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1 **Evaluation of the efficacy of a full-spectrum medicinal cannabis plant extract with less than**
2 **0.3% Δ9-tetrahydrocannabinol in *in vitro* models of inflammation and excitotoxicity**

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14 **Running title** Efficacy of full-spectrum medicinal cannabis extract in preclinical models

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27 **Abstract**

28 The rapid development of research on the therapeutic benefits of medicinal cannabis, in parallel with
29 an increased understanding of the endocannabinoid system, has driven research of *Cannabis sativa*
30 constituents for managing neurological conditions. While most studies have focused on the therapeutic
31 potential of the major components of cannabis plant extract isolated or combined, limited research has
32 explored the pharmacological benefits of whole cannabis plant extract. In this study, we investigated
33 the potential anti-inflammatory and neuroprotective effects of NTI-164, a novel full-spectrum cannabis
34 extract with negligible Δ9-tetrahydrocannabinol (THC), compared with cannabidiol (CBD) alone in
35 BV-2 microglial and SHSY-5Y neuronal cells. The inflammation-induced upregulation of microglial
36 inflammatory mediators, being tumour necrosis factor α (TNFα), granulocyte-macrophage colony-
37 stimulating factor (GM-CSF), inducible nitric oxide synthase (iNOS), and Arginase-1 (Arg-1), were
38 significantly attenuated by NTI-164. This immunomodulatory effect was not observed upon treatment
39 with isolated CBD. Compared to CBD alone, NTI-164 prevented elevated mitochondrial activity while
40 normalising cell numbers in immune-activated microglia cells. NTI-164 also promoted the proliferation
41 of undifferentiated neurons and the survival of differentiated neurons under excitotoxic conditions.
42 Overall, our work shows that the anti-inflammatory and neuroprotective effects of NTI-164 as a full-
43 spectrum cannabis extract are enhanced relative to that of CBD alone, highlighting the potential
44 therapeutic efficacy of NTI-164 for the treatment of neuropathologies such as autism spectrum disorder
45 (ASD) and related neuropathologies. This study has further shown that understanding the synergistic
46 effect of phytocannabinoids is integral to realising the therapeutic potential of full-spectrum cannabis
47 extract to inform the design of botanical-derived treatments for managing neurological disorders.

48

49 **Keywords:** Autism, botanical synergy, cytokines, full-spectrum cannabis extract,
50 neurodevelopmental disorders

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51 **Introduction**

52 Despite differences in clinical profiles, genetics, and symptoms among neurological diseases, many
53 central nervous system (CNS) disorders share substantial similarities in cerebrovascular impairments
54 and immune dysregulation associated with neuroinflammation as the underlying pathophysiological
55 mechanism of disease [1, 2]. In particular, the role of the neuroimmune axis in the pathogenesis of
56 neurodevelopmental disorders, including autism spectrum disorder (ASD), has been the focus of intense
57 research during the past decade [3-6]. The hallmarks of ASD are social and behavioural deficits, often
58 associated with intellectual disabilities, hyperactivity, anxiety, epilepsy and cognitive impairments [7,
59 8]. Despite the rising prevalence of this neurodevelopmental disorder [9, 10], significant economic
60 burden [11, 12], and ongoing research efforts, the underlying etiopathogenesis of ASD remains unclear.
61 The aetiology of ASD involves intricate interactions between multiple genetic and environmental risk
62 factors during the critical period of nervous system development, resulting in aberrant neuroplasticity
63 with long-term alterations in brain physiology and behavioural processes [13, 14]. The complex clinical
64 profile of ASD, compounded with significant genetic heterogeneity, has further hindered efforts to
65 dissect underlying disease mechanisms and develop effective pharmacological therapies [15, 16].
66 Consequently, there are no effective medications to address the core symptoms of ASD, with the current
67 pharmacotherapies targeted at ASD comorbidities frequently associated with adverse side effects and
68 relatively low efficacy [17, 18].

69 Accumulating evidence highlights the potential role of neuroglial cells and, in particular, disruption of
70 homeostatic microglial function directly contributing to ASD etiopathogenesis [5, 6]. Microglia are
71 myeloid cells of mesodermal origin and the only glial cells to enter the developing brain preceding
72 neurogenesis during the perinatal period [19, 20]. Microglia have a diverse array of physiological
73 functions in adulthood and mediate key developmental processes, including astrocyte formation, early
74 synaptogenesis, synaptic plasticity, synaptic remodelling (pruning) plus the regulation of adult
75 neurogenesis [5, 19, 21, 22]. In response to signs of homeostatic disturbance, microglia shift activity
76 states and transform from homeostatic to an 'activated state', such as immune activation, which can
77 result in distinct response phenotypes [23]. Depending on the pathological condition and its impacts on

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78 the local microenvironment, microglia contribute various immune-related activities through the
79 differential release of molecules, including pro-inflammatory mediators, anti-inflammatory cytokines,
80 or neurotrophins [5]. The activation states of microglia in response to brain insults have historically
81 been broadly divided into the two reactive phenotypes of an M1 pro-inflammatory response and an M2
82 neuroprotective response [22]. Although an oversimplification [24], these terms are helpful for basic
83 function descriptions in simplified systems, such as *in vitro* paradigms like those in this study.

84 Consistent with human and animal studies that have demonstrated glial abnormalities in ASD-related
85 phenotype [3, 13, 25-28], several imaging studies have further reported the presence of markedly
86 immune-activated microglia cells with morphological alterations in multiple brain regions of young
87 ASD patients [29-32]. The role of microglia in neuroinflammation in these individuals is also supported
88 by the observation of differential expression of inflammatory cytokines in different brain regions of
89 children with ASD [33, 34].

90 Endogenous endocannabinoids, together with their receptors (CB1 and CB2) and associated metabolic
91 enzymes (the endocannabinoid system, ECS), are critical regulators of early neuronal plasticity [35]
92 and behavioural processes [36]. In immune-activated microglia, CB2 receptors modulate synaptic
93 activity and neuroinflammatory responses [37]. A large body of evidence further supports the
94 involvement of ECS in ASD-related neurodevelopmental processes, as aberrant endocannabinoid
95 signalling pathways are linked to ASD pathogenesis [38-40]. Moreover, recent clinical studies in
96 children with ASD have demonstrated altered endocannabinoid-CB2 cellular signalling [41] and
97 circulating endogenous cannabinoids [42, 43], underpinning the importance of the ECS as a potential
98 therapeutic target for ASD and related neuroinflammatory diseases.

99 The mounting evidence for the involvement of ECS in ASD-related neurodevelopmental processes, in
100 parallel with the well-researched therapeutic potential of medicinal cannabis, has heightened interest in
101 using cannabis-derived compounds to treat neurodevelopmental disorders. For centuries, the
102 therapeutic benefits of *Cannabis sativa* L. extracts have been widely recognised in the treatment of a
103 broad spectrum of nervous system-related conditions ranging from common neurological and
104 hyperexcitability disorders such as epilepsy, affective disorders (e.g. anxiety) to neurodegenerative

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105 disorders such as Alzheimer's disease [44]. The role of the most abundant non-psychoactive
106 phytocannabinoid cannabidiol (CBD) in the modulation of inflammatory and immune responses has
107 been extensively documented [45-47]. Most current research on treating common neurological and
108 psychiatric disorders is focused on the application of CBD alone or combined with the major
109 psychoactive cannabis component Δ9-tetrahydrocannabinol (THC) [48-50]. Several lines of evidence
110 from experimental models suggest that individual phytocannabinoids derived from the *Cannabis sativa*
111 plant modulate microglial functions by blocking excitotoxic-induced activation [51], inhibiting
112 intracellular calcium increase [52] and modulating inflammatory responses [53, 54].

113 Clinical evidence is emerging that to improve the safety profile and enhance the therapeutic effects of
114 cannabis-based treatments, we should be administering CBD combined with other phytocannabinoids
115 for treating children with neurodevelopmental disorders such as ASD and behavioural-related
116 symptoms [55-58]. Nearly 25 years ago, the term' entourage effect' was coined to describe the
117 synergistic contributions of the multitude of endogenous endocannabinoids in cannabis [59]. The
118 benefits of the entourage effect over single isolates have been demonstrated in preclinical models of
119 neurological disorders [60-62] and cancer [63-65]. Increasingly, specific breeding programs are
120 generating cannabis lines with low THC, often known as hemp plants, but with higher levels of other
121 cannabinoids.

122 Therefore, this study investigated the entourage effect of a full-spectrum medicinal hemp strain
123 cannabis plant extract with only 0.08% THC (NTI-164) on inflammatory and excitotoxic responses in
124 well-established preclinical *in vitro* models.

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125 **Materials and Methods**

126 **Isolation of NTI-164**

127 NTI-164 sativa plant was grown and harvested following the natural process of open pollination. After
128 harvesting, we ground the dried plant using a commercial herb grinder until the particulates were
129 approximately 1mm in size. This was to increase the surface area of the plant for subsequent isolation
130 of plant extract. The ground plant was then mixed at a ratio of 1g to 10 mL with 100% ethanol, and then
131 this mixture was placed on a rocker at 50 rpm for 4 hours. We then aspirated the mixture into a new
132 tube, and the remaining particulates were removed via centrifugation at 300*G* for 10 minutes. We stored
133 the final extract at -20°C until use, and an aliquot was sent for analysis with ultra-high-performance
134 liquid chromatography (U-HPLC) to assess the purity and composition of the final extract.

135 **Ultra-high-performance liquid chromatography (U-HPLC)**

136 We undertook experiments using an integrated U-HPLC system and a single quadrupole mass
137 spectrometer (MS) detector with an electrospray ionization (ESI) interface. The following ten
138 cannabinoids were combined: CBD, cannabidivarin (CBDV), cannabidiolic acid (CBDA),
139 cannacannabigerol (CBG), tetrahydrocannabivarin (THCV), cannabinol (CBN), Δ9-
140 tetrahydrocannabinol (Δ9-THC), Δ8-tetrahydrocannabinol (Δ8-THC), cannabichromene (CBC). We
141 prepared a mixture to contain 10 parts per million (ppm) of each of the ten cannabinoids in methanol.
142 All solvents were liquid chromatography-MS grade with standards prepared by diluting with 90%
143 mobile phase B and 10% deionized water. Reference standard solutions previously shown to be suitable
144 for generating calibration curves were obtained as pre-dissolved solutions from Novachem, Cerilliant
145 Corporation (TX, USA).

146 **Microglial BV2 cell culture**

147 The immortalised microglia cell line, BV2 (EP-CL-0493, Elabscience), was cultured in Rosewell Park
148 Memorial Institute (RPMI) 1640 media containing 2mM L-glutamine (Gibco, Cat# 11875) containing
149 0.1% gentamycin (Thermo, Cat# 15750060) and supplemented with heat-inactivated newborn calf

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150 serum (NCS; Gibco, Cat# 26010-074) at a concentration of 10% for expansion and 5% when plated for
151 experiments. All cells were from between passage numbers 5 and 10. Cells were plated at 45,000
152 cells/mm^2 and treated 24 hours after plating. Here, cells were treated with 4μL of either phosphate-
153 buffered saline (PBS; Gibco, Cat# 100100) to serve as a control (i.e., unstimulated cells) or interleukin-
154 1β (IL-1β; 50ng/mL; Miltenyi Biotec, Cat#130-101-682) plus interferon-γ (IFNγ; 20ng/mL; Miltenyi
155 Biotec, Cat# 130-105-785) to induce inflammation (i.e., immune activation). We based the cell
156 culturing and immune activation techniques described here on established protocols from our lab [66-
157 68]. After 1 hour of post-treatment with either PBS or IL-1β+ IFNγ, we applied a 10μL dose of either
158 NTI-164 extract or CBD (1mg/mL), and we undertook the analysis at 24 hours post-treatment. As NTI-
159 164 extract and CBD were dissolved in ethanol for this investigation, all control (PBS-treated) wells
160 also contained a matched ethanol concentration of 0.1%. An independent replicate (n=1) is cells plated
161 and treated from one stock flask per day.

162

163 **Neuronal SHSY-5Y cell culture**

164 The immortalised neural precursor cell line, SHSY-5Y (The American Type Culture Collection, Cat#
165 CRL-2266), was used to assess the effect of NTI-164 or CBD treatment on either neuronal
166 differentiation or in response to excitotoxic injury. For expansion, we cultured cells in Dulbecco's
167 Modified Eagle Medium (DMEM, Gibco, Cat# 11995) that contained 1g/L D-glucose, 584mg/L L-
168 glutamine, 110mg/L sodium pyruvate, and phenol red, further supplemented with 0.1% penicillin-
169 streptomycin (Sigma-Aldrich, Cat# P4458) and 10% NCS. We plated SHSY-5Y cells at 45,000
170 cells/mm^2 for experiments.

171 For work on differentiated cells, we altered the media to contain 1% NCS and daily delivery of 10uM
172 all-trans-retinoic acid (Sigma-Aldrich, Cat#R2625 dissolved in PBS). Specifically, we differentiated
173 the cells for five days with daily half-volume media changes before the excitotoxicity assays. We
174 exposed the SHSY-5Y to 3 mM glutamate (Sigma-Aldrich, Cat# G815) dissolved in PBS, delivered as
175 a 10uL dose. After 1 hour, cells were treated with NTI-164 or CBD as previously described for BV2

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176 experiments, with analysis conducted 24 hours post-treatment. For undifferentiated culture conditions,
177 cells were plated in DMEM as described above. An independent replicate (n=1) is cells plated and
178 treated from one stock flask per day.

179 **Multiplex Cytokine/Chemokine Assay**

180 In separate sterile 96-well plates, 300uL of 10% bovine serum albumin (BSA) dissolved in PBS (BSA
181 buffer) was added (to prevent any cytokine loss to the plastic), and we incubated plates for 30 minutes
182 at room temperature, then discarded the contents, and air-dried the plates. We then used these protein-
183 blocked plates to collect BV2 culture media at 24 hours following the initiation of the treatment
184 protocol. Media was immediately centrifuged to remove particulate at 300*G* for 10 minutes with
185 supernatant, then collected in an additional BSA-blocked plate and stored at -80°C until use in multiplex
186 experiments. We measured the cytokine and chemokine levels in the media using a Bio-Plex 200 as per
187 the manufacturer's instructions (Bio-Rad, Cat# M60000007A). Cytokines and chemokines measured
188 included IL-2, IL-10, IL-5, GM-CSF, and TNFα, as per our previous work [69, 70]. We ran all samples
189 in duplicate with data analysed via the Bio-Plex Manager software from BioRad.

190

191 **Immunohistochemistry**

192 Plated cells were fixed for 10 minutes with 2% paraformaldehyde (PFA; VWR Chemicals, Cat#
193 28794.364) in phosphate buffer and washed for 3x5 minutes with PBS. All washes were for 3x5 minutes
194 throughout the protocol. Plates were stored at 4°C in 150uL PBS containing 0.02% sodium azide
195 (Sigma- Aldrich, Cat# S-2002). Immediately before staining, cells were washed and then incubated for
196 30 minutes with 100uL of BSA buffer containing 0.01% Triton X (Sigma-Aldrich, Cat# X100).
197 Following this, we removed the 75uL of BSA buffer and added 25uL of primary antibody diluted in
198 PBS to obtain the final concentration, as detailed in Table 1. We incubated the plates overnight at 4°C
199 and washed them the following day. Afterwards, 50uL of the corresponding fluorescent secondary
200 antibody (see Table 1) was applied for 1 hour at room temperature and then washed. To visualise cell
201 nuclei, 4',6-diamiino-2-phenylindole (DAPI; Invitrogen, Cat# D21490) diluted 1:1000 in PBS was

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202 applied for 15 minutes at room temperature with plates subsequently washed. For imaging,
203 photomicrographs were taken using the EVOS M5000 (Invitrogen, Cat# AMF5000) in three fields of
204 view per well from duplicate wells and analysed using Fiji 2 to determine the area coverage of each
205 marker. We undertook the staining and analysis per our previous work [71, 72].

206

207 **Table 1. Primary and secondary antibodies**

208

Antibody	Concentration	Supplier
Mouse α-ARG1	1:250	Abcam, Cat# AB239731
Rabbit α-COX2	1:500	Abcam, Cat# AB15191
Rabbit α-iNOS	1:250	Abcam, Cat# AB178945
Mouse α-Beta III Tubulin	1:500	R & D, Cat# MAB1195
Goat α-Rabbit IgG, Alexa Fluor 555	1:500	Invitrogen, Cat# A-21428
Goat α-Mouse IgG, Alexa Fluor 488	1:500	Invitrogen, Cat# A-11001

209 **Abbreviations**. α-: anti; Arg-1: Arginase-1; COX-2: cyclooxygenase-2; IgG: Immunoglobulin G;
210 iNOS: inducible nitric oxide synthase

211

212 **Cell Viability (Mitochondrial Activity) Assay**

213 We assessed mitochondrial activity using the 3-(4,5-dimethylthiazol-2-yl-)-2,5-diphenyl-2H-
214 tetrazolium bromide (MTT) assay (Sigma-Aldrich, Cat# M6494). As a tetrazolium dye, MTT is bio-
215 reduced by the mitochondria into a formazan product that is insoluble in the tissue culture medium.
216 Upon being dissolved, we quantified the formazan content via a colourimetric analysis. In brief, we
217 added MTT to the wells at a final concentration of 250µg/mL. After 30 minutes, we carefully removed
218 and discarded the media, then dissolved the formazan within the wells in dimethylsulfoxide (DMSO;
219 Sigma-Aldrich, Cat# D2650) and absorbance measured at 490nm using a spectrophotometer (Glomax
220 Multi+; Promega, UK).

221

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222

223 **Statistics**

224 We averaged the data for replicates within experiments, and then data from at least three independent
225 experiments was analysed using GraphPad Prism software (GraphPad Software, Inc.). We have outlined
226 the replicate numbers for each experiment and the specifics of the statistics for each analysis in the
227 figure legends. We expressed all data as mean ± standard error of the mean (SEM) with significant
228 differences between groups set at a p-value of less than 0.05 ($P<0.05$)

229

230 **Results**

231 **Chemical characterisation of NTI-164 constituents by U-HPLC**

232 The U-HPLC analysis revealed that NTI-164 was rich in the acidic precursor of CBD, CBDA as the
233 main constituent of the extract (up to 60% wt/wt). The extraction procedure also generated a solution
234 of up to 14% wt/wt CBD, 0.44% wt/wt CBG, 0.06% CBDV and 0.08% wt/wt THC content. The HPLC
235 chromatogram was recorded at 229 nm, and we have shown an example in Figure 1.

236 **Effect of NTI-164 on energy production and number of BV-2 microglia**

237 In unstimulated BV2 microglial cells (treated with PBS + EtOH excipient), NTI-164 or CBD treatment
238 had no significant effect on mitochondrial activity as assessed via the MTT assay (Figure 2A). In line
239 with prior findings, we observed that mitochondrial activity was significantly increased in immune-
240 activated cells stimulated with IL-1B+IFN-y (+EtOH excipient). While NTI-164 prevented this
241 increase, CBD was not (Figure 2B). In line with this, exposure to NTI-164 or CBD in unstimulated cells
242 did not significantly alter overall cell number as assessed via area-coverage analysis of DAPI staining
243 (Figure 2C). Interestingly, CBD significantly increased the area coverage of DAPI staining in immune-
244 activated cells. We did not observe any impacts of NTI-164 treatment on DAPI staining in the immune-
245 activated cells (Figure 2D).

246

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247

248 **Effect of NTI-164 on the expression of inflammatory markers in BV-2 microglial**

249 In immune-activated microglia, NTI-164 treatment resulted in a significant decrease in the expression
250 of inducible nitric oxide synthase and arginase-1, which was not observed in CBD-treated cells (Figure
251 3A, B). Furthermore, NTI-164 and CBD were both able to significantly reduce the expression of COX-2
252 from the immune-activated microglia (Figure 3C).

253

254 **Effects of NTI-164 on microglia cytokine production**

255 In immune-activated BV2 cells, treatment with NTI-164 or CBD led to no significant change in the
256 levels of the anti-inflammatory cytokines IL-4 and IL-10 (Figure 4A, B). However, treatment with NTI-
257 164 significantly reduced the expression of the growth and differentiation-inducing chemokine GM-
258 CSF, but this finding was not observed in CBD-treated cells (Figure 4C). NTI-164 and CBD
259 significantly decreased the production of the pro-inflammatory IL-2 (Figure 4D), with no significant
260 alteration to levels of inflammatory cytokine IL-5. Finally, NTI-164 but not CBD was able to reduce
261 levels of TNFα significantly (Figure 4F).

262 **Effect of NTI-164 and CBD on neuronal responses**

263 In undifferentiated neurons, NTI-164 treatment significantly increased the area coverage of DAPI
264 staining, while CBD treatment resulted in no detectable change to DAPI staining (Figure 5A). However,
265 neither treatment induced spontaneous differentiation as assessed via expression of Beta III Tubulin
266 (Figure 5B). In differentiated neurons, neither NTI-164 nor CBD altered the number of cells or the
267 amount of de-differentiation (Figure 5 C, D). In a paradigm of excitotoxicity, NTI-164 significantly
268 increased the mitochondrial activity (survival) of neurons, while CBD could not rescue the
269 mitochondrial activity (Figure 5E).

270

271

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272

273 **Discussion**

274 The current study sought to determine the impact of a full-spectrum medicinal cannabis plant extract
275 (NTI-164) using *in vitro* models of neuroinflammation and neuroinjury. In our study, NTI-164
276 normalised inflammation-induced changes in immune-activated microglia cells by preventing increased
277 mitochondrial activity and promoting the survival of differentiated neurons under excitotoxic
278 conditions. As a common feature of neurodegenerative and neurodevelopmental disorders,
279 neuroinflammation typically involves activation of resident immune cells of CNS (glia cells) and
280 chronic production of cytokines together with reactive oxygen intermediates resulting in immune cell
281 infiltration, excitotoxicity with subsequent damage cells and to the blood-brain-barrier integrity [73].
282 In particular, consistent evidence supports the contribution of microglia-mediated neuroinflammatory
283 response in the pathogenesis of ASD [74]. In children with ASD, an abnormal cytokine profile has been
284 reported [75], with elevated levels of pro-inflammatory cytokines predominantly in children with more
285 aberrant behaviours [76]. In parallel, the ECS is involved in modulating microglial phenotype and
286 mediating inflammatory signalling pathways in neuropathology [77]. Compared to neurotypical
287 children, an abnormal profile of ECS has been reported in the peripheral blood of children with ASD
288 [41-43]

289 Phytocannabinoids or exogenous cannabinoids are abundant in the *Cannabis sativa* plant and have been
290 found to influence microglia regulation of inflammatory response through various biological
291 mechanisms [51, 52]. The therapeutic benefits of combined phytocannabinoids have been consistently
292 demonstrated in several experimental models [52, 78-81] and clinical research studies [82], in particular
293 for the treatment of ASD-related symptoms [56, 57, 83-85]. In an amyotrophic lateral sclerosis animal
294 model, daily treatment of mice with a phytocannabinoid-enriched botanical extract (Sativex) for 20
295 weeks resulted in a significant decrease in the progression of neurological impairment [81]. In another
296 multiple sclerosis model, treatment with Sativex for ten days reduced microglial immune-related
297 activity and expression of pro-inflammatory cytokines [80]. In a more recent experimental study, THC
298 and CBD combination treatment (but not either compound alone) in a murine multiple sclerosis model

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299 led to reduced levels of pro-inflammatory cytokines such as IL-6 and TNF-α while increasing
300 production of anti-inflammatory cytokines [62]. The authors proposed the underlying anti-
301 inflammatory and neuroprotective mechanism of the combined phytocannabinoids to involve cell cycle
302 arrest, apoptosis and induction of anti-inflammatory cytokines in immune-activated brain cells through
303 regulation of miRNA-mediated signalling pathways [62].

304 In this study, the full-spectrum cannabis extract NTI-164 effectively attenuated elevated mitochondria
305 activity and normalising cell numbers in immune-activated microglia cells and CBD alone did not. We
306 also found that NTI-164 was able to prevent the increase of inflammatory mediators TNFα, GM-CSF,
307 iNOS and Arg-1 and significantly reduced their levels under inflammation-induced conditions.
308 Microglial activation is an important neuroglial response to injury or any pathological event, as
309 microglia are the chief cells engaged in immune surveillance [86]. Animal models and human studies
310 consistently demonstrate an abnormal microglial function associated with ASD-related symptoms. In
311 an inflammatory animal model, microglia activation resulted in ASD-like neurobehavioral deficit [87],
312 which has been linked to impaired ECS signalling, including elevated levels of endocannabinoids and
313 its metabolic enzymes in the amygdala region of the brain [88]. In children with ASD, studies have
314 shown activation of microglia and altered inflammatory gene expressions in multiple brain regions,
315 providing strong evidence supporting that neuroinflammation is involved in ASD pathogenesis [33, 34,
316 89]. Evidence from a recent systematic review which revealed morphological alterations of activated
317 microglia in various brain regions of individuals with ASD provides additional support for microglia-
318 mediated neuroinflammation in the development of ASD [74]. Disruption in microglia function
319 mediated by neuroactive substances can lead to marked neuronal impairment, contributing to neuronal
320 dysfunction in neurodevelopmental disorders such as ASD [90]. Cannabis extracts may provide
321 potential therapeutic benefits by targeting microglia-induced neuroinflammation in neuropathology.

322 Both NTI-164 and CBD significantly decreased IL-2 and COX-2 levels in immune-activated cells.
323 Similarly, previous studies have shown that whole cannabis extract [91] and the acidic cannabidiol
324 CBDA [92] exhibit anti-inflammatory effects by inhibiting COX-2 activity in cell culture. Notably,
325 NTI-164 and CBD did not affect IL-5, IL-4 or IL-10 levels, which contrasts with previous studies

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326 demonstrating changes in the production of these cytokines induced by CBD under inflammatory
327 conditions [93]. The variability in our findings and previous studies could be due to the differences in
328 physiological responses to cannabis extracts, such as bell-shaped dose or dose-dependent responses
329 [94], which needs to be explored in future studies. The neuroprotective and anti-inflammatory profiles
330 of NTI-164 will be further characterised using *in vivo* models of neuroinflammation.

331 Although the underlying positive mechanisms of phytocannabinoids are challenging to characterise due
332 to the complexity and diversity of the pharmacodynamic profile, a number of mechanisms for
333 attenuation of inflammation have been proposed. A recent study showed that the anti-inflammatory
334 effect of CBD involved inhibiting oxidative stress-activated NF-κB-dependent signalling pathway
335 through regulation of NADPH production and glucose uptake [95]. In addition to the potential
336 modulation of intracellular antioxidant pathways, other pharmacological anti-inflammatory
337 mechanisms include modulating calcium signalling [96] and through increased peroxisome proliferator-
338 activated receptor γ-dependent activation [97].

339 Apart from its anti-inflammatory effect, NTI-164 treatment significantly increased the proliferation of
340 neural progenitors and promoted the survival of neurons under a cytotoxic state, whereas CBD had no
341 effect. Congruent with our findings, CBD alone did not enhance neural progenitor cell proliferation
342 [98], which could either indicate the observed effect was due to botanical synergy or due to variability
343 of CBD dose-response. However, Luján and Valverde (2020) suggest that CBD exerts pro-neurogenic
344 effects only after the generation of newborn neurons [99]. Nevertheless, the effect of phytocannabinoids
345 on neural progenitor cell proliferation requires further investigation. Several biological mechanisms
346 involved in CBD-mediated neuroplasticity protection and survival have been investigated as candidates
347 [100], including extracellular-signal-regulated kinases [101], glycogen synthase kinase 3β signalling
348 [102, 103], and mammalian target of rapamycin pathways [104]. Given the interaction between
349 phytocannabinoids and the ECS, molecular mechanisms underlying the synergistic effect of cannabis
350 constituents on neurogenesis in preclinical models need further investigation.

351 Together, these findings suggest that the anti-inflammatory effect of NTI-164 is likely due to the
352 synergistic interaction of the cannabis extract derivatives rather than isolated CBD supporting the

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353 entourage effect of full-spectrum cannabis derivatives and suggesting a role for botanical synergy in the
354 phenotypic transition of microglial cells. These results need to be confirmed and warrant further
355 investigation of phytocannabinoid interaction in preclinical *in vivo* models to explore the potential for
356 distinct effects of full-spectrum cannabis extracts compared to isolated compounds.

357

358 **Conclusions**

359 Preclinical studies set the foundation for designing clinical trials using medicinal cannabis extract to
360 treat various neuropathologies. In this study, the anti-inflammatory and neuroprotective effects of the
361 chemically characterised full-spectrum cannabis extract NTI-164 were explored in immune-activated
362 cells. NTI-164 showed higher efficacy than isolated CBD in attenuating inflammatory cytokines and
363 promoting neuronal survival in the cytotoxic paradigm. Overall, these findings provide additional
364 support for full-spectrum medicinal cannabis plant extract as a valid and safe therapeutic intervention
365 for treating ASD-related and other neurological disorders. The growing evidence for the therapeutic
366 benefit of whole-plant cannabis extracts in treating complex neurological disorders, which require a
367 multi-mechanistic approach, is promising and supports further research. Future studies need to
368 characterise the effects of NTI-164 on key anti-inflammatory and immunomodulatory mechanisms
369 involved in neurological pathogenesis and progression *in vivo*. Also, the synergistic effect of
370 phytocannabinoids in modulation of microglial cell functions warrants further investigation. Further
371 examination of the optimal dose of NTI-164 and treatment duration may also enhance efficacy and
372 expected outcomes.

373

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376

377

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378 **Figure Legends**

379 **Figure 1.** A representative HPLC chromatogram of the NTI-164 extract was recorded at 229 nm,
380 according to the method used to analyse cannabinoids. Representing the main component, the CBD
381 acidic precursor, CBDA eluted at 3.41 minutes, CBD eluted at 3.08 minutes, CBN at 4.57 minutes,
382 THC at 5.35 minutes and THCA at 8.45 minutes. Abbreviations**.** CBD: cannabidiol; CBDA:
383 Cannabidiolic acid; CBN: cannabinol, THCA: tetrahydrocannabinolic acid; THC:Δ9-
384 tetrahydrocannabinol

385 **Figure 2.** Effect of NTI-164 and CBD on MTT outputs in BV-2 microglia in **A)** unstimulated condition,
386 **B)** immune-activated condition and overall DAPI-positive cell number in **C)** unstimulated condition,
387 **D)** immune-activated condition. Data expressed as Mean ± SEM, n=6-10. Significance set at *, p<0.05;
388 **, p<0.01; ***, p<0.001.

389 **Figure 3.** Effect of NTI-164 and CBD on the expression of inflammatory markers in BV-2 microglia
390 cells. **A)** inducible-nitric oxide synthase (iNOS), **B)** arginase-1 (Arg1), and **C)** cyclo-oxygenase-2
391 (COX2) protein expression normalised to DAPI-positive cell number in the control (PBS only) group.
392 Data expressed as Mean ± SEM, n=6-8. Significance set at *, p<0.05; **, p<0.01; ***, p<0.001.

393 **Figure 4.** Impact of NTI-164 and CBD on inflammation-induced cytokine release from BV2 microglia.
394 In **A)** interleukin-4 (IL-4), **B)** interleukin-10 (IL-10), **C)** Granulocyte-macrophage colony-stimulating
395 factor (GM-CSF), **D)** interleukin-2 (IL-2), **E)** interleukin-5 (IL-5) and **F)** tumour necrosis factor-alpha
396 (TNF-α) analysed by multiplex immunoassay and expressed as fluorescent units. Mean ± SEM, n=6-7.
397 Significance set at *, p<0.05; **, p<0.01; ***, p<0.001.

398 **Figure 5.** Impacts of NTI-164 and CBD on SHSY-5Y neurons**.** In **A)** impacts on numbers of DAPI-
399 positive cells in undifferentiated cultures, and in **B)** impacts on the expression of beta-tubulin (BTUB)
400 as a proposition of DAPI-positive cells as an indicator of spontaneous differentiation in undifferentiated
401 cells. In **C)** impacts on numbers of DAPI-positive cells in differentiated cultures, and in **D)** impacts on
402 BTUB expression in differentiated cells, and **E)** impacts on the MTT outputs (mitochondrial activity

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403 assay) in differentiated cells in the glutamate exposure excitotoxicity assay. Mean ± SEM, n=6-10.

404 Significance set at *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

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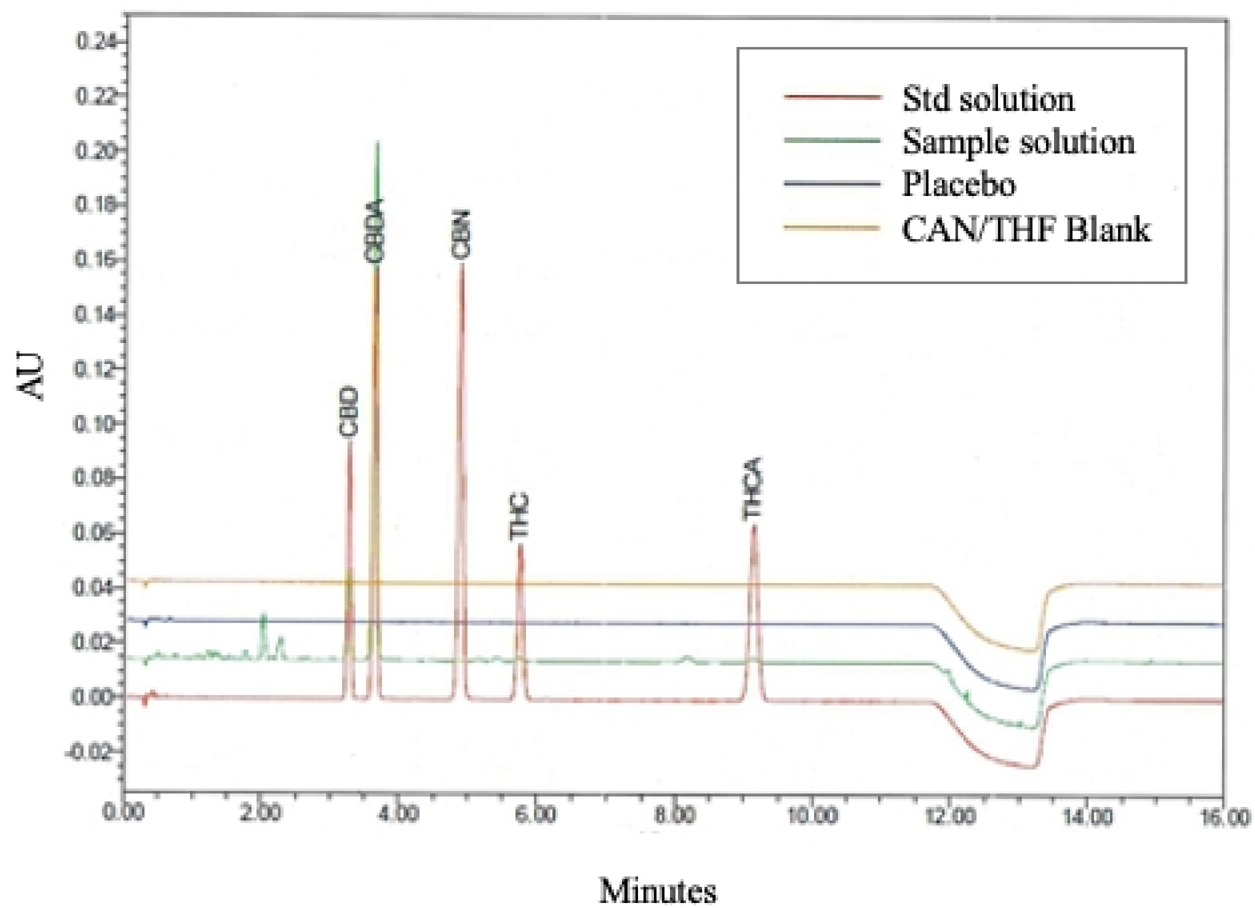
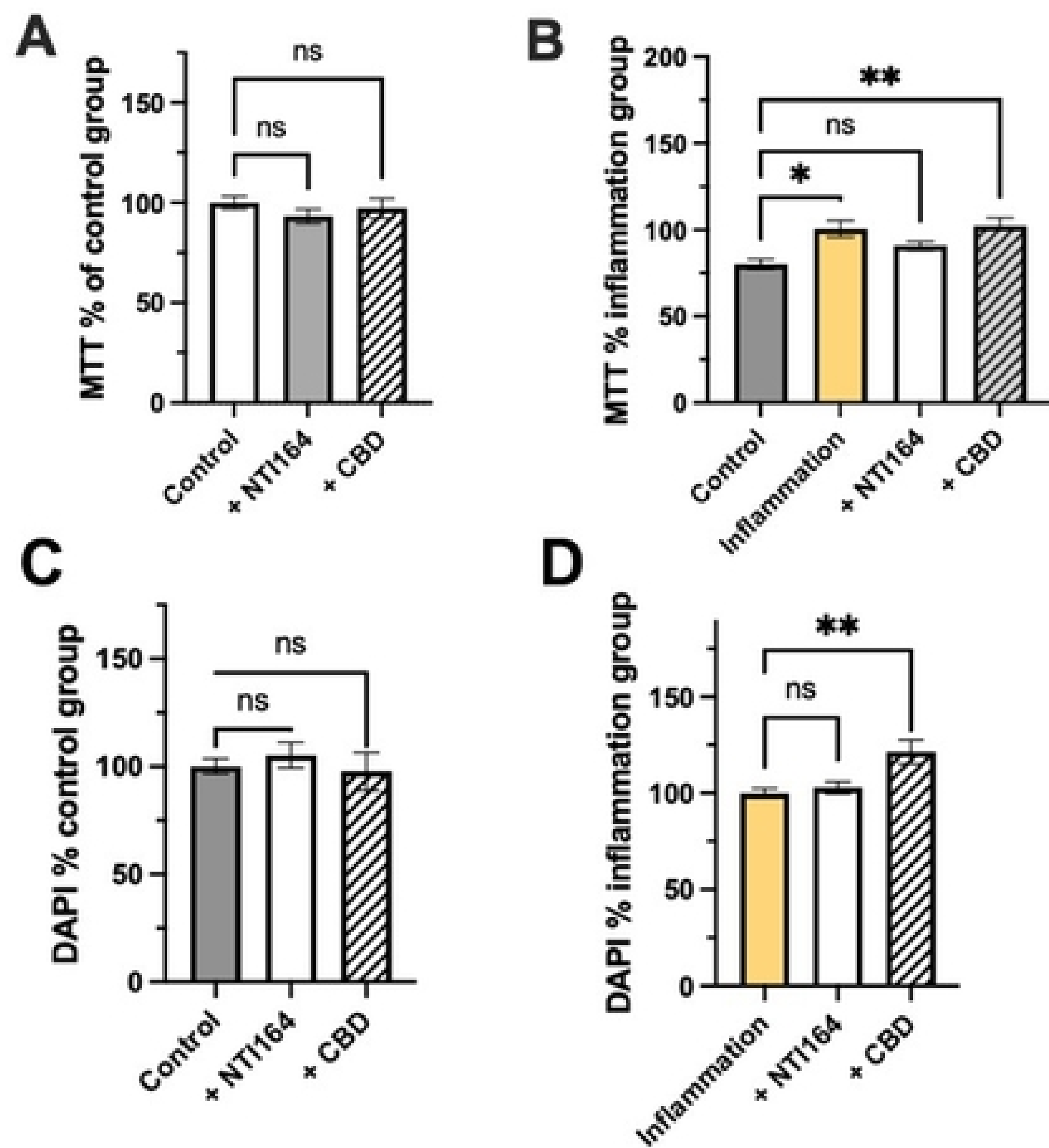


Figure 1



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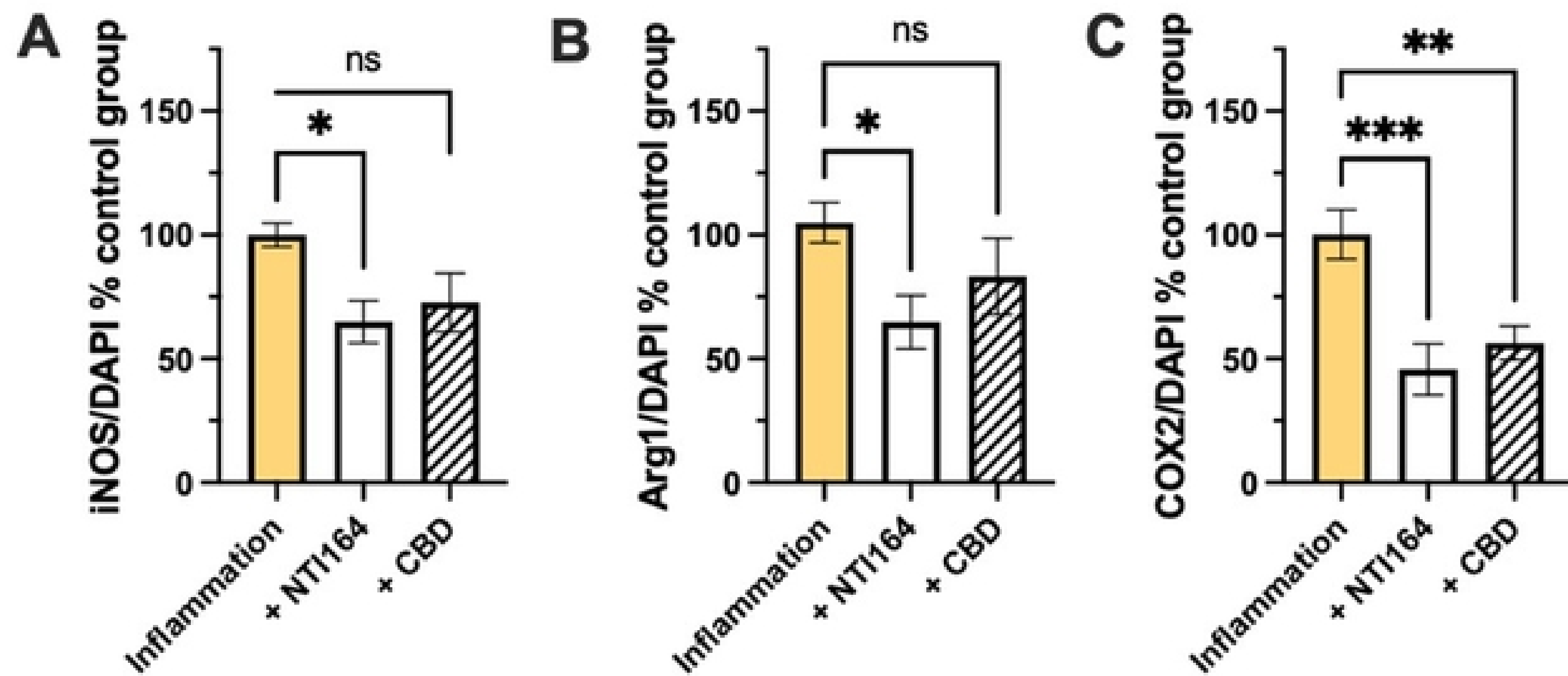

Figure 3

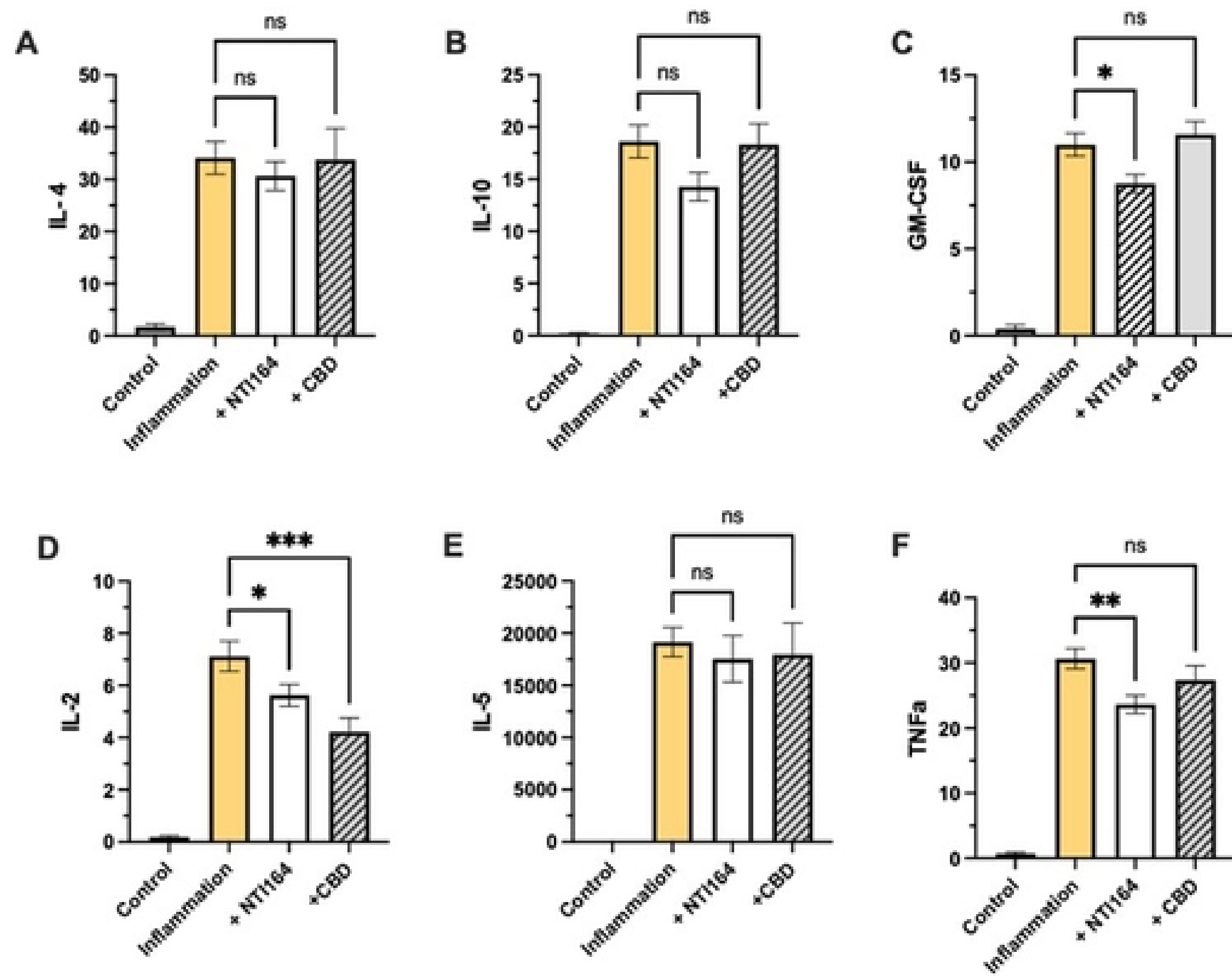

Figure 4

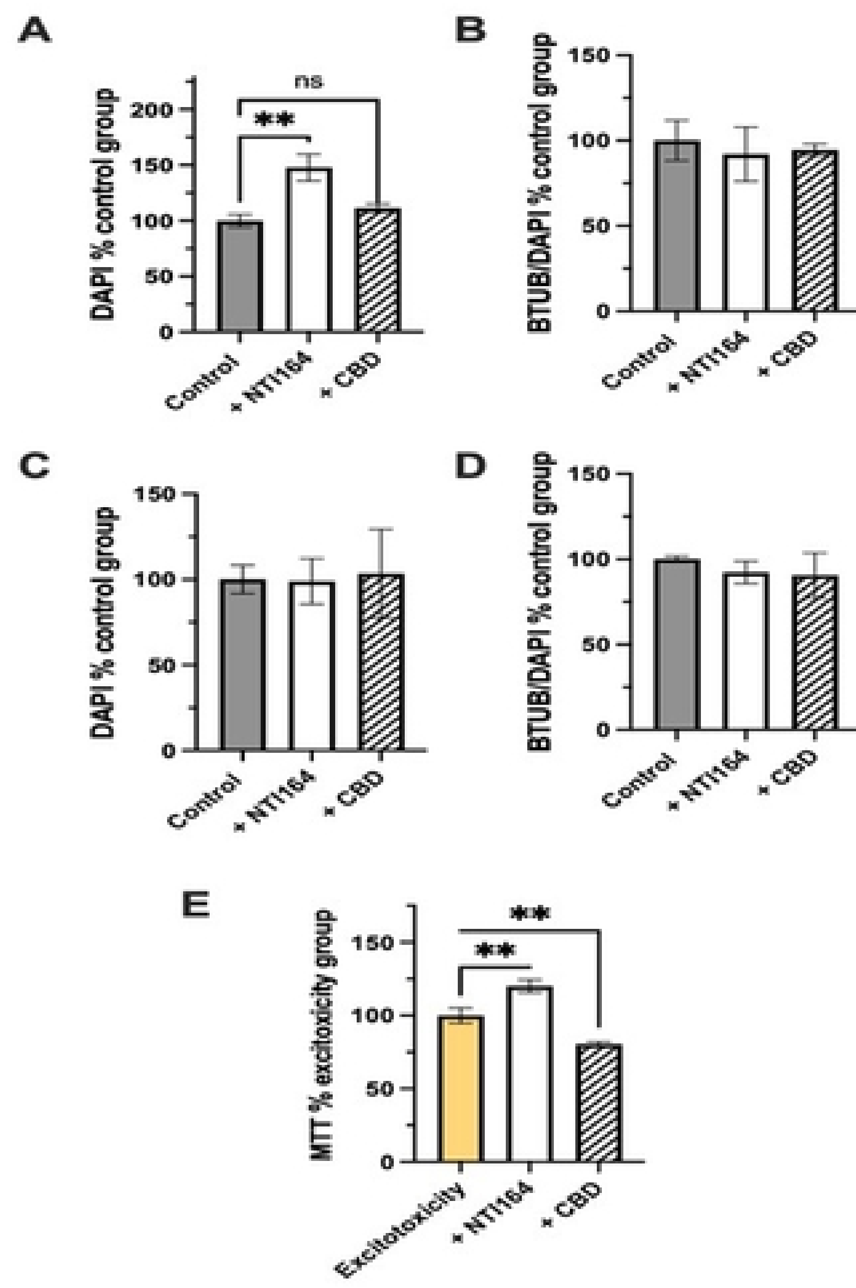

Figure 5