1000 mg/kg-bw/d [34]. The review of Epidiolex's non-clinical safety summarized an *in vivo* study, reporting no MNi induction MNi in rats after exposure to a CBD isolate up to 500 mg/kg-bw/d [35]. However, the study did not provide reasoning behind the selection of the maximum dose at 500 mg/kg and lacks confirmation of whether CBD reached the bone marrow compartment. Negative findings for the CBMN were reported also by Marx et al., who tested a hemp extract containing approximately 25 % CBD, in mice at concentrations of up to 2000 mg/kg-bw/d [36].

In addition to the evaluation of the above-described endpoints, toxicogenomic analyses were performed to identify gene expression changes associated with a (geno)toxicological outcome. Recently, it has been proposed that gene expression profiles could serve as valuable tools for assessing human health risks. They offer not only qualitative but also quantitative insights into the specific mechanism of action) triggered by the test compound [59]. Compounds having similar biological activities, such as genotoxicity, can deregulate the expression of particular genes. This attribute can thus aid in distinguishing between various molecular mechanisms of action among the tested compounds. In the present study, the expression of selected DNA damage-responsive genes (*TP53, MDM2, GADD45a, CDKN1A, CHEK2, MYC and OGG1*) and genes involved in apoptosis (*BAX, BCL2* and *BBC3)* and cell proliferation (*Ki67*) upon exposure to CBD CP and CBD EX was evaluated. These genes encode proteins that play vital roles in the DNA damage response. Upon sensing DNA damage, TP53, known as the "guardian of the genome," activates a cascade of downstream targets that induce cell cycle arrest, DNA repair, and other pro-survival pathways, and on the other hand, elimination of damaged cells [60,61]. MDM2 modulates TP53 activity and degradation, GADD45α assists in repair and cell cycle regulation, while *CDKN1A*, the major TP53-regulated gene, encodes $P21^{Cip1/Waf1}$ that halts cell cycle progression, allowing time for DNA repair. CHEK2 is one of the checkpoint kinases that initiate cell cycle arrest post-damage. MYC, which is also involved in the regulation of GADD45α, influences repair pathways, while OGG1 aids in repairing oxidative DNA damage [62]. BBC3 is involved in TP53-mediated apoptosis regulation in response to DNA damage and interacts with the anti-apoptotic protein BCL2 and potentially also with pro-apoptotic proteins such as BAX [63]. Ki67, on the other hand, serves as a proliferation marker, indicating active cell division [64]. Together, these genes intricately coordinate to maintain genomic integrity, orchestrating responses to DNA damage and cell cycle regulation. None of the tested genes was significantly deregulated in HepG2 cells following exposure to CBD CP or CBD EX (Fig. 8 and Table S8), providing further confirmation that the tested CBD samples did not induce DNA damage in HepG2 cells under the applied conditions.

In conclusion, our comprehensive assessment of the mutagenicity and genotoxicity of CBD CP and CBD EX indicates that both CBD samples were non-mutagenic in the Ames assay and non-genotoxic and did not affect genomic instability at non-cytotoxic concentrations in HepG2 cells under the conditions applied in the study. Collectively, the obtained information offers crucial insights essential for the assessment of the safe consumption of CBD in food and dietary supplements.

Ethics

No human participants and/or animals were involved in the study. The human liver-derived cell line HepG2 (HB-8065™), obtained from the ATCC-Cell bank, was used in the study.

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CRediT authorship contribution statement

Matjaž Novak: Supervision, Methodology, Investigation, Formal analysis, Data curation. **Alja Štern:** Writing – original draft, Visualization, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Bojana Žegura:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Conceptualization. **Metka Filipič:** Writing – review & editing, Resources, Funding acquisition. **Tjaša Šentjurc:** Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Sonja Žabkar:** Software, Methodology, Investigation, Formal analysis, Data curation. **Jurij Trontelj:** Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Katja Kološa:**

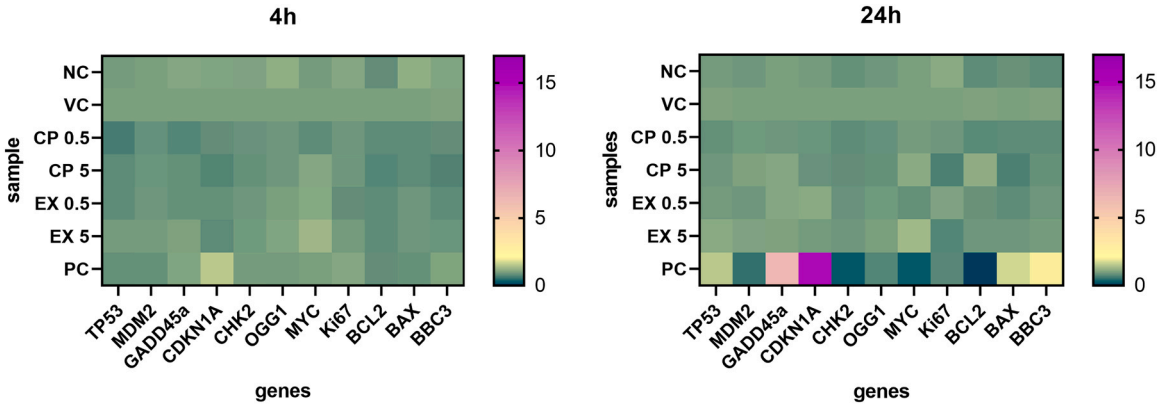

Fig. 8. Heatmap showing the induction of selected genes involved in DNA damage response, apoptosis induction and proliferation. Fold change deregulation of gene expression after exposure to CBD CP and CBD EX (0.5 and 5 μg/ml) for 4 (left) and 24 h (right) in comparison to the vehicle control (VC; 0.2 % EtOH) is presented in the form of heatmaps. NC is the negative control (growth medium), and PC is the positive control (30 μg/ml BaP). VC fold-change value is 1 and is represented in light green color. Down-regulation is indicated by darker green color, while up-regulation is indicated by lighter green colour and changes to jelow and purple, as shown in the scale bar.

Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation.

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.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.116969.

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