Neuroprotective effect of a pharmaceutical extract of cannabis with high content on CBD against rotenone in primary cerebellar granule cell cultures and the relevance of formulations

Short running title: Neuroprotective effect of Epifractan

Carolina Echeverry[1], Analía Richeri[2], Jimena Fagetti[2], Gaby F. Martínez[2], Federico Vignolo[1], Giselle Prunell[1], Leticia Cuñetti[3], Marcela Martínez Busi[4], Sandra Pérez[4], Verónica Sánchez de Medina[5], Carlos Ferreiro[5], Cecilia Scorza[2*]

[1]Laboratorio de Mecanismos de Neurodegeneración y Neuroprotección, Instituto de Investigaciones Biológicas Clemente Estable, Uruguay; [2]Departamento de Neurofarmacología Experimental, **Instituto de Investigaciones Biológicas Clemente Estable, Uruguay**; [3]**Instituto de Nefrología y Urología, Uruguay**, [4]Plataforma de Química Analítica, IIBCE, Uruguay, [5]Phytoplant Research S.L, Spain.

*Corresponding author: Cecilia Scorza, Ph.D. Departamento de Neurofarmacología Experimental, **Instituto de Investigaciones Biológicas Clemente Estable, Uruguay**. E-mail: cscorza@iibce.edu.uy / scorzacecilia@gmail.com

Abstract

Preclinical research supports the benefits of pharmaceutical cannabis-based extracts for treating different medical conditions (e.g., epilepsy); however, their neuroprotective potential has not been widely investigated. Using primary cultures of cerebellar granule cells, we evaluated the neuroprotective activity of Epifractan (EPI), a cannabis-based medicinal extract containing a high level of cannabidiol (CBD), components like terpenoids and flavonoids, and trace levels of ∆9-tetrahydrocannabinol and the acid form of CBD. We determined the ability of EPI to counteract the rotenone-induced neurotoxicity by analyzing cell viability and morphology of neurons and astrocytes by immunocytochemical assays. The effect of EPI was compared with XALEX, a plant-derived and highly purified CBD formulation (XAL), and pure CBD crystals (CBD).

The results revealed that EPI induced a significant reduction in the rotenone-induced neurotoxicity in a wide range of concentrations without causing neurotoxicity *per se*. EPI showed a similar effect to XAL suggesting that no additive or synergistic interactions between individual substances present in EPI occurred. In contrast, CBD did show a different profile to EPI and XAL since a neurotoxic effect *per se* was observed at the higher concentrations assayed. Medium-chain triglyceride (MCT) oil used in EPI formulation could explain this difference.

Our data support a neuroprotective effect of EPI which may provide neuroprotection in different neurodegenerative processes. The results highlight the role

of CBD as the active component of EPI but also support the need for an appropriate formulation to dilute pharmaceutical cannabis-based products, which could be critical to avoid neurotoxicity at very high doses.

Keywords: Cannabis sativa, Epifractan, Xalex, cannabidiol, astrocytes, neurotoxicity.

Introduction

The therapeutic activity of cannabidiol (CBD), a non-psychotomimetic component extracted from *Cannabis sativa,*[1,2] is widely accepted in a wide range of medical conditions.[3,4] Accordingly, several formulations or cannabis-based extracts containing CBD have emerged in the pharmaceutical market.[5] The beneficial effects of these products are fundamentally dependent on the purity, extract preparation, the concentration of CBD, and possibly the presence of other components (e.g., ∆9-tetrahydrocannabinol, Δ9-THC, other cannabinoids, terpenoids, and flavonoids). An example of these pharmaceutical cannabis-based products is Sativex® (GW Pharmaceuticals plc), which contains an equimolecular combination of ∆9-THC and CBD. It has been approved for its use in patients with multiple sclerosis-related spasticity and neuropathic pain.[6,7] Another example is a purified preparation of CBD, also available from GW Pharmaceuticals plc under the name of Epidiolex® (> 98% CBD), prescribed for refractory epilepsy.[8,9] It has been suggested that cannabis extracts could provide advantages over a single phytocannabinoid (i.e., CBD), offering beneficial entourage effects;[5,10] however, more investigation about this effect is required.

Epifractan (EPI), a cannabis-based medicine extract containing a high level of cannabidiol (CBD), terpenoids, flavonoids, and trace levels of ∆9-THC and the acid form of CBD (CBDA), has been approved and recorded by the Ministry of Public Health in Uruguay.[11] Based on the evidence supported by Epidiolex,[8,9] EPI is prescribed for refractory epilepsy in children and adolescents. However, the potential therapeutic use of EPI for the treatment of neurodegenerative processes has not been evaluated so far.

Mitochondrial dysfunction is one of the main mechanisms involved in the neuronal death of several neurodegenerative diseases.[12] Rotenone is a potent natural neurotoxin[13] that has been extensively used to mimic cellular alterations that occur in many neurodegenerative disorders, like Parkinson's disease.[14] Exposure to rotenone results in the inhibition of the mitochondrial electron transport chain of cells leading to a bioenergetic dysfunction, activation of the apoptotic cascade, and cell death.[15] Based on these considerations, the first purpose of the study was to investigate the neuroprotective effect of EPI on an experimental model of mitochondrial dysfunction induced by rotenone in cultures of primary cerebellar granule cells.[16] The second aim of this study was to identify if the neuroprotective activity of EPI was primarily due to its CBD content or to additive or synergistic interactions between other individual components. To assess this, a comparison was performed between the activity of EPI and XALEX (XAL), a plant-derived and highly purified CBD formulation. A purified and crystallized CBD sample (named CBD to clarify) was taken as a reference compound for the study.

Materials and Methods

Animals

Wistar rat pups 6-8 days old were employed (IIBCE animal facilities, Montevideo). Pups were housed with their mothers in a light- and temperature-controlled room with a 12-h light/dark cycle (lights on at 7:00 am). All animals procedures were carried out following the IIBCE Bioethics Committee's requirements following the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978), and under the current ethical regulations of the national law on animal experimentation Nº18.611. Adequate measures were taken to minimize the discomfort or stress of the animals, and all efforts were made to use the minimal number of animals necessary to produce reliable scientific data.

Pharmaceutical cannabis-based products and CBD

Epifractan (EPI; a cannabis-based medicinal extract containing a high level of CBD, terpenoids, and flavonoids, and trace levels of the CBDA and ∆9-THC), and XALEX (XAL; a plant-derived, highly purified CBD formulation) were kindly provided by RAMM Pharma Corp., Uruguay. Both pharmaceutical products were registered by the Ministry for Public Health in Uruguay. EPI (5 %) contains 50 mg/ml of CBD, less than 2 mg/ml of Δ9-THC-THCA, and other undetermined compounds of Cannabis sativa (e.g., terpenoids and flavonoids). XAL (10 %) contains 100 mg/ml of CBD without Δ9-THC-THCA. **EPI and XAL are commercially sold in medium-chain triglyceride (MCT) oil, a lipid source commonly used for preparing medical cannabis-derived products. MCT oil was also kindly provided by RAMM Pharma Corp., Uruguay**. In addition, a purified and crystallized CBD was kindly provided by Phytoplant Research Spain (www.phytoplantresearch.com). **CBD was purified from GOYA variety following a direct crystallization method described previously[17]. The purity was set at > 95% (data provided by Phytoplant Research). Pure CBD was prepared in**

DMSO. Pharmaceutical products (EPI, XAL) and pure CBD were prepared with an equivalent content of CBD, and then dilutions were made in DMSO plus media at 20% in each experimental session (vehicle), to finally obtain a concentration of DMSO in each well of < 1.5 %. For comparative purposes, and in an independent set of experiments, pure CBD was prepared in MCT oil or DMSO. Then, dilutions were made in DMSO plus media at 20% to finally obtain a concentration of DMSO in each well of < 1.5 % (Supplementary data).

General Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock concentrated solutions (10 mM of Rotenone) were prepared in DMSO and conveniently diluted in media culture in each experimental session to finally reach a DMSO < 0.1 %.

Chromatographic conditions: HPLC-DAD

The purity and concentration of CBD were analyzed and quantified in EPI and XAL samples. Both pharmaceutical products were properly diluted in mobile phase, centrifuged at 10.000 rpm for 5 min at 4°C, and supernatants were injected on to the HPLC system. Separation of constituents was achieved by reverse-phase HPLC using a C18 column (Kinetex, USA) with 5 µm particle size. A binary HPLC pump (Waters 1525) with a 717 plus autosampler Waters and a photodiode array detector (DAD) Waters 2998 linked to Empower 2 (Waters) chromatography data software was utilized. The temperature of the column was set at 30°C. The mobile phase used was (A) 89.9 % acetonitrile, 10% water, 0.1% formic acid (B) 99.9 % water, 0.1% formic acid. At 1mL/min. The gradient system consisted of (min/%A): 0/90, 10/60, 12/90, and 30/90. The eluent was monitored by photodiode array detection at a wavelength of 210 nm, specific to determine cannabinoids.[18]

Primary cerebellar granule cell cultures

Cultures of primary cerebellar granule cells were obtained from 6-8 days Wistar rats[19,20] and seeded in poly-L-ornithine pre-coated **96** (for immunocytochemical assays) or **24** wells (for biochemical MTT assays) plates at a density of 200.000 or 900.000 cells per well, respectively. They were kept in Basal Medium Eagle supplemented with fetal bovine serum (PAA Laboratories, Austria), at 10 %, 20 mM KCl and 25 mM glucose in a humidified chamber at 37 ºC in a 5 % CO_2 atmosphere. Cell division was inhibited by the addition of cytosine arabinoside (10 µM).

Experimental procedure

The experimental procedures for neurotoxicity and neuroprotective assays were designed based on our previous studies.[16,20] Briefly, to evaluate the neurotoxicity *per se* of EPI, XAL, or CBD, different concentrations of these compounds were added to the culture on the 7th day *in vitro* (DIV7) during 24 hours and then the cell viability was evaluated. To evaluate the neuroprotective effect of EPI, XAL, or CBD, cultures were treated with rotenone (40 nM) at DIV7 during 24 hours to obtain a significant loss of cell viability of about 50 % compared with the control condition. Pre-treatments with EPI, XAL, CBD, or MCT oil (vehicle control) were performed during 1 hour before rotenone. Cell viability was evaluated at DIV8 24 hours after the toxic insult.

Determination of Cell Viability by MTT Assay

The neuronal mitochondrial activity, as an indicator of cell viability, was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) assay5.[21] After the different mentioned conditions, cell culture was incubated for 45 min at 37ºC with MTT (0.1 mg/ml final concentration). Formazan crystals were dissolved with DMSO, and the absorbance was recorded in a microplate reader (Varioskan Flash, Thermo Scientific) using a reference wavelength of 630 nm and a test wavelength of 570 nm. Results were presented as the percentage of MTT reduction, assuming that the absorbance of control cells was 100 %.

Immunofluorescence and quantitative assessment of neurites and astrocytes

Cells on coverslips were fixed with 4 % paraformaldehyde at 37 ºC for 20 min. After three washes with phosphate-buffered saline (PBS, pH 7.5), cells were permeabilized with Triton X-100 0.1% (v/v) in PBS during 30 min and blocked with BSA 5% for 1h, followed by overnight incubation at 4 °C with primary antibodies anti-Tyr-tubulin (mouse 1/1000; Clone TUB-1A2; Sigma-Aldrich, Cat. Number T9028) and anti-GFAP (rabbit 1/1000; Sigma-Aldrich, Cat. Number G9269). After that, cells were washed with PBS and incubated with secondary antibodies goat anti-mouse IgG conjugated with Alexa Fluor 488 (1/1000, Invitrogen A32723) and goat anti-rabbit IgG conjugated with Alexa Fluor 568 (1/1000, Invitrogen A11011), for 60 min at room temperature. Finally, three washes were performed, and Hoechst 33258 (1 ng/mL) was added in the last wash to visualize the nuclei. Immunolabelled cultures were observed with a confocal microscope (LSM ZEISS 800) using 20x objective lens, and images were captured with ZEN Blue 2.3 software. In each resulting image, the area occupied by neurites, astrocytes, and the level of fluorescence intensity of GFAP staining were determined using the FIJI-ImageJ software. A mask was created within which the area and intensity of cytoplasmic GFAP labelling were measured. Another mask used to quantify the neurite area was calculated by subtracting astrocytes labeled with GFAP and the nuclei labeled with Hoechst from Tyr-tubulin-IR area acquired with the 488 laser. Following evidence that astrocytes change their morphology,[22] and this could affect the area apart from a change in the number of astrocytes, the fluorescence intensity in these cells was measured as integrated optical density (ID= mean optical density per area unit; OD x cytoplasmic area).[23]

Statistical analyses

Data are given as Mean ± Standard Error of the Mean (SEM) and were analyzed by One-way (treatment) analysis of variance (ANOVA) of independent

measures followed by post hoc Tukey multiple comparison test. Statistical significance was set at $P < 0.05$.

Results

Chemical profile of EPI based on the CBD content

Figure 1 shows a comparative profile of the chemical content of EPI and XAL, taking CBD as a reference sample. The HPLC-DAD analysis revealed CBD as the main component present in EPI, but other compounds like CBDA and ∆9-THC were detected in a very low proportion compared to CBD (not quantified). As expected, CBD is the only detected compound in the XAL sample.

Neurotoxicity and neuroprotective effects of EPI analyzed by the biochemical MTT assay

A comparison of the cell viability of cerebellar granule cell cultures exposed to different concentrations of EPI, XAL, or CBD (1.25 to 20 µM), for 24 hours was performed. Figure 2 a-c illustrates the results of the neurotoxicity assays. It can be observed that EPI and XAL, tested at an equivalent content of CBD, elicited a similar curve pattern without inducing neurotoxicity *per se* at any concentration assayed (Fig. 2a and b). In contrast, the highest concentrations of CBD (15 and 20 µM) evoked a significant reduction in cell viability compared with the control group (0 µM; $p < 0.001$; Fig. 2c) and with the lower concentrations (1.25 to 10 µM). A significant potentiation of

this neurotoxic effect was observed at 20 μM compared with the elicited by 15 μM of CBD ($p < 0.001$; Fig. 2c). Next, we characterized the neuroprotective effect of EPI in comparison with XAL and CBD. Figure 3 a-c illustrates the results obtained from the pre-treatment of EPI, XAL, and CBD against rotenone-induced neurotoxicity, respectively. As expected from our previous studies,[16] the incubation of the cerebellar granule cells with rotenone for 24 hours resulted in approximately 40-50 % loss of cell viability compared with the control condition (Fig. 3a-c). Similar curves of neuroprotection were observed after the EPI and XAL assays. EPI and XAL 1-hour pre-treatment significantly attenuated cell death induced by rotenone from 2.5 to 20 μM ($p < 0.001$; Fig. 3a and b). However, a quite interesting difference was observed after 1 hour of CBD pre-treatment (Fig. 3c). While CBD was effective in preventing the reduction of cell viability induced by rotenone in the range of 2.5 to 10 μM ($p < 0.001$; Fig. 3c), this effect was not maintained with the highest concentrations of CBD (15 and 20 μM). A significant reduction of the cell viability compared with the rotenone group was observed at CBD 15 and 20 μM ($p < 0.01$ and $p < 0.001$, respectively). In addition, statistical differences were observed along the concentrations of EPI, XAL, or CBD, denoting a concentration-dependence profile, especially for EPI and XAL. In contrast, a significant difference was observed between the higher concentrations (15 and 20 μM) and the lower 1.25 to 10 μM of CBD (Fig. 3c; to clarify, significance values are shown in the figure legend). This profile indicates that EPI (and XAL) has a broader range of active doses than CBD. **MCT oil (**the vehicle of EPI and XAL), did not alter *per se* the cell viability and did not attenuate the damage elicited by rotenone (Supplementary **Figure 1**). **In addition, the two highest concentrations of pure CBD (15 and 20 μM) prepared in DMSO evoked a significant decrease in cell viability compared with the same concentrations of pure CBD prepared in MCT oil ($p < 0.001$) (Supplementary Figure 2).**

Neuroprotective effect of EPI in culture assayed by immunocytochemistry

The neuroprotective effect of EPI was further addressed with immunocytochemistry (Fig. 4). After a pre-treatment with EPI, and the treatment with rotenone, the neurite area of the cultured neurons was quantified as an indicator of neuronal damage (Fig. 4). Taking into account that the primary cerebellar cell cultures used in this study were mainly composed of granular neurons, but also by a small proportion of astrocytes,[16] we also explored the impact of rotenone on astrocytes. Therefore, primary cerebellar cell cultures were immunolabelled with the glial fibrillary acidic protein (GFAP). Results showed that in control primary cerebellar cell cultures, granular neurons extended bundled neurites (Fig. 4a; arrow), while in rotenone-treated cell cultures, a drastic decrease in the area occupied by neurites compared with the control group was observed. This finding is consistent with the neuronal damage provoked by this drug (Fig. 4b and e; $p < 0.0001$). Rotenone did not significantly modify the GFAP-immunoreactive area (GFAP IR-area) nor the fluorescence intensity (GFAP-ID) of this astrocytic marker (Fig. 4f and g, respectively). The treatment with EPI did not alter *per se* the neurite area (Fig 4c; e) but attenuated the neuritic damage elicited by rotenone (Fig. 4d and e; $p < 0.001$). Moreover, EPI treatment significantly increased GFAP-IR-area and the levels of fluorescence intensity of GFAP (Fig. 4f and g; $p < 0.05$, respectively). The combination of EPI and rotenone denoted a significant difference compared with the rotenone-treated cell cultures (Fig. 4f and g; $p < 0.05$), suggesting an interaction between both treatments.

Discussion

This study reports the neuroprotective effect of EPI, demonstrated using an experimental neurotoxicity model, induced by rotenone in cultures of primary cerebellar granule cells. The ability of EPI to counteract the rotenone-induced neurotoxicity was analyzed biochemically (cell viability) and morphologically on neurons and astrocytes

by immunocytochemical assays. EPI showed a neuroprotection concentration curve similar to XAL, suggesting that CBD, but not the other components present in EPI, may account for its beneficial action. On the other hand, a significant reduction in cell viability was observed at very high doses of CBD alone (15 and 20 μM), suggesting that other factors, in addition to CBD, collaborate in the neuroprotective action of EPI. These findings indicate that some features of the formulation (MCT oil) of both **commercial** cannabis products would counteract the neurotoxicity of CBD present in EPI and XAL. **In line with this idea, we tested cell viability at the two neurotoxic highest concentrations of CBD not only in DMSO but also diluted in MCT oil. As expected, we observed a significant difference between both experimental conditions (Supplementary Figure 2).**

It has recently been reported that formulations are critical in the bioavailability to achieve the optimal therapeutic effect of CBD alone, combined with other cannabinoids (e.g., ∆9-THC) or pharmaceutical cannabis-based extracts.[24] Also, the anti-inflammatory effects of cannabinoids seem to vary, depending on the selected formulation.[25] MCT oil includes lipids with a carbon chain length of 6-12 carbon atoms, making MCTs easier to absorb and metabolize than long-chain fatty acids (LCTs). Due to these features, MCTs have been proposed as drug vehicles for lipophilic drugs.[25,26] Our results agree with Ramella and colleagues (2020), who reported that MCT could represent an excellent lipid source for the formulation of pharmaceutical cannabis-based extracts.[26] Alternatively, it could be thought that a low solubility of MCT in the culture medium could require a more extended exposition time of EPI pretreatment (in MCT oil) to enter the cell and exert its effects. Taking this issue into account, preliminary experiments showed that extending the EPI exposition time from 24 to 48 hours (in MCT oil) did not induce a reduction in the cell viability, compared with CBD under the same schedule, while CBD at higher concentrations (15 and 20 μM) elicited

once again a decrease in the cell viability (unpublished data). This result highlights the role of the MCT oil (i.e., formulation) in attenuating the CBD neurotoxicity observed at high concentrations.

In opposition to this interpretation, a possible entourage effect[27,28] in EPI action could explain the absence of neurotoxicity at high concentrations, according to the EPI chemical profile. The entourage effect refers to the idea that multiple compounds can act in concert or synergically to produce different outcomes.[10,28] In fact, Russo and McPartland[29] and other reports proposed the entourage effect in relation to the clinical contribution of CBD, other cannabinoids, terpenoids, and flavonoids in pharmaceutical-medical cannabis.[10,30] However, our results did not support a possible synergistic or entourage effect, since EPI showed similar toxicity (Fig. 2a and b) and neuroprotective action curves compared to XAL (Fig. 3 a and b). Our findings would a priori rule out the participation of other individual substances present in EPI, highlighting the benefit of the formulation of MCT oil in the neuroprotective EPI's effect.

Additionally, the neuroprotective effect of EPI was morphologically confirmed by immunocytochemistry, focused on neurons and astrocytes. GFAP is a marker of mature astrocytes, and its abnormal expression occurs in neuroinflammation or neurodegenerative processes, among others.[22] Since a glia-mediated mechanism might be involved in the neuroprotective effects exerted by EPI,[31,32] we investigated if EPI was able to prevent putative impairments induced by rotenone on astrocytes. Rotenone can activate glial cells in cultures eliciting phenotypic changes which could be toxic to neurons.[33,34] In contrast, rotenone did not modify any parameter in our paradigm (Fig. 4f and g). Rotenone inhibits the mitochondrial electron transfer chain complex I enhancing the formation of reactive oxygen species (ROS). These events lead to a depletion of ATP and mitochondrial dysfunction, eventually ending in apoptotic cell death.[15,35] A differential sensitivity between neurons and astrocytes to rotenone may explain our results. It may be possible that the mitochondrial dysfunction and oxidative stress induced by rotenone cannot be counteracted by neurons because

of their high-energy consumption and fewer energy resources than astrocytes. In turn, although astrocytes have deficient mitochondrial respiration, they can survive as glycolytic cells.[36] Also, astrocytes have a higher buffering capacity against ROS than neurons.[37] The increase in intensity levels of GFAP in EPI-pretreated cultures was an unexpected result that could be interpreted as an opposite effect regarding its neuroprotective effect described below. Increased GFAP expression is associated with alterations in astrocyte morphology and reactivity triggered by any alteration in brain homeostasis.[38] Astrocytes are equipped with several receptors and intracellular signaling cascades to respond quickly to environmental changes.[38,39] While some signals can mediate harmful effects; others can trigger protective and adaptive consequences.[40] Thus, the phenotype of a reactive astrocyte cannot always be predicted from the expression of a single marker like GFAP. It has been reported that reactive astrogliosis or astrocyte proliferation can also be neuroprotective since, under this condition, the glial cells could provide factors that promote cell survival against severe injury or degenerative insults.[41,42] Thus, some of these events could underly the neuroprotective effect of EPI on GFAP intensity in this group.

The sites of action that explain the beneficial effect of EPI (i.e., mainly due to CBD content) on mitochondrial dysfunction induced by rotenone remain to be identified in this study, although some hypotheses could be proposed. Several studies have suggested mitochondria as targets for cannabinoids,[43] as the mitochondrial CB1 (mCB1) receptor, plays a relevant role in cell metabolic activity.[44] However, it is unlikely that the mCB1 receptor could mediate the neuroprotective effect of CBD on rotenone-induced neurotoxicity since the CB1 antagonist (AM251) did not block the neuroprotective effect of CBD on rotenone-induced neurotoxicity in our previous study.[16] We also tested the participation of CB2, 5-HT1A receptors,[16] and the PPAR gamma receptor activity, and none of these targets mediated the beneficial action of CBD in the same paradigm. Alternatively, non-receptor-mediated mechanisms involving the inhibition of

mitochondrial respiration,[45] or mitochondrial biogenesis,[46] could be involved. Finally, it could be possible that CBD acts as a "neuron conditioner" or "neuron protective agent," conferring tolerance to an oxidant insult like that induced by rotenone.[47]

Conclusions

The neuroprotective effect of EPI was observed in a wide range of concentrations without neurotoxic effects. A similar action profile between EPI and XAL strongly suggests that the beneficial property of EPI is mainly due to its CBD content, discarding an entourage effect between the different compounds present in this cannabis extract. Moreover, while EPI diminished neuronal damage and restored morphology, a neuroprotection mechanism involving astrocytes could be hypothesized. Our results agree with several preclinical studies showing the potential of CBD as a neuroprotective agent.[16,42,48,49] While a few clinical trials of the benefits of CBD on neurological conditions have been carried out,[50,51] further studies are necessary to introduce pharmaceutical CBD-based extracts in different neurodegenerative disorders clinical trials. Our results highlight the potential benefits of EPI in neurodegenerative diseases involving mitochondrial dysfunction. When considering the clinical use of pharmaceutical cannabis-based extracts, the purity and concentration of CBD could be critical factors. This study also demonstrated that MCT could represent a good lipid source for the formulation of medical cannabis-based oils. Therefore, selecting an appropriate pharmaceutical formulation could be crucial for the product's efficacy in clinical trials.

Acknowledgments

We are grateful to Dr Raquel Peyraube and Dr Edelma Ros for their remarkable help in the Cannabis field and chemical concepts. This study has a license from IRCCA, Uruguay. **We also thank the Ibero-American Cyted-CannaLatan network.**

Funding Information

This study was partially supported by ANII-FCE_3_2020_1_162440 and PEDECIBA (Uruguay).

References

1. Mechoulam R, Shvo Y. Hashish I. The structure of cannabidiol. Tetrahedron. 1963;19(12):2073-2078. doi:10.1016/0040-4020(63)85022-x

2. Echeverry C, Reyes-Parada M, Scorza C. Constituents of Cannabis sativa. Adv Exp Med Biol. 2021;1297:1-9. doi:10.1007/978-3-030-61663-2_1

3. Campos AC, Fogaça MV, Sonego AB, et al. Cannabidiol, neuroprotection, and neuropsychiatric disorders. Pharmacol Res. 2016;112:119-127. doi:10.1016/j.phrs.2016.01.033

4. Peres FF, Lima AC, Hallak JEC, et al. Cannabidiol as a Promising Strategy to Treat and Prevent Movement Disorders?. Front Pharmacol. 2018;9:482. doi:10.3389/fphar.2018.00482

5. Arzimanoglou A, Brandl U, Cross JH, et al. Epilepsy and cannabidiol: a guide to treatment. Epileptic Disord. 2020;22(1):1-14. doi:10.1684/epd.2020.1141

6. Feliú A, Moreno-Martet M, Mecha M, et al. A Sativex(®) -like combination of phytocannabinoids as a disease-modifying therapy in a viral model of multiple sclerosis. Br J Pharmacol. 2015;172(14):3579-3595. doi:10.1111/bph.13159

7. Keating GM. Delta-9-Tetrahydrocannabinol/Cannabidiol Oromucosal Spray (Sativex®): A Review in Multiple Sclerosis-Related Spasticity. Drugs. 2017;77(5):563-574. doi:10.1007/s40265-017-0720-6

8. Devinsky O, Marsh E, Friedman D, et al. Cannabidiol in patients with treatment-resistant epilepsy: an open-label interventional trial. Lancet Neurol. 2016 Mar;15(3):270-8. doi:10.1016/S1474-4422(15)00379-8

9. European Medicines Agency Epidiolex (Cannabidiol) [(accessed on 10 September 2020)]; Available online:https://www.ema.europa.eu/en/medicines/human/EPAR/epidyolex#authorisation-details-section.

10. McPartland J, Russo E. Cannabis and Cannabis Extracts: Greater Than the Sum of Their Parts?. J Cannabis Therapeutics. 2001;1:103-132. doi:10.1300/J175v01n03_08.

11. MedicPlast. Epifractan 2%. Montevideo: MedicPlast; 2017. Available in: http://medicplast.com.uy

12. Wang Y, Xu E, Musich PR, et al. Mitochondrial dysfunction in neurodegenerative diseases and the potential countermeasure. CNS Neurosci Ther. 2019;25(7):816-824. doi:10.1111/cns.13116

13. Blesa J, Phani S, Jackson-Lewis V, et al. Classic and new animal models of Parkinson's disease. J Biomed Biotechnol. 2012;2012:845618. doi:10.1155/2012/845618

14. Mouhape C, Costa G, Ferreira M, et al. Nicotine-Induced Neuroprotection in Rotenone In Vivo and In Vitro Models of Parkinson's Disease: Evidences for the Involvement of the Labile Iron Pool Level as the Underlying Mechanism. Neurotox Res. 2019;35(1):71-82. doi:10.1007/s12640-018-9931-1

15. Giordano S, Lee J, Darley-Usmar VM, et al. Distinct effects of rotenone, 1-methyl-4-phenylpyridinium and 6-hydroxydopamine on cellular bioenergetics and cell death. PLoS One. 2012;7(9):e44610. doi:10.1371/journal.pone.0044610

16. Echeverry C, Prunell G, Narbondo C, et al. A Comparative In Vitro Study of the Neuroprotective Effect Induced by Cannabidiol, Cannabigerol, and Their Respective Acid Forms: Relevance of the 5-HT_{1A} Receptors. Neurotox Res. 2021;39(2):335-348. doi:10.1007/s12640-020-00277-y

17. Nadal X. Methods of purifying cannabinoids, compositions and kits thereof. U.S. Patent No 9765000 Washington, DC: U.S. Patent and Trademark Office (2016).

18. Hazekamp A, Peltenburg A, Verpoorte, R, et al. Chromatographic and Spectroscopic Data of Cannabinoids from Cannabis sativa L. J. Liq Chromatographic Relat Techno. 2005, 28: 2361-2382. doi:10.1080/10826070500187558

19. García O, Massieu L. Strategies for neuroprotection against L-trans-2,4-pyrrolidine dicarboxylate-induced neuronal damage during energy impairment in vitro. J Neurosci Res. 2001;64(4):418-428. doi:10.1002/jnr.1093

20. Echeverry C, Arredondo F, Abin-Carriquiry JA, et al. Pretreatment with natural flavones and neuronal cell survival after oxidative stress: a structure-activity relationship study. J Agric Food Chem. 2010;58(4):2111-2115. doi:10.1021/jf902951v

21. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods. 1986;89(2):271-277. doi:10.1016/0022-1759(86)90368-6

22. Li D, Liu X, Liu T, et al. Neurochemical regulation of the expression and function of glial fibrillary acidic protein in astrocytes. Glia. 2020;68(5):878-897. doi:10.1002/glia.23734

23. Richeri A, Bianchimano P, Mármol NM, et al. Plasticity in rat uterine sympathetic nerves: the role of TrkA and p75 nerve growth factor receptors. J Anat. 2005;207(2):125-134. doi:10.1111/j.1469-7580.2005.00435.x

24. Lucas CJ, Galettis P, Schneider J. The pharmacokinetics and the pharmacodynamics of cannabinoids. Br J Clin Pharmacol. 2018;84(11):2477-2482. doi:10.1111/bcp.13710

25. Robaina Cabrera CL, Keir-Rudman S, Horniman N, et al. The anti-inflammatory effects of cannabidiol and cannabigerol alone, and in combination. Pulm Pharmacol Ther. 2021;69:102047. doi:10.1016/j.pupt.2021.102047

26. Ramella A, Roda G, Pavlovic R, et al. Impact of Lipid Sources on Quality Traits of Medical Cannabis-Based Oil Preparations. Molecules. 2020;25(13):2986. Published 2020 Jun 30. doi:10.3390/molecules25132986

27. Ben-Shabat S, Fride E, Sheskin T, et al. An entourage effect: inactive endogenous fatty acid glycerol esters enhance 2-arachidonoyl-glycerol cannabinoid activity. Eur J Pharmacol. 1998;353(1):23-31. doi:10.1016/s0014-2999(98)00392-6

28. Russo EB. Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. Br J Pharmacol. 2011;163(7):1344-1364. doi:10.1111/j.1476-5381.2011.01238.x

29. Russo EB, McPartland JM. Cannabis is more than simply delta(9)-tetrahydrocannabinol. Psychopharmacology (Berl). 2003;165(4):431-434. doi:10.1007/s00213-002-1348-z

30. Mechoulam R, Ben-Shabat S. From gan-zi-gun-nu to anandamide and 2-arachidonoylglycerol: the ongoing story of cannabis. Nat Prod Rep. 1999;16(2):131-143. doi:10.1039/a703973

31. di Giacomo V, Chiavaroli A, Recinella L, et al. Antioxidant and Neuroprotective Effects Induced by Cannabidiol and Cannabigerol in Rat CTX-TNA2 Astrocytes and Isolated Cortexes. Int J Mol Sci. 2020;21(10):3575. Published 2020 May 18. doi:10.3390/ijms21103575

32. Kozela E, Juknat A, Vogel Z. Modulation of Astrocyte Activity by Cannabidiol, a Nonpsychoactive Cannabinoid. Int J Mol Sci. 2017;18(8):1669. Published 2017 Jul 31. doi:10.3390/ijms18081669

33. Swarnkar S, Goswami P, Kamat PK, et al. Rotenone-induced apoptosis and role of calcium: a study on Neuro-2a cells. Arch Toxicol. 2012;86(9):1387-1397. doi:10.1007/s00204-012-0853-z

34. Yu YX, Li YP, Gao F, et al. Vitamin K2 suppresses rotenone-induced microglial activation in vitro. Acta Pharmacol Sin. 2016;37(9):1178-1189. doi:10.1038/aps.2016.68

35. Li N, Ragheb K, Lawler G, Sturgis J, Rajwa B, Melendez JA, Robinson JP. Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. J Biol Chem. 278 (2003) 8516-25.

36. Supplie LM, Düking T, Campbell G, et al. Respiration-Deficient Astrocytes Survive As Glycolytic Cells In Vivo. J Neurosci. 2017;37(16):4231-4242. doi:10.1523/JNEUROSCI.0756-16.2017

37. Schmuck G, Röhrdanz E, Tran-Thi QH, Kahl R, Schlüter G. Oxidative stress in rat cortical neurons and astrocytes induced by paraquat in vitro. Neurotox Res. 2002;4(1):1-13. doi:10.1080/10298420290007574

38. Buffo A, Rolando C, Ceruti S. Astrocytes in the damaged brain: molecular and cellular insights into their reactive response and healing potential. Biochem Pharmacol. 2010;79(2):77-89. doi:10.1016/j.bcp.2009.09.014.

39. Burda JE, Sofroniew MV. Reactive gliosis and the multicellular response to CNS damage and disease. Neuron. 2014;81(2):229-248. doi:10.1016/j.neuron.2013.12.034

40. Sofroniew MV, Vinters HV. Astrocytes: bio]logy and pathology. Acta Neuropathol. 2010;119(1):7-35. doi:10.1007/s00401-009-0619-8

41. T.C. Mohn, A.O. Koob, Adult Astrogenesis and the Etiology of Cortical Neurodegeneration. J Exp Neurosci. 9 (2015) 25-34. doi: 10.4137/JEN.S25520

42. Kim J, Choi JY, Seo J, et al. Neuroprotective Effect of Cannabidiol Against Hydrogen Peroxide in Hippocampal Neuron Culture. Cannabis Cannabinoid Res. 2021;6(1):40-47. Published 2021 Feb 12. doi:10.1089/can.2019.0102

43. Athanasiou A, Clarke AB, Turner AE, et al. Cannabinoid receptor agonists are mitochondrial inhibitors: a unified hypothesis of how cannabinoids modulate mitochondrial function and induce cell death. Biochem Biophys Res Commun. 2007;364(1):131-137. doi:10.1016/j.bbrc.2007.09.107

44. Xu Z, Lv XA, Dai Q, Ge YQ, Xu J. Acute upregulation of neuronal mitochondrial type-1 cannabinoid receptor and it's role in metabolic defects and neuronal apoptosis after TBI. Mol Brain. 2016;9(1):75. doi:10.1186/s13041-016-0257-8

45. Fišar Z, Singh N, Hroudová J. Cannabinoid-induced changes in respiration of brain mitochondria. Toxicol Lett. 2014;231(1):62-71. doi:10.1016/j.toxlet.2014.09.002

46. Chan JZ, Duncan RE. Regulatory Effects of Cannabidiol on Mitochondrial Functions: A Review. Cells. 2021;10(5):1251. doi:10.3390/cells10051251

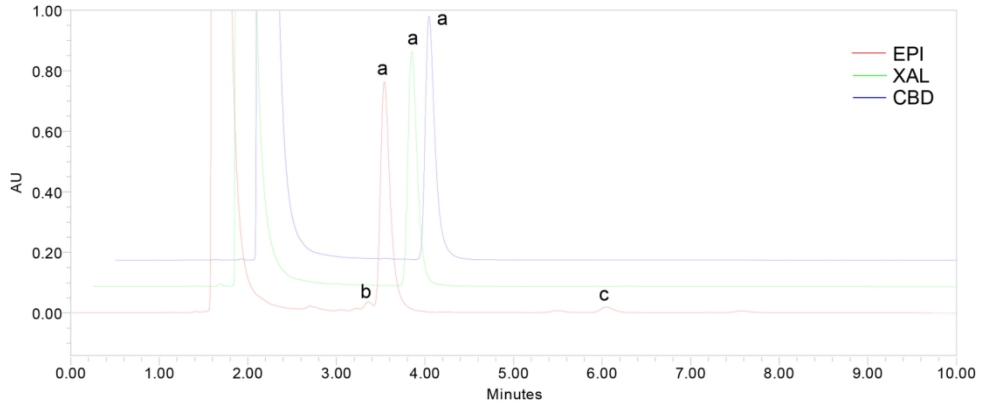
47. Oliveira MF, Geihs MA, França TFA, Moreira DC, Hermes-Lima M. Is "Preparation for Oxidative Stress" a Case of Physiological Conditioning Hormesis?. Front Physiol. 2018;9:945. doi:10.3389/fphys.2018.00945

48. Martín-Moreno AM, Reigada D, Ramírez BG, et al. Cannabidiol and other cannabinoids reduce microglial activation in vitro and in vivo: relevance to Alzheimer's disease. Mol Pharmacol. 2011;79(6):964-973. doi:10.1124/mol.111.071290

49. Yang S, Du Y, Zhao X, et al. Cannabidiol Enhances Microglial Beta-Amyloid Peptide Phagocytosis and Clearance via Vanilloid Family Type 2 Channel Activation. Int J Mol Sci. 2022;23(10):5367. Published 2022 May 11. doi:10.3390/ijms23105367

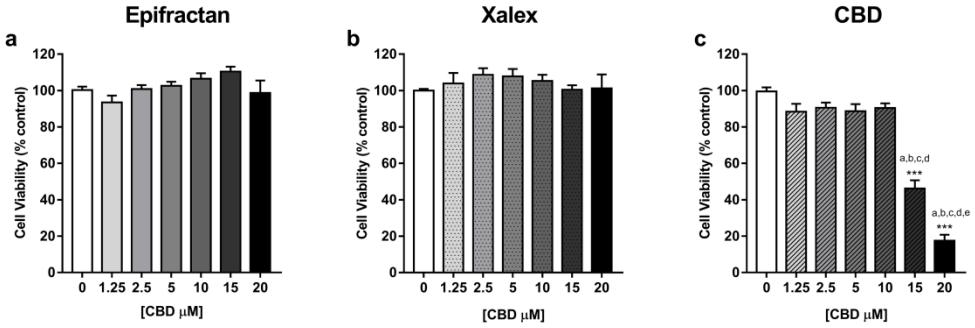
50. Zuardi AW, Crippa JA, Hallak JE, et al. Cannabidiol for the treatment of psychosis in Parkinson's disease. J Psychopharmacol. 2009;23(8):979-983. doi:10.1177/0269881108096519

51. Zuardi AW, Crippa JA, Hallak JE, et al. Cannabidiol for the treatment of psychosis in Parkinson's disease. J Psychopharmacol. 2009;23(8):979-983. doi:10.1177/0269881108096519.



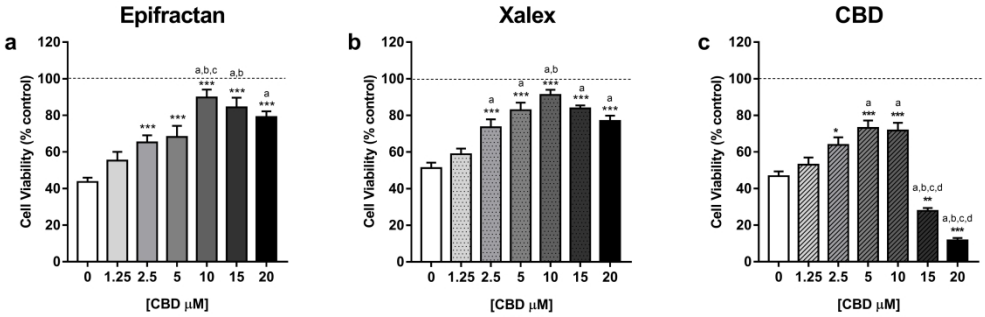
Representative HPLC-DAD chromatogram of EPI (red) and XAL (green), taking the purified and crystallized CBD (blue) as a reference sample. The wavelength was set at 210 nm. CBD (a), CBDA (b) and THC (c) are indicated.

139x57mm (300 x 300 DPI)



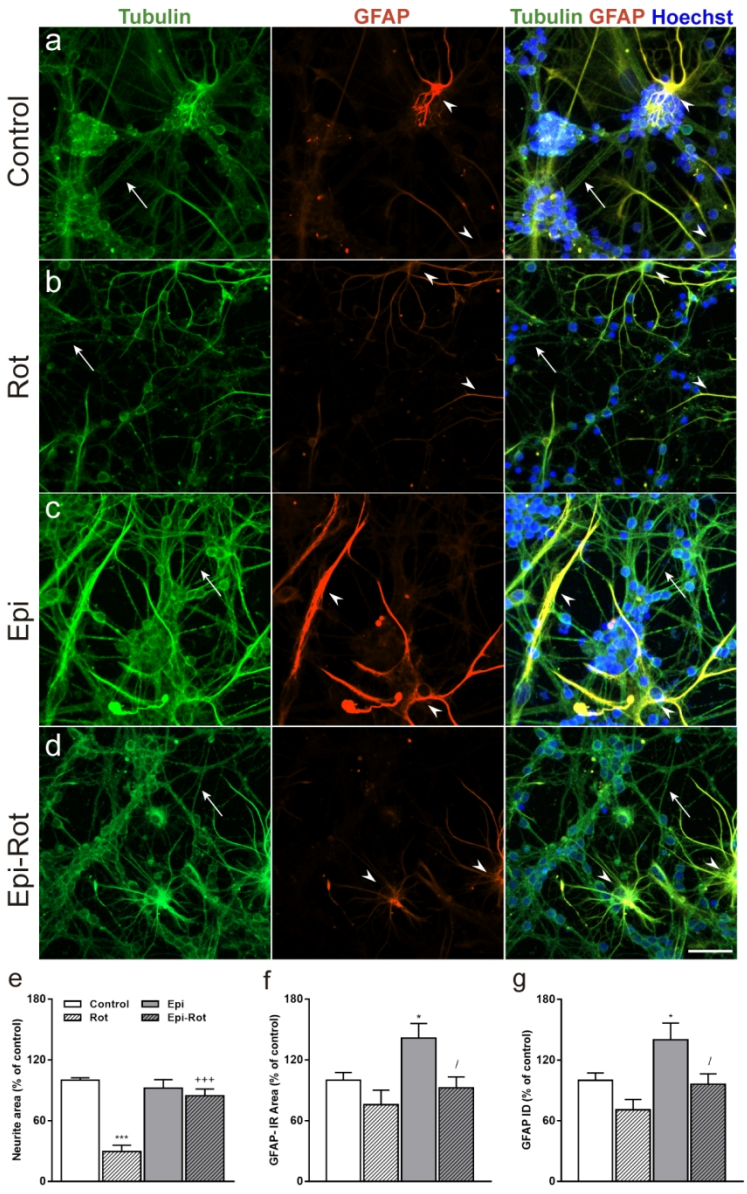
Neurotoxicity curves of the effect of Epifractan (a), Xalex (b), and CBD (c) exposed for 24 h on the cell viability in cerebellar granule cell cultures. Data were expressed as mean ± SEM of cell viability (% of control) with respect to different concentrations of CBD content. One-Way ANOVA followed by post hoc Tukey multiple comparison test. * = compared with the control group (0 μM). *** = $P < 0.001$ for 10 and 20 μM vs. control group. To simplify, a, b, c, d, and e represent the significant differences compared to 1.25, 2.5, 5.0, 10, and 15 μM of CBD, respectively.

179x62mm (600 x 600 DPI)



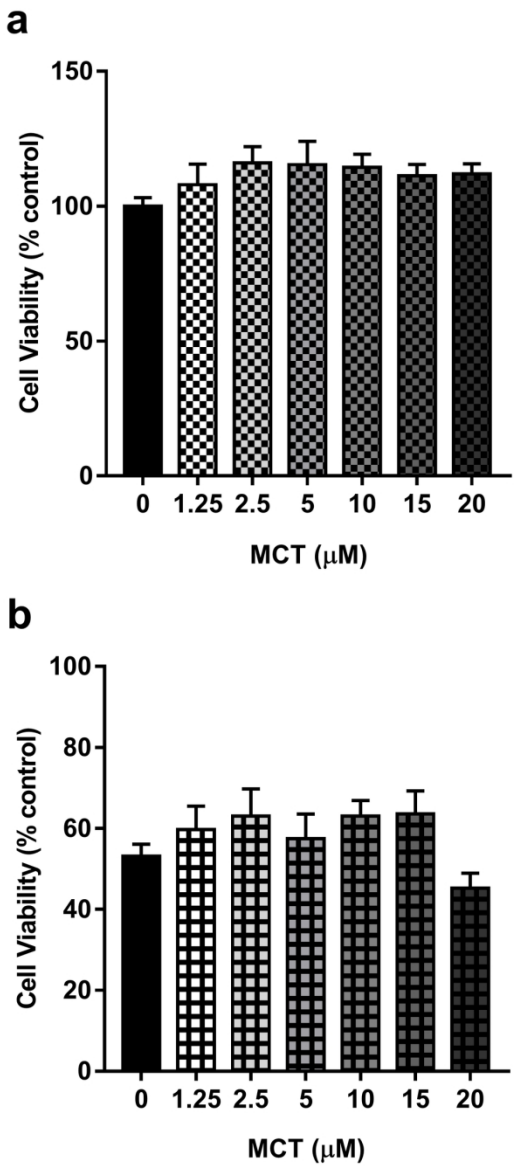
Neuroprotective effect induced by 24 h pre-treatment with Epifractan (a), Xalex (b), and CBD (c) against rotenone. Data were expressed as mean ± SEM of cell viability (% of control) with respect to different concentrations of CBD content. One-Way ANOVA followed by post hoc Tukey multiple comparison test. * = compared with the control group (0 μM). ** = P < 0.01; *** = P < 0.0001. To simplify, a, b, c, d, and e represent the significant differences compared to 1.25, 2.5, 5.0, 10, and 15 μM of CBD, respectively.

180x60mm (600 x 600 DPI)

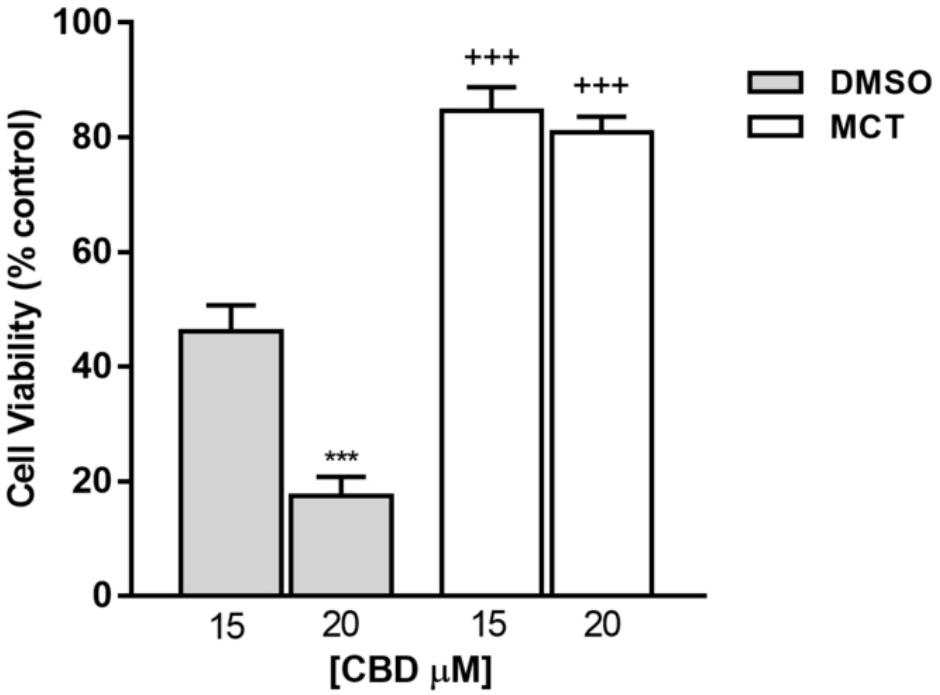


Neuroprotective effect of EPI at the morphological level. Representative images of (a) control (b) rotenone-exposed, (c) one-hour pre-treated with EPI (2.5 μM), and (d) 1-h pre-treated with EPI followed by rotenone-exposed cerebellar granule cell cultures. Cytoskeleton marker anti-Tyr tubulin (green), astrocytic marker GFAP (orange). Overlapping images with both markers and nuclei stained with Hoechst 33258 dye (blue) are shown in the right column. Plots (e-g) show a quantitative assessment of the neurite area (calculated as Tubulin-IR area – nuclei - GFAP-IR area), the GFAP-IR area, and the integrated density (ID) of GFAP fluorescence, respectively. Data were expressed as mean ± SEM (% of control). One-Way ANOVA followed by post hoc Tukey multiple comparison test. * = compared with the control group; + = compared with the rotenone group. / = compared with the EPI group. *,/ = $P < 0.05$; ***,+++ = $P < 0.0001$. Arrows, neurites; arrowheads, astrocytes. Scale bar = 50 μm.

104x162mm (300 x 300 DPI)



49x102mm (600 x 600 DPI)



60x46mm (300 x 300 DPI)

Figure legends

Figure 1: Representative HPLC-DAD chromatogram of EPI (red) and XAL (green), taking the purified and crystallized CBD (blue) as a reference sample. The wavelength was set at 210 nm. CBD (a), CBDA (b) and THC (c) are indicated.

Figure 2. Neurotoxicity curves of the effect of Epifractan (a), Xalex (b), and CBD (c) exposed for 24 h on the cell viability in cerebellar granule cell cultures. Data were expressed as mean ± SEM of cell viability (% of control) with respect to different concentrations of CBD content. One-Way ANOVA followed by post hoc Tukey multiple comparison test. * = compared with the control group (0 μM). *** = $P < 0.001$ for 10 and 20 μM vs. control group. To simplify, a, b, c, d, and e represent the significant differences compared to 1.25, 2.5, 5.0, 10, and 15 μM of CBD, respectively.

Figure 3. Neuroprotective effect induced by 24 h pre-treatment with Epifractan (a), Xalex (b), and CBD (c) against rotenone. Data were expressed as mean ± SEM of cell viability (% of control) with respect to different concentrations of CBD content. One-Way ANOVA followed by post hoc Tukey multiple comparison test. * = compared with the control group (0 μM). ** = $P < 0.01$; *** = $P < 0.0001$. To simplify, a, b, c, d, and e represent the significant differences compared to 1.25, 2.5, 5.0, 10, and 15 μM of CBD, respectively.

Figure 4. Neuroprotective effect of EPI at the morphological level. Representative images of (a) control (b) rotenone-exposed, (c) one-hour pre-treated with EPI (2.5 μM), and (d) 1-h pre-treated with EPI followed by rotenone-exposed cerebellar granule cell cultures**. Cytoskeleton marker anti-Tyr tubulin (green), astrocytic marker GFAP (orange). Overlapping images with both markers and nuclei stained with Hoechst 33258 dye (blue) are shown in the right column.** Plots (e-g) show a quantitative

assessment of the neurite area (calculated as Tubulin-IR area – nuclei - GFAP-IR area), the GFAP-IR area, and the integrated density (ID) of GFAP fluorescence, respectively. Data were expressed as mean ± SEM (% of control). One-Way ANOVA followed by post hoc Tukey multiple comparison test. * = compared with the control group; + = compared with the rotenone group. / = compared with the EPI group. $^{*,/}$ = P < 0.05; $^{***,+++}$ = P < 0.0001. *Arrows*, neurites; *arrowheads*, astrocytes. Scale bar = 50 μm.

Supplementary Figures

Figure 1. Cell viability in cerebellar granule cell cultures exposed for 24 h to different concentrations of MCT alone (a) or combined with rotenone (b). Data were expressed as mean ± SEM of cell viability (% of control). One-Way ANOVA followed by post hoc Tukey multiple comparison test. Non-statistical differences were observed.

Figure 2. Cell viability in cerebellar granule cell cultures exposed for 24 h to the highest concentrations of CBD dissolved in DMSO or MCT. One-Way ANOVA followed by post hoc Tukey multiple comparison test. * = compared between concentrations of CBD; + = compared between vehicles. $^{***,+++}$ = P < 0.0001.