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A NEW APPROACH TO FULL-SPECTRUM CANNABIDIOL TESTING: *IN VITRO* AND *IN VIVO* TOXICITY STUDIES

O NOUĂ ABORDARE A TESTĂRII ÎNTREGULUI SPECTRU DE CANABIDIOL: STUDII DE TOXICITATE *IN VITRO* ȘI *IN VIVO*

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ABSTRACT | REZUMAT

Cannabidiol oil (CBD) has a wide array of uses in both human and veterinary medicine, as it has anti-epileptic, analgesic, anxiolytic, and cardioprotective effects, along with its potential to mediate oncological side effects. Its use in veterinary medicine is becoming more and more prevalent as more studies are conducted on the various properties of CBD, especially in oncological therapies. It is of utmost importance to be certain that a product is safe for the patient by characterising the product and conducting both *in vivo* and *in vitro* toxicity studies, as well as a heavy metal analysis. This study was aimed at *in vitro* cytotoxicity studies on cell cultures as well as *in vivo* toxicity studies on rats in order to determine the potential toxicity of a novel cannabidiol product. The *in vitro* cytotoxicity study was conducted on rat cardiac myocyte and fibroblast cultures. For the assessment of the proliferation potential and cytotoxic effect of CBD oil, confluent cultures were treated with 5 different dilutions of CBD full spectrum oil (5, 10, 20, 25, and 30 µl). After 24 hours of exposure, cell viability was determined by an MTT test. This test reflects the normal functioning of the mitochondria and, hence, cellular viability. The acute toxicity study was conducted on 7 rats, who were given fixed doses ranging from 5 mg/kg to 2000 mg/kg and monitored for 14 days. The results obtained from the *in vitro* study indicated that no concentration showed any signs of cytotoxicity; at 20 and 25 µg dilutions, statistically significant cellular proliferation was observed, and at a 30 µg dilution, both cellular viability and proliferation were observed. Heavy metals were found to be minimal, under the threshold of allowed quantities. From the *in vivo* study, it was concluded that even at extremely high doses, the product does not cause any acute toxicity. This cannabidiol product, based on these toxicity results, is deemed to be well tolerated with no acute toxicity.

Keywords: acute toxicity, cannabidiol, cytotoxicity, MTT, heavy metals

Uleiul de canabidiol (CBD) are o gamă largă de utilizări atât în medicina umană, cât și în medicina veterinară, deoarece are efecte antiepileptice, analgezice, anxiolitice și cardioprotectoare, alături de potențialul său de a media efectele secundare oncologice. Utilizarea sa în medicina veterinară devine din ce în ce mai răspândită, pe măsură ce se realizează mai multe studii privind diferitele proprietăți ale CBD, în special în terapiile oncologice. Este extrem de important să avem certitudinea că un produs este sigur pentru utilizarea in beneficiul pacientului, prin caracterizarea produsului și efectuarea de studii de toxicitate *in vivo* și *in vitro*, precum și a unei analize a metalelor grele. Acest studiu a vizat studii de citotoxicitate *in vitro* pe culturi celulare, precum și un studiu de toxicitate *in vivo* pe șobolani, pentru a determina potențialul toxic al unui nou produs cu canabidiol. Studiul de citotoxicitate *in vitro* a fost efectuat pe culturi de miocite cardiace și fibroblaste de șobolan. Pentru evaluarea potențialului de proliferare și a efectului citotoxic al uleiului de CBD, culturile confluente au fost tratate cu 5 diluții diferite de ulei CBD cu spectru complet (5, 10, 20, 25 și 30 µl). După 24 de ore de expunere, viabilitatea celulară a fost determinată printr-un test MTT. Acest test reflectă funcționarea normală a mitocondriilor și, prin urmare, viabilitatea celulară. Metalele grele s-au dovedit a fi minime, sub pragul cantităților admise. Studiul de toxicitate acută a fost efectuat pe 7 șobolani, cărora li s-au administrat doze fixe cuprinse între 5 mg/kg și 2000 mg/kg și au fost monitorizați timp de 14 zile. Rezultatele obținute în urma studiului *in vitro* au indicat faptul că nicio concentrație nu a prezentat semne de citotoxicitate; la diluțiile de 20 și 25 µg, s-a observat o proliferare celulară semnificativă din punct de vedere statistic, iar la o diluție de 30 µg, s-au observat atât viabilitate cât și proliferare celulară. Din studiul *in vivo*, s-a concluzionat că, chiar și la doze extrem de mari, produsul nu provoacă nicio toxicitate acută. Pe baza acestor rezultate de toxicitate, se consideră că acest produs cu canabidiol este bine tolerat, fără toxicitate acută.

Cuvinte cheie: toxicitate acută, cannabidiol, citotoxicitate, MTT, metale grele

Cannabidiol (CBD), the primary non-psychoactive constituent of the *Cannabis sativa* plant, has shown an extensive variety of therapeutically promising pharmacological impacts, either on its own or as an adjuvant therapy to different medications. CBD is not to be confused with tetrahydrocannabinol (THC), which is the main psychoactive constituent of the *Cannabis sativa* plant and still continues to be a banned substance in most countries. CBD does not contain addictive properties and, therefore, cannot be

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considered a substance of abuse (2). At the present time, there is an extraordinary interest in the many possible clinical applications of cannabidiol. A useful pharmacological examination on CBD happened during the 1970s by Jones et al, and has been strengthened as of late with numerous disclosures about the endocannabinoid framework (18). Various preclinical and clinical examinations prompted FDA-endorsement of Epidiolex®, a refined CBD medication planned for the treatment of juvenile epileptic conditions, by the US Food and Drug Administration in 2018 and then again in 2020 for another juvenile epileptic condition. Preclinical and clinical examinations likewise report potential benefits, therapeutic uses as well as, adverse effects (AEs), and intoxications following CBD consumption in various pathologies (1, 6, 29). The use of CBD is becoming more and more widespread, especially in veterinary medicine (10, 27). Doses in veterinary medicine vary greatly in correlation with the pathology. They are better documented in dogs than in other species. The starting doses are generally small and are titrated to a clinical dose-dependent therapeutic effect. The dose is not collectively agreed upon in the literature; however, 2-2.5 mg/kg as a starting dose seems to be used in studies concerning osteoarthritis and epilepsy in dogs (19).

In Romania, there are less than 10 products on the market of CBD produced locally, none of which are destined for veterinary use, and the demand is high for concentrated products that require lower quantities of product to be administered. Many veterinarians use CBD products destined for human use "off-label" for animal patients (15). This study highlights the potential acute safety of a concentrated cannabidiol oil product for animals. It is an exciting time for cannabidiol, as studies in the literature have stated its therapeutic potential in a broad range of pathologies, both in human and veterinary medicine, as an antiepileptic (14), anxiolytic (9), antioxidant and anti-inflammatory (1), analgesic in osteoarthritic pain (27) and neuropathic pain (6), cardioprotector (12), and neuroprotector (18). In the literature, there are many studies advocating for the use of CBD as mono or combined therapies; however, there are also studies stating potential toxicities and adverse effects (15), such as neurotoxicity and hepatotoxicity, hypotension, and reproductive system alterations in various species, including humans. However, the results in animal studies are limited, and none have evaluated a full-spectrum CBD product. Thus, the main aim of this research project is to determine the potential toxicity of a highly concentrated CBD product by means of heavy metal analysis and *in vitro* and *in vivo* toxicity studies.

MATERIALS AND METHODS

Characterization and heavy metal analysis

The characterization of the CBD product (Seva Elementals SRL, Romania) was undergone in a specialised laboratory in Venlo, Holland, at Brightlabs, by means of High-Performance Liquid Chromatography with ultraviolet spectroscopy (HPLC-UV), whereas the heavy metal analysis was performed in Bucharest, Romania at the Eurofins Evic Product Testing Romania SRL laboratory by means of Inductively Couples Plasma Mass Spectroscopy.

In vitro cell cultures

The *in vitro* toxicity study was conducted on cardiac myocytes and fibroblasts, cell lines that were harvested from foetal rats from pregnant females at 12.5 days of gestation, as stated in the literature. For the assessment of the proliferation potential and cytotoxic effect of CBD oil, confluent cultures were treated with five different dilutions of CBD. The tests were carried out on plates of 96 wells in triplicate, and the concentration of cells was $1x10^5$. After 24 hours of exposure, cell viability was determined by an MTT test. The negative control was the untreated culture. The MTT test is based on the detection of the reduction of a chemical compound called MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide] by mitochondrial dehydrogenase with the formation of formazan. The process reflects the normal functioning of the mitochondria, and hence, cellular viability. To obtain cell suspensions, the cells were treated with 0.25% trypsin-EDTA, and after centrifugation (1500 rpm for 5 min), there were $1x10^4$ cells/well seeded on plates with 96 wells in 200µl full propagation medium. After 24 hours, the predetermined concentrations of CBD mentioned above were added. The control samples were represented by untreated cells. The tests were carried out in triplicate.Cell proliferation was analysed after 24 hours, the culture medium was removed, and an amount of 100 µl of MTT solution (1 mg/ml) was added (Sigma-Aldrich, St. Louis, MO, USA). After 3 hours of incubation (myocytes) and 4 hours of incubation (fibroblasts) at 37 °C in the dark, the MTT solution was removed from each well, and 150 µl of DMSO (dimethyl sulfoxide) solution (Fluka) was added. The intensity of the chromogenic reaction was measured, spectrophotometrically at 450 nm using the BioTek Synergy 2 spectrophotometer (Winooski, VT, USA). Cytotoxicity was expressed as a percentage of the control. The results obtained from the MTT test were processed as an average value ± standard deviation and subsequently presented as a viability percentage (%), which is the ratio of the mean value of the optical densities recorded for each product tested at the mean optical density determined for the control (untreated cells, grown under the same standard conditions).

In vivo experiment: Experimental Protocol

The toxicity study was an acute, fixed-dose study that followed OECD 420 guidelines (26). 1 rat received paraffin oil as a control, while the other 6 rats received doses of 5 mg/kg, 50 mg/kg, 300 mg/kg, 600 mg/kg, 1000 mg/kg, and 2000 mg/kg of CBD on the first day of the study by oral gavage. The animals were monitored for 30 minutes, 2 hours, 24 hours, and then daily after receiving cannabidiol. They were then humanely euthanized after 14 days, in accordance with European directives. Necropsies were performed on each subject, and specific tissue samples were obtained (brain, spleen, liver, and kidneys).

Animals and Bioethics

The animals used in this experiment were procured from an accredited institution (Cantacuzino Institute, Bucharest, Romania). In the week prior to the experiment, the animals were left to acclimatise to the new conditions. Their environment was standard in terms of temperature and humidity, with a 12-hour light/dark cycle. The rats received a standardised diet (Cantacuzino Institute, Bucharest, Romania) and fresh water ad libitum. The experimental protocol is in accordance with European directives set in place, with the approval number 366/09.06.2023.

Clinical

The animals were examined clinically (weight, temperature, respiratory rate, and cardiac frequency) before the doses of cannabidiol, after 30 minutes of administration, after 2 hours, and after 24 hours. This is per the OECD toxicity protocol. The rats were also weighed daily throughout the 14-day period, because a weight loss greater than 10% of total body weight is an indication of toxicity (7).

Histopathology

Tissue samples were harvested according to the recommendations of the Society of Toxicologic Pathology from Europe (ESTP). Hepatic samples from the left lateral lobe and right medial lobe were taken; half of the right and, respectively, left kidneys were also harvested; and half of the spleen and a sagittal section from the temporal lobe of the brain were also harvested. The samples were fixed in 10% buffered neutral formalin and embedded in paraffin. The sections were made at 4 µm, and the slides were stained with the Haematoxylin-Eosin (H&E) method. The slides were examined under a BX51 Olympus microscope (Olympus America Inc., Melville, NY, USA), and images were taken with an Olympus UC 30 digital camera (Olympus America Inc., Melville, NY, USA) and processed using the Olympus basic stream software. All tissue sections have been examined by an independent observer blinded to the experimental protocol.

Haematology and Biochemistry

On day 14 of the experiment, the rats were humanely euthanized by dislocation at the cervical level. A necropsy was performed to reveal possible macroscopic lesions visible in various organs. Blood samples were also collected from the retro-orbital sinus for haematological and biochemical analyses. For the biochemical examination, spectrophotometric dosing with the MasterTouch (Hospitex International) spectrophotometer was performed. The enzymes were dosed spectrophotometrically in the ultraviolet field at a wavelength of 396 nanometres with kits from AMP Diagnostics (Graz) by the enzymatic method. Urea was also dosed by the fixed-time spectrophotometric method in the ultraviolet field with a kit of the same origin, and creatinine was dosed in the visible field with a corresponding kit of the same origin. The haematological examinations were performed using whole blood with the Abacus Junior Vet 5 machine (Diatron, Hungary).

RESULTS AND DISCUSSIONS

Characterization

The 20% full-spectrum cannabidiol product contains 203.7 mg of CBD per 1 ml of suspension, and thus 10.185 mg of CBD per one drop of suspension, making it highly concentrated compared to other products on the market. By HPLC-UV examination, concentrations of all of the phytocannabinoids contained in the product were determined (Table 1). The concentration stated on the bottle is the concentration contained in the product.

Table 1

Complete HPLC characterization of the phytocannabinoids

Compound	Results (%)
CBDV (cannabidivarin acid)	0.07
CBDA (cannabidiol acid)	<0.005
CBGA (cannabigerol acid)	<0.005
CBG (cannabigerol)	0.11
CBD (cannabidiol)	20.37
THCV (tetrahydracannabivarin)	<0.005
CBN (cannabinol)	0.20
DELTA9-THC (Δ9-tetrahydrocannabinol)	0.01
DELTA8-THC (Δ8-tetrahydrocannabinol)	<0.005
CBC (cannabichromene)	0.39
THCA (tetrahydrocannabinol acid)	<0.005

Heavy metal analysis

The accumulation of heavy metals in plant-based pharmaceutical preparations is of concern, as those heavy metals can then be amassed in the body, especially with chronic administrations of plant-based supplements, causing potential side effects. It was therefore important to test the product for the presence of heavy metals, especially a cannabidiol product known to bioaccumulate heavy metals. As noted in Table 2, all of the tested heavy metals are well under the maximum allotted limits as set at a European level under EU Directive 2021/1323.

Table 2

Heavy metal analysis

Heavy Metal Tested	Result	Units
Aluminum (Al)	<2.5	mg/kg
Arsenic (As)	<0.01	mg/kg
Cadmium (Cd)	<0.005	mg/kg
Mercury (Hg)	<0.005	mg/kg
Nickel (Ni)	<0.01	mg/kg
Lead (Pb)	<0.01	mg/kg

In vitro cell cultures

Cardiac Myocytes

An MTT test was conducted after 24 hours with CBD oil in dilutions of 5, 10, 20, 25, and 30 µg. As seen in Fig. 1, no dilution showed any signs of cytotoxicity, whereas at 20 and 25 µg dilutions, statistically significant cellular proliferation was observed, at 107.25% and 107.64%, respectively ($p<0.0001$). Moreover, at a 30 µg dilution, both cellular viability and proliferation were observed (106.11%). Therefore, it can be stated that CBD oil presents little to no *in vitro* cytotoxicity on cardiomyocytes; moreover, it actually enhances cellular growth. The preliminary *in vitro* safety of this product tested on rat embryonic heart cells has not only been confirmed in favour of the absence of toxicity of the product, which would also appear to be responsible for an increase in the proliferation of the cell population tested.

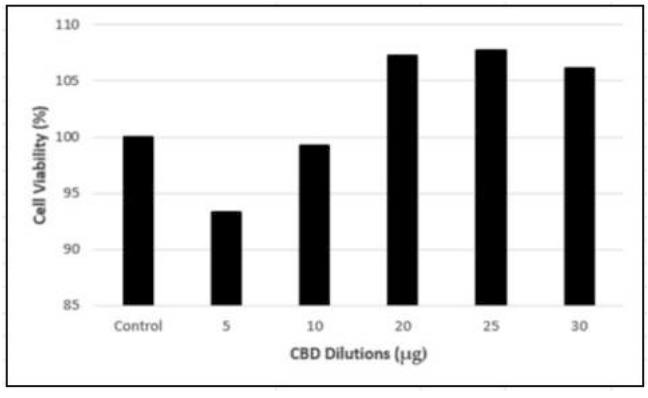

Fig. 1. Cellular viability results of CBD on cardiac myocyte cell cultures expressed as percentages for 5 different dilutions of CBD oil

Fibroblasts

At 10,15,20, and 25 µg dilutions, statistically significant cellular proliferation was observed, at 118.73%, 117.43%, 113.22%, and 114.92%, respectively ($p< 0.05$).

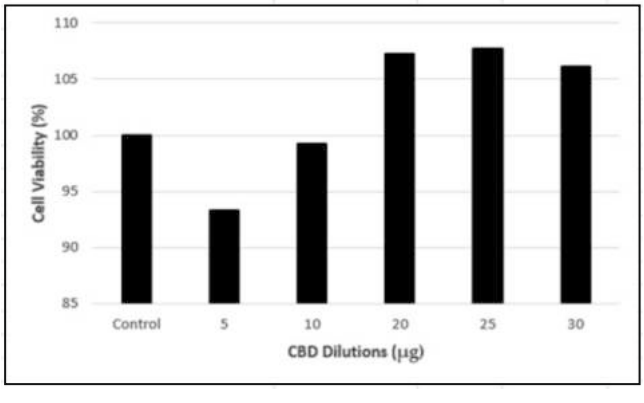

Fig. 2. Cellular viability results of CBD on fibroblast cell cultures expressed as percentages for 5 different dilutions of CBD oil

At all dilutions mentioned above, except 5µg, both cellular viability and proliferation were observed (Fig. 2). At the 5µg dilution, cellular viability rules out the pre-sence of product toxicity; however, the test did not show cellular proliferation. This confirms the absence of toxicity in the product, which would also appear to be responsible for an increase in the proliferation of the cell population tested.

In vivo experiment

Clinical examination

Prior to the administration of CBD, baseline values for weight and vitals were taken for each animal. Behaviour was also noted for each rat. The doses were calculated according to weight and administered by oral gavage. Two hours post-administration, some rats showed somnolence and decreased appetite, specifically the subjects that received the highest doses. By 24 hours, the animal's behaviour and appetite had returned to normal. The body weights of the rats decreased slightly within the first 24 hours, possibly due to the stress of manipulation, and then generally showed an upward trend from 24 hours onwards until the end of the experiment.

Haematology and biochemistry

No pathological changes to the haematological parameters compared to the reference ranges were observed in correlation with the administration of CBD oil. All parameters were within normal limits. Furthermore, based on the results obtained in Table 3, no changes were observed in the biochemical parameters that would be related to the administration of CBD oil. All parameters were within normal limits.

Table 3

Biochemistry results taken at 14 days

	ALAT (U/l)	ASAT (U/l)	GGT (U/l)	ALP (U/l)	BUN (mg/dl)	CREAT (mg/dl)	Dose Received
Control	29	82.4	3.6	402.6	16.1	0.48	-
S1	36.6	88.12	4.3	201.1	18.2	0.55	5 mg/kg
S2	18.3	79	3.5	231.8	16.6	0.54	1000 mg/kg
S3	13.8	38.9	7.2	230	18.4	0.59	300 mg/kg
S4	35.7	44.3	2.2	463	17.9	0.52	600 mg/kg
S5	32	72.7	1.4	326.2	15.8	0.43	2000 mg/kg
S6	25.7	75.4	6.3	508.3	19.3	0.49	50 mg/kg

*Normal Values: ALAT – alanine aminotransferase (19-48 U/L), ASAT - aspartate-transaminase (63-175 U/L), GGT - gamma-glutamyl transaminase (0,5-5,3), ALP–alkaline phosphatase (95-611 U/L),BUN –blood urea nitrogen (10.7-20 mg/dL), CREAT –creatinine (0.2-0.7 mg/dL)

Histopathology

The organs were removed and examined and showed no outward signs of organ damage, nor did they show any visible increase in size. In the samples of the brain, spleen, liver, and kidneys (Figures 3-6) that were stained with an H&E stain and examined microscopically, there was no evidence of any lesions that could signal the tested CBD oil's possible organ toxicity. This means that there were no lowered cell numbers specific to each organ, nor was there presence of fibrosis and/or necrosis within the organs.

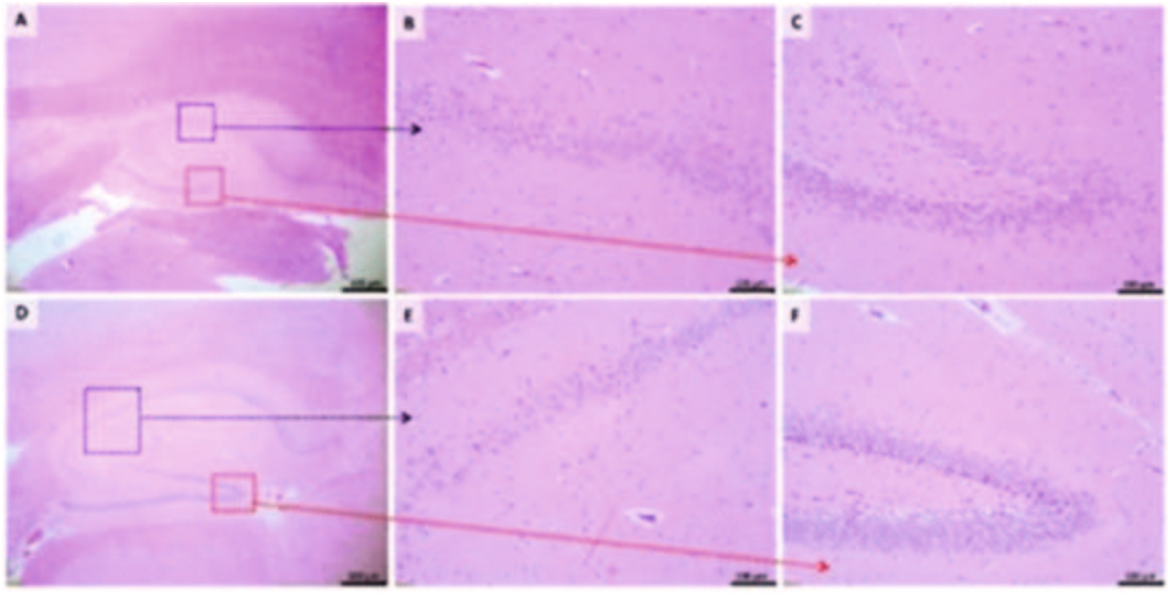

Fig. 3. Histology of the hippocampus of the brain (H&E stain). A, B, C denote samples from the rat that received a dose of 2000 mg/kg, compared to D, E, and F, which denote samples from the rat that received 50 mg/kg.

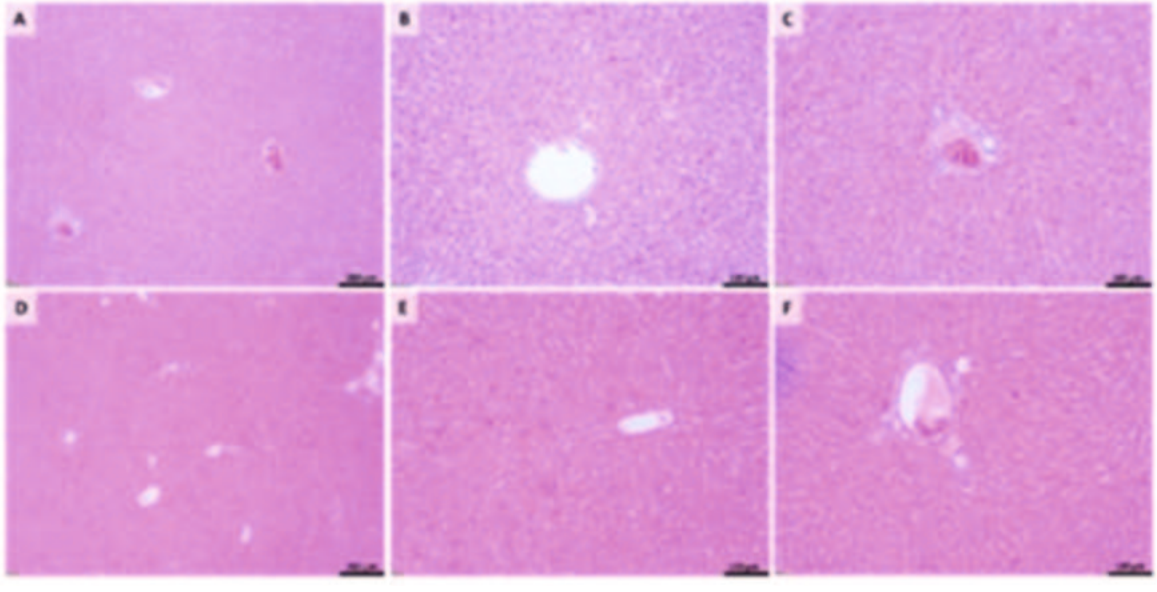

Fig. 4. Histology of the liver (H&E stain). A, B, and C denote samples from the rat that received a dose of 2000 mg/kg, compared to D, E, and F, which denote samples from the rat that received 50 mg/kg.

Discussion

As the medical communities, both human and veterinary, begin to use cannabidiol more and more, especially in the last decade, new products are being developed rapidly. In order to have peace of mind as a practitioner, safety studies need to be conducted on the products used. In most countries, cannabidiol is classified as a supplement and therefore not as tightly regulated as conventional medications. The exception to this is in the United States of America, where Epidiolex is the only cannabinoid-based medication that has been approved by the FDA and is under tighter regulations. The characterization of products is of the utmost importance, especially in countries where THC-based products are illegal and the quantity of THC in a predominantly CBD-based product must be under a specific threshold. The characterization performed in this study showed that the product contains accurate quantities of cannabidiol, which is the molecule of interest, and very trace amounts of THC. Studies conducted by Miller et al. in 2022 analysed 11 cannabidiol oil products for conformity to labelling and saw many discrepancies between what the stated and actual concentrations of the products were (21). Another study was conducted by Wakshlag in 2020 on commercially available cannabidiol products specifically for veterinary use, and the results showed even more discrepancies between what was stated on the label and what the reality was (29). In this particular study by Wakshlag, heavy metal

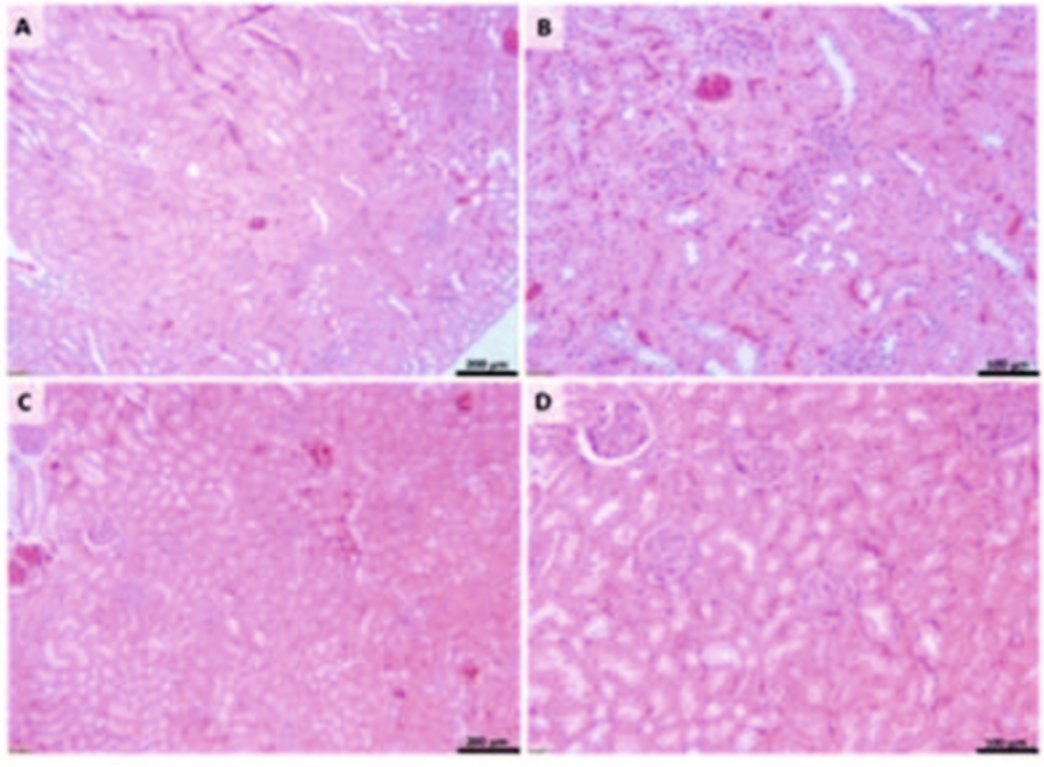

Fig. 5. Histology of the kidney (H&E stain). A and B denote samples from the rat that received a dose of 2000 mg/kg, compared to C and D, which denote samples from the rat that received 50 mg/kg

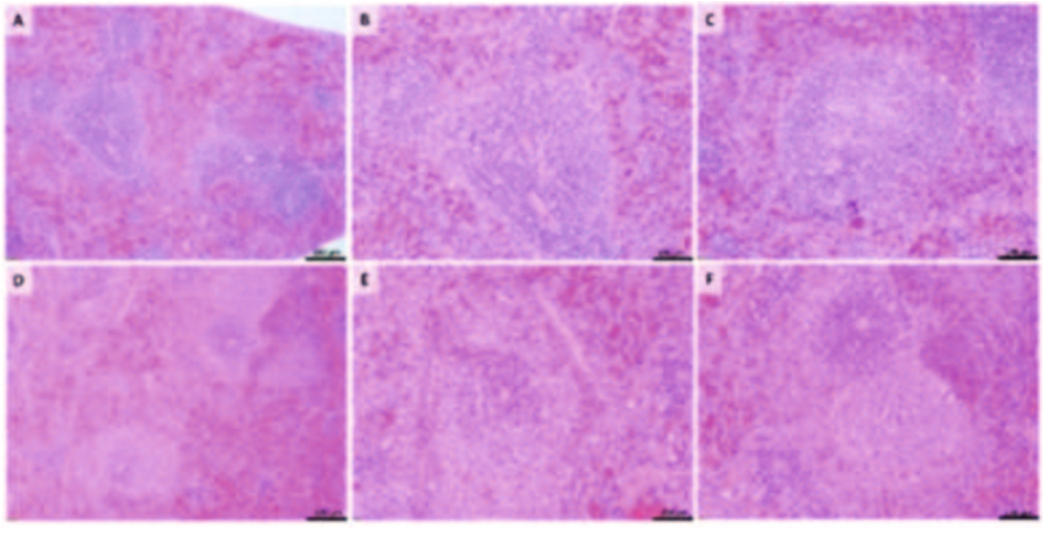

Fig. 6. Histology of the spleen (H&E stain). A, B, and C denote samples from the rat that received a dose of 2000 mg/kg, compared to D, E, F, which denote samples from the rat that received 50 mg/kg

analyses were also conducted, and four of the 29 products analysed were found to be contaminated with heavy metals. Contamination of plant-based products destined for consumers with heavy metals has serious clinical implications, especially in plants such as *Cannabis* spp., which are known to bioaccumulate metals such as arsenic (As), lead (Pb), and mercury (Hb) (3). CBD and THC are used as adjuvant treatments in many serious pathologies, such as cancer and chronic pain (24) where the body is already weakened due to the primary pathology. Heavy metal intoxication from cannabinoid products would have much more severe implications for such patients. Therefore, it is of utmost importance for any manufacturers of cannabinoid products to perform heavy metal analysis and ensure the application of agronomic best practices in order to eliminate metal contaminants (4, 5).

When first developing a new product destined for the pharmaceutical sphere in both human and veterinary medicine, the first step after characterization would be *in vitro* toxicity studies on cell cultures. This method gives

researchers a very good idea of whether or not the molecule can advance to animal/human testing. If a compound is cytotoxic in cell cultures, there are great chances of the product being toxic in real life. Cell cultures are chosen based on the intended route of administration and the target organ(s) of the molecule being tested. For example, in Nirwana's 2021 *in vitro* study on gingival fibroblasts, calcium hydroxide and ellagic acid are tested for cytotoxicity because calcium hydroxide is very often used in endodontic procedures (23). To our knowledge, no cytotoxicity studies such as the present study have been published for CBD on normal cells. Cannabidiol, however, has been shown to be cytotoxic against cancer cells (8, 22). *In vivo* studies on therapeutic molecules are the hallmark for developing new treatment protocols. The literature states that cannabidiol is used in many pathologies with different mechanisms of action. Our interest in the molecule, being an academic institution rather than industrial research centre, is in using the product in the treatment of neuropathy (30), osteoarthritis (13) and epilepsy in dogs (20, 25). However, the literature also states potential side effects in clinical studies, both human and animal (16). Most of these side effects occurred in studies where cannabidiol was not the only substance being used, especially in the case of epilepsy; therefore, some adverse events are the result of drug-drug interactions (11). In clinical studies, CBD appears to increase liver transaminases and be hepatotoxic; however, no evidence of such effects was noted in the present study or our previous clinical study on a dog (16, 25). Because the product tested is a highly concentrated oil, it is much easier to administer to both animals and humans and also much easier to dose correctly. With this study, we confirmed that the cannabidiol product does not present acute toxicity or heavy metal contamination and is relatively safe to use, and these results are in line with our previous clinical study (25).

The limitations of this experiment are mainly time and dosing. Because this was an acute study, it gives us a good idea of the acute safety of the cannabidiol product; however, a longer, chronic study with serial dosing that mimics a real-life dosing regimen would be indicated in order to see if there are adverse effects associated with said product. In veterinary medicine, the general dosing guideline is 2-5mg /kg of body weight, and in human medicine, depending on the pathology, doses range from 25-600 mg/kg. The doses used in this acute study far surpass these regular doses and thus highlight the safety and tolerability of the product.

CONCLUSIONS

The aim of our acute toxicology study was to confirm or deny the safety of use of the full-spectrum CBD product. This study is conclusive that this highly concentrated, full-spectrum CBD product does not show acute *in vitro* or *in vivo* cytotoxicity, even at very large doses. The studies on cell cultures showed both viability and cell proliferation. Furthermore, heavy metals were detected within the allowed range. The post-mortem histopathological study carried out on the liver and kidneys as well as other organs of the various subjects did not show the presence of either storage or morpho-functional alterations of the necrotic type, among others. The safety of the CBD product regardless of the dose, without association with the appearance of undesirable side effects or mortality in the various study subjects, was observed in this study. It was also noted that 100% of the subjects gained weight without any change in clinical status or behavioural parameters. No mortality was revealed, including at the maximal dose of 2000 mg/kg; no toxicity at the organ level was demonstrated by our study at this dosage, indicating relatively high safety of *in vivo* use of the product.

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