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ORIGINAL ARTICLE

Preliminary evaluation of the neuroprotective activity of Cannabidiol in combination with some current medicinal drugs *in vitro*

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Abstract

Objective: *The objective of this study was to evaluate the neurotoxicity and neuroprotective activity of cannabidiol (CBD), valproate, furosemide, metformin, and bilobalide, individually and mixed with CBD in PC12 cells exposed to Glutamate. In addition, antioxidant activity, reactive oxygen species (ROS) production, and caspase-3 activity were evaluated.* **Methods:** *Neurotoxicity and neuroprotection were evaluated with the MTT assay; antioxidant activity, and production of ROS with 2,2-diphenyl-1-picryl-hydrazyl-hydrate, and DCFDA methods, respectively; the apoptosis with caspase-3 activity assay.* **Results:** *CBD 5 μM, and bilobalide 15.3 μM showed neuroprotective activity by ROS inhibition and decreased caspase-3 activity. Metformin 1548.4 μM did not provide significant neuroprotection but decreased ROS generation.* **Conclusions:** *Combining CBD with the study drugs did not improve neuroprotection than the neuroprotection of individual drugs.*

Keywords: *Cannabidiol. Neuroprotection. Cell death.*

Evaluación preliminar de la actividad neuroprotectora del Cannabidiol en combinación con algunos medicamentos in vitro

Resumen

Objetivo: *Evaluar la neurotoxicidad y la actividad neuroprotectora del Cannabidiol, valproato, furosemida, metformina y bilobalida, individualmente y mezclados con Cannabidiol en células PC12 expuestas a Glutamato. Además, se evaluó la actividad antioxidante, la producción de especies reactivas de oxígeno y la actividad de la caspasa-3.* **Métodos:** *La neurotoxicidad y la neuroprotección se evaluaron con el ensayo MTT; la actividad antioxidante, y la producción de ROS con los métodos DPPH, y DCFDA respectivamente; la apoptosis con el ensayo de actividad caspasa-3.* **Resultados:** *El cannabidiol 5 μM, y el bilobalide 15,3 μM mostraron actividad neuroprotectora por inhibición de las especies reactivas del oxígeno y disminución de la actividad de la caspasa-3. La metformina 1548,4 μM no proporcionó una neuroprotección significativa, pero disminuyó la generación de especies reactivas de oxígeno.* **Conclusiones:** *La combinación de Cannabidiol con los fármacos del estudio no mejoró la neuroprotección que la neuroprotección de los fármacos por separado.*

Palabras clave: *Cannabidiol. Neuroprotección. Muerte celular.*

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Introduction

Neuroprotection is the prevention of neuronal cell death by intervening and inhibiting the pathogenetic process that causes neuronal dysfunction and death[1]. Compounds with this activity have the potential to treat neurodegenerative diseases. Although some therapies, such as pharmaceutical, herbal agents, surgical methods, exercise, ultrasound, and photobiomodulation[2], have been approved for managing and caring for these diseases, until now there is no cure.

Compounds like bilobalide and the current medicinal drugs valproic acid, furosemide, and metformin have shown neuroprotective effects in some studies, so they have the potential to be pharmacologically repositioned for other therapeutic use. On the other hand, cannabidiol (CBD), the main non-psychoactive phytocannabinoid of marijuana, has been proposed as an alternative for the treatment of several neuropsychiatric and neurological disorders[3]. This molecule has shown a neuroprotective effect through several mechanisms such as anti-inflammatory, immunomodulatory, decreasing inflammatory cytokines.[4]

The search for neuroprotective compounds is needed considering that there is a lack of medicinal drugs that limit neuronal damage; the potential neuroprotection of CBD; and the increased use of non-prescribed CBD products for treating different conditions. The objective of this study was to evaluate the neuroprotective activity of CBD individually and mixed with valproate, furosemide, bilobalide, and metformin in an *in vitro* model of neurotoxicity.

Materials and methods

CBD was purchased from Spex CertiPrep (Metuchen, NJ, USA), valproic acid, bilobalide, metformin, bilobalide, Trolox, glutamate, DCFDA, xanthine oxidase, 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH), dimethyl sulfoxide, MTT, H_2O_2 from Sigma-Aldrich, (St Louis, MO, USA). Furosemide from PiSA Laboratories (Santa Catarina, Mexico), RPMI-1640 medium, and kit EnzChek® Caspase-3 assay Kit #2 from Thermo Fisher Scientific. The PC12 cell line is part of the UANL molecular pharmacology and biological models laboratory catalog.

Cell culture

The PC12 cell was grown in RPMI-1640 medium, 20% fetal bovine serum, and 1% antibiotics (5 mg/mL penicillin, 5 mg/mL streptomycin). They were cultured in 25 cm^2 tissue-culture flasks at 37°C, 5% CO_2, and humidified atmospheric conditions. The medium was replaced every 2 days. Confluent cultures were washed with phosphate-buffered saline and detached with trypsin/ethylenediaminetetraacetic acid solution. Third passage cells with 80% confluence were used for experiments.

The concentrations used for each compound were as follows: CBD 5 μM[5], valproate 433.4 μM (therapeutic dose for seizure in patients with epilepsy and mood disorders)[6], bilobalide 15.3 μM (is the IC_{50} value)[7], furosemide 604.7 μM (is the ED_{50})[8], and metformin 1548.4 μM (neuroprotective against H_2O_2)[9]. Each compound was dissolved in an FBS-free medium.

Neurotoxicity assay

Ten thousand cells were placed in 96-well plates and incubated for 24 h. Compounds were added alone and in a binary combination with CBD at the concentrations previously mentioned and incubated for 24 h. The medium was replaced with [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) solution (0.5 mg/mL) and incubated for 3 h. Formazan crystals were solved with a solution of isopropyl alcohol/HCl (10%), and after 30 min, absorbance was measured at 550 nm in a microplate enzyme-linked immunosorbent assay reader ELx800 (Biotek instruments). Results were expressed as a percentage of cell viability[10]. Glutamate (25 mM) was the positive control for neurotoxicity, and glutamate-free cells as a control of 100% cell viability.

Neuroprotection assay

Ten thousand cells were placed in 96-well plates and incubated for 24 h. Then, a 2 h pre-treatment[11] with the compounds alone and mixed with CBD was added. Thereafter, 0.1 mL of glutamate 25 mM[12] was added, and incubated for 22 h. The medium was removed and replaced with MTT solution (0.5 mg/mL). The same protocol for MTT assay was performed. Results were expressed as a percentage of cell viability. Trolox® 50 μM was the positive neuroprotection control[13].

Two different concentrations of each drug, including CBD, were used for the preliminary evaluation of the combinations of CBD with the other studied drugs. The first concentration was the same used for the neuroprotective and neurotoxic assays; the second concentration was ½ of the concentrations previously

mentioned. The increase or decrease in cell viability was compared between the CBD combinations and the individual drugs.

Antioxidant activity

0.1 mL of each drug, alone and mixed with CBD 5 μM was placed in a 96-well plate, then 0.1 mL of 100 μM DPPH[14] solution was added. The microplate was incubated in darkness at room temperature for 15 min, then absorbance was measured at 517 nm. The negative control was wells containing DPPH solution. Results were expressed as a percentage of DPPH inhibition.

Inhibition of reactive oxygen species (ROS) production

Ten thousand cells/well were pretreated with CBD alone and mixed with the other molecules. Then, H_2O_2 (0.1 mM) was added, and incubated for 1 h. Then, 2',7'-dichlorofluorescein diacetate (H_2DCFDA)[15] solution (0.05 mg/mL) was added and incubated for 20 min. Fluorescence was measured at 485 nm of excitation and 530 nm of emission. Negative control was cells treated with H_2O_2 0.1 mM.

Caspase-3 activity

The EnzChek® Caspase-3 Assay Kit#2 was used according to the manufacturer's instructions. The assay was performed only for bilobalide and metformin alone and mixed with CBD. First, 1,000,000 cells were placed in a 35-mm petri dish and exposed to bilobalide and metformin alone and mixed with CBD. Glutamate 25 mM was added and incubated for 2 h. The medium was removed, cells harvested, washed with PBS, lysed, and centrifuged. To 0.05 mL of supernatant, 0.05 mL of the caspase-3 substrate Z-DEVD-R110 was added and set in darkness for 30 min at room temperature. Fluorescence was measured at 496 nm/520 nm excitation and emission in Fluoroskan Ascent (Thermo Fisher). Trolox® 50 μM was the positive control of inhibition of caspase-3, and glutamate 25 mM was the negative control.

Statistical analysis

Data are expressed as mean ± standard error of the mean of three independent experiments performed in triplicate (n = 9). Statistical comparisons were performed using one-way analysis of variance with Tukey's multiple comparisons test. The statistical software GraphPad Prism 5 was used for the analysis. A $p < 0.05$ was considered significant.

Results

Neurotoxicity assay

Individually, CBD and other drugs did not show neurotoxic activity, their cell viability ranging from 77.3 to 106.5%. Furosemide had significantly lower cell viability than CBD (77.3 vs. 106.5%; $p < 0.0001$). Combinations of CBD with other drugs did not show neurotoxicity, and the cell viability was above 80%. All drugs, both individually and combined with CBD, significantly increased cell viability compared to the neurotoxic control, glutamate 25 mM (cell viability 47%; $p < 0.05$); however, the cell viability of the combination of CBD with the other compounds was not better than individual drugs (Fig. 1).

Neuroprotection activity of individual molecules

The pre-treatment of PC12 cells with CBD, and subsequent exposition to glutamate, 25 mM, significantly increases 22.6% cell viability than non-pretreated cells (cell viability = 47%; $p < 0.05$). This neuroprotective activity, however, was lesser than the positive control of neuroprotection Trolox 50 μM (cell viability = 103.4%). Bilobalide (15.3 μM) showed neuroprotection (cell viability = 97.6%) similar to Trolox. The other studied compounds did not show neuroprotective activity (Fig. 2).

Neuroprotection activity of combined molecules

Only the pre-treatment of CBD combined with bilobalide showed neuroprotective activity since significantly increased the viability (108.3%) of glutamate-exposed cells ($p < 0.05$). However, this neuroprotection was not different than neuroprotection of individual compounds (Fig. 2).

Antioxidant activity

All drugs, individually and combined with CBD, showed lower antioxidant activity than Trolox 50 μM (71.35%) ($p < 0.0001$). The values of antioxidant activity ranged from 3.87% to 9.3% with no significant difference among them.

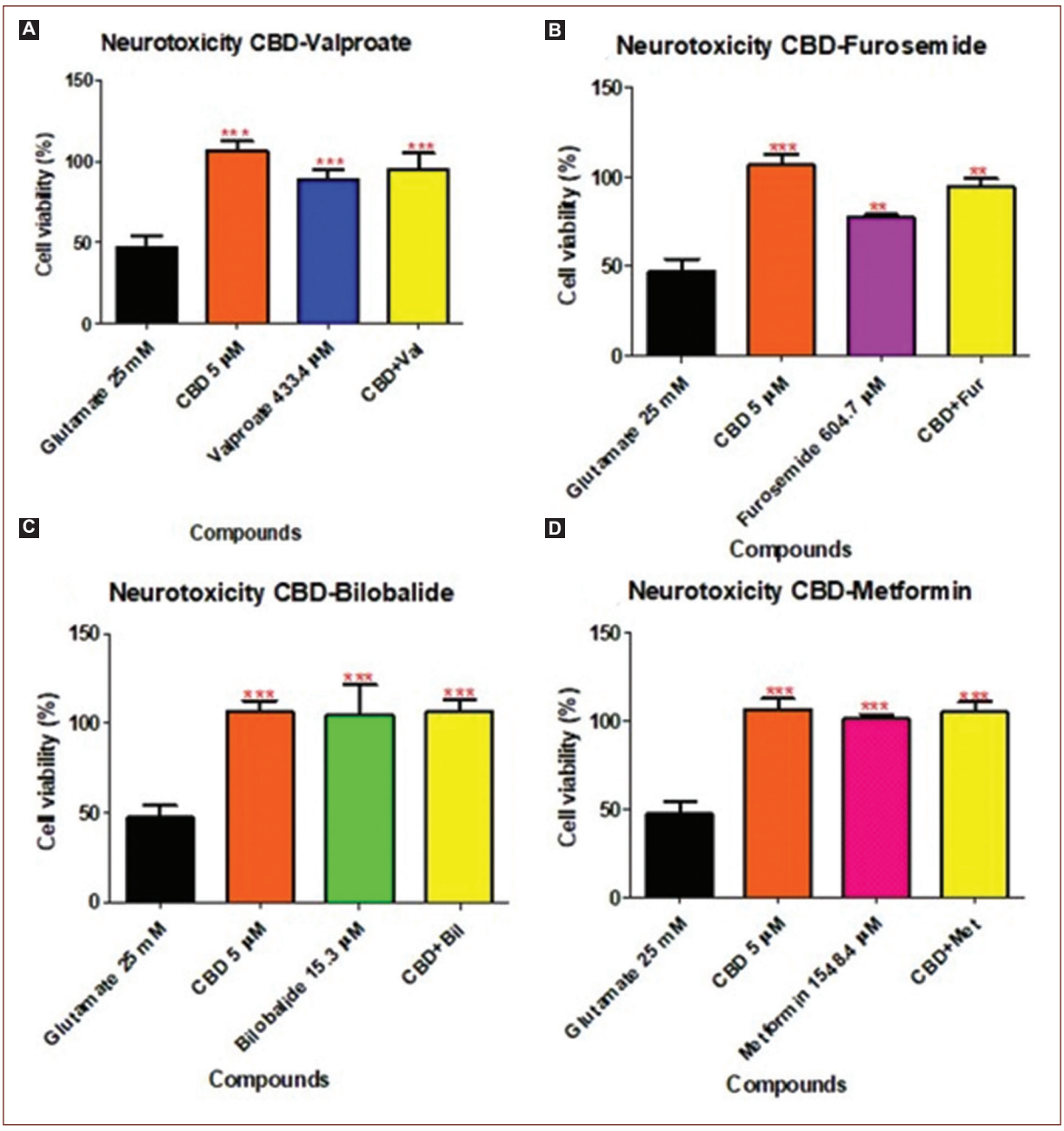

Figure 1. (A-D) Neurotoxic activity of CBD, alone and mixed with current medicinal drugs. ***p < 0.05 with respect to control; CBD: cannabidiol; Val: valproate; Bil: bilobalide: Met: metformin.

Inhibitory activity of ROS production

Only CBD, bilobalide, and metformin decreased the production of ROS (38.2%, 50.1%, and 39.5%, respectively) compared with H_2O_2 0.1 mM. However, these values were minor than Trolox 50 µM (78%) ($p < 0.0001$). The capacity of CBD to inhibit ROS production did not improve when it was mixed with the other molecules (Fig. 3).

Caspase-3 activity

Only CBD, alone and mixed with bilobalide, significantly decreased caspase-3 activity (40.9 and 38.5%, respectively). Although the combination of bilobalide plus CBD significantly decreased caspase 3 activity, this activity was not better than the activity of individual drugs (Fig. 4). There was no difference between the control (Trolox), CBD, and the combination CBD plus bilobalide.

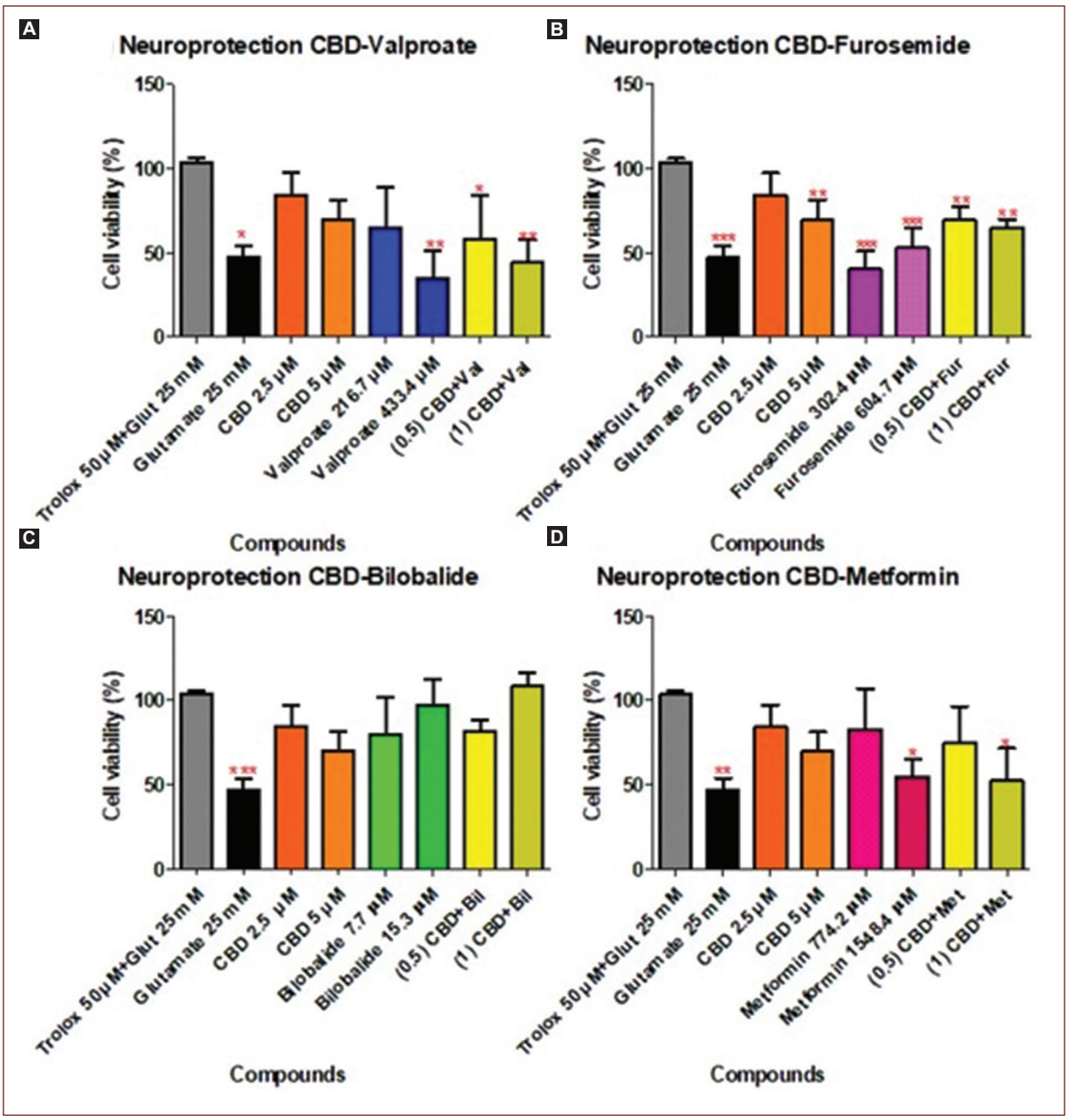

Figure 2. (A-D) Neuroprotection activity of CBD alone and mixed with current medicinal drugs. ***p < 0.05 with respect to control; CBD: cannabidiol; Val: valproate; Bil: bilobalide; Met: metformin.

Discussion

Individually, CBD and the other molecules studied did not showed neurotoxicity in PC12 cells. Previous reports indicate that CBD (10 μM) and valproate (1-5 mM) are neurotoxic in higher doses than used in the present study[16,17]. Bilobalide and metformin (2 mM) do not have neurotoxic activity in the PC12 cells[18,19] and SH-SY5Y cells,[20] respectively, which is similar to the present study. In the case of furosemide, there are no previous studies.

The pre-treatment of CBD showed neuroprotection that was similar to previous studies that use different cells and neurotoxic agent[5]. The mechanisms reported include decreased ROS accumulation, lipid peroxidation, caspase-3, DNA fragmentation, attenuation of intracellular calcium, and inhibition of iNOS and NO production[21,22]. The inhibition of ROS production, and

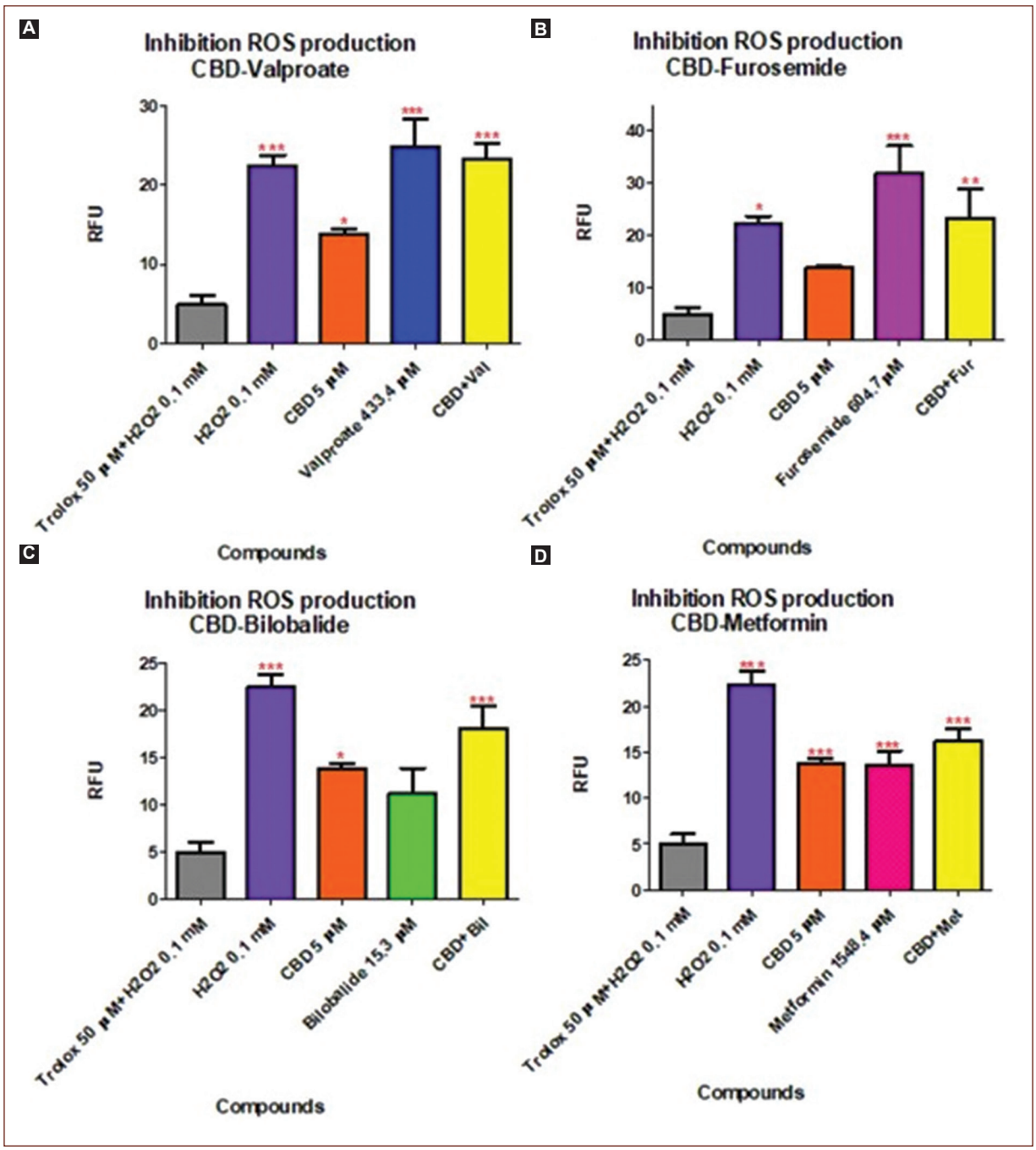

Figure 3. (A-D) Reactive oxygen species production inhibitory activity of CBD alone and in combination with valproate, furosemide, bilobalide, and metformin. ***p < 0.05 with respect to control: CBD: cannabidiol; Val: valproate; Bil: bilobalide; Met: metformin.

decrease in caspase-3 activity (40.9%) observed in the present study also has been reported in SH-SY5Y and PC12 cells[23,24].

Valproate does not show neuroprotective activity, which is contrary to previously reported, this could be due to the different dose and neurotoxic agents used. In a study, valproate (50-400 µM) increases cell viability (> 75%) of PC12 cells exposed to aluminum maltolate (1000 µM), the mechanism was by minor apoptosis, decreased ROS, catalase activity, and reduced mitochondrial membrane potential[25]. In another study with motor neurons treated with glutamate 100 µM,

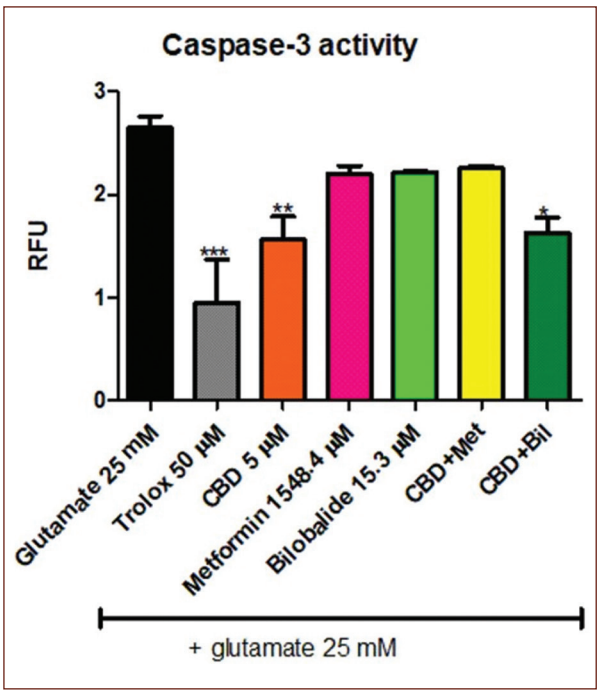

Figure 4. Caspase-3 activity of CBD alone and combined with bilobalide and metformin. **p < 0.05; CBD: cannabidiol; Bil: bilobalide.

valproate increased cell viability through the inhibition of histone deacetylase[26] and acetylation of factor transcription SP1[27,28].

In the present work, furosemide was studied for the 1st time in PC12 cells and did not show neuroprotective activity. This is contrary to reported previously in microglial cell line SIM-A9, where it decreased the proinflammatory microglia phenotype M1 and favored the anti-inflammatory phenotype M2 on cells exposed to lipopolysaccharides (5 ng/mL)[29]. The antioxidant activity was similar to that previously reported, but the inhibition of ROS production was different than reported in SIM-A9 cells[29,30].

As has been previously reported in PC12 cells (25-100 µM), and primary culture of astrocytes (100 µM), bilobalide has neuroprotective activity. Mechanisms include inhibition of ROS production by glucose and oxygen deprivation[31], decreased apoptosis by BAX, and caspase-3 activation induced by ROS[19]. The inhibition of ROS production is similar to that obtained in the present study.

Metformin (1.54 mM) did not show neuroprotective activity which is contrary to other studies and is explained by the different doses used. Higher doses (2 mM) demonstrated neuroprotective activity in PC12 cells and primary culture of hippocampal neurons exposed to H_2O_2 100 µM. The mechanism includes decreased apoptosis and necrosis, less intracellular ROS production, and protected mitochondrial membrane potential by AMPK activation[9]. Antioxidant activity (DPPH inhibition) was lower (5.14% at 1.54 mM) than previously reported, and it is explained by the higher doses used (31% at 20 mM)[32]. The decreased ROS production by metformin (39.4%) is similar to previously reported[9]. Contrary to other studies metformin does not significantly decrease caspase-3 activity and is explained by the different cells and concentrations used[33].

Results showed that CB combined with the studied drugs neither improve nor worsen the neuroprotection of each individual drug. Although with limitations, these results imply that the combinations studied do not produce a beneficial synergic effect in PC12 cells. It is necessary to confirm this with an isobolographic analysis.

There are no studies about the neuroprotective activity of CBD combined with these drugs *in vitro* or *in vivo* models; however, there are some anecdotal findings. In the case of valproate, a clinical case reports that CBD does not modify the antiepileptic effect of valproate[34]. In a clinical trial, CBD plus valproate increased hepatic aminotransferase enzymes[35]. Although the coadministration of CBD and furosemide has not been evaluated, some neuroprotective activity is possible since furosemide has a neuroprotective activity similar to CBD in an *in vivo* binge model induced by ethanol in rats. The combination of CBD plus bilobalide could have pharmacokinetic interaction since both molecules interact with the CYP1A2 isoform. The same could be for the combination of CBD plus metformin since both decrease CYP3A4 expression[36].

Limitations

Only two concentrations of each combination were used in this study. This approach does not permit concluding the type of interactions, such as synergism, summation, potentiation, or antagonism. However, our study permits discarding, in a preliminary way, a beneficial synergic effect of the CBD combination with the study drugs *in vitro*.

Conclusion

CBD and bilobalide present neuroprotective activity in PC12 cells exposed to glutamate 25 mM. The

neuroprotective activity of CBD did not improve when combined with furosemide, valproate, and metformin. Bilobalide, CBD, and metformin decrease ROS production. Only CBD decreases caspase-3 activity.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article. Furthermore, they have acknowledged and followed the recommendations as per the SAGER guidelines depending on the type and nature of the study.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Use of artificial intelligence for generating text. The authors declare that they have not used any type of generative artificial intelligence for the writing of this manuscript nor for the creation of images, graphics, tables, or their corresponding captions.

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