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Anti-inflammatory effects of single cannabinoids and in combinations

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Abstract

Cannabinoids, such as phytocannabinoids and endocannabinoids, inhibit inflammatory responses in vitro and in vivo. Endocannabinoids act through the cannabinoid receptors (CBRs), whereas phytocannabinoids may also act through other targets than CBRs.

Here we used a SIM-A9 microglial cell line to investigate the anti-inflammatory effects of the two major phytocannabinoids (Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD)) and anandamide (AEA) with the aim to elucidate their potential synergistic or entourage effect.

All three cannabinoids reduced lipopolysaccharide (LPS)-induced inflammation in a concentration-dependent manner as determined using nitric oxide (NO) measurements. When AEA and CBD were co-applied, they produced a greater effect together than would be expected from their individual effects across all concentrations. The same was seen with combinations of THC and CBD, only at relatively high concentrations, and dependent on the order of stimulation. In summary, these results indicate that cannabinoids are interacting via multiple targets and produce both synergisms and antagonism dependent on the combination, concentrations, and order of application.

INTRODUCTION

The *Cannabis sativa* (*C. sativa*) plant has been used for medical purposes before modern age for antiemetic, analgesic, muscle relaxant, and anticonvulsant properties (Iversen, 2019; Kopustinskiene et al., 2022). The two most abundant phytocannabinoids in the plant are Δ-9-tetrahydrocannabinol (THC) acid and cannabidiolic acid, which are applied in their decarboxylated active forms as THC and cannabidiol (CBD). The entourage effect is defined as the impact of all compounds in the cannabis plant is greater than the sum of its parts (Ben-Shabat et al., 1998; Christensen et al., 2023). This concept may have clinical implications, as specific combinations of phytocannabinoids that are different from their ratios in the plant, may produce therapeutic benefits while reducing unwanted side effects (Christensen et al., 2023; Paes-Colli et al., 2022). The mechanism behind the entourage effect is unknown, partly because the target(s) of the cannabinoids is not entirely clear either.

In this study, we took a pharmacological approach to evaluate the effects of the endocannabinoid anandamide (AEA), THC, and CBD individually or in combination using the microglial cell line SIM-A9, originally isolated by Nagamoto-Combs et al. (2014). SIM-A9 cells display microglial morphologies and secrete TNF-α and increase the production of nitric oxide (NO) in response to inflammatory stimuli such as lipopolysaccharide (LPS) (Nagamoto-Combs et al., 2014).

Microglia are the brains resident macrophage-like immune cells, playing a central role in neuroinflammation (Guo et al., 2022; Skrzypczak-Wiercioch and Salat, 2022). When microglia are activated by infectious stimuli, including LPS they release pro-inflammatory mediators such as NO, interleukin-6 (IL-6), IL-1β and tumor necrosis factor alpha (TNF-α) (Guo et al., 2022; Skrzypczak-Wiercioch and Salat, 2022). While inducible NO synthase (iNOS)-mediated NO production is crucial for

host defense, excessive or prolonged activation of iNOS and the subsequent production of NO contributes to the inflammatory cascade which sustains chronic tissue damage (Cinelli et al., 2020).

Increasing evidence suggests that the cannabinoid type 2 receptor (CB2R) mediates anti-inflammatory effects of various cannabinoids (Correa et al., 2010; Dos Santos et al., 2023; Mecha et al., 2015; Rodrigues et al., 2024; Young and Denovan-Wright, 2021). CB2R, and not the cannabinoid type 1 receptor (CB1R), is expressed in immune tissues (Galiegue et al., 1995; Munro et al., 1993). It is believed that THC acts as a partial agonist at both receptors, whereas CBD acts as a partial agonist at the CB2R (Tham et al., 2019), supporting this receptor as the main target.

However, similar mechanisms of action via a single receptor target are inconsistent with the concept of the entourage effect. We hypothesize that interactions among multiple phytocannabinoids occur via their actions at diverse molecular targets including CB2R, may underlie the entourage effects. We used SIM-A9 cells to explore the effects of cannabinoids and to elucidate the pharmacology of their actions, as well as their interactions, with particular emphasis on synergistic and/or antagonistic effects.

EXPERIMENTAL PROCEDURES

Cells and compounds

The SIM-A9 cells were purchased from American Type Culture Collection (ATCC®, CRL-3265). AEA (cat# T14046) [(5Z,8Z,11Z,14Z)-N-(2-Hydroxyethyl) icosa-5,8,11,14-tetraenamide] was purchased from Targetmol Chemicals (Wellesley Hills, MA 02481, USA), and CBD and THC were purchased from Sigma-Aldrich: CBD (cat# C-045), THC (cat# T-005). The compounds were kept in stock diluted in methanol.

The SIM-A9 cells (ATCC®) were cultured in 75 cm^2 Nunclon™ delta surface flasks (7342066, ThermoFisher Scientific) under controlled conditions (5% CO_2, 37°C). The culture medium consisted of 74% (v/v) DMEM/F-12 (#D8437, Sigma Aldrich) and 10% heat inactivated (HI) Fetal Bovine Serum (FBS, #F9665, Sigma-Aldrich), 5% (v/v) Inactivated horse serum (HS, #26050088 ThermoFisher Scientific) and 1% (v/v) Penicillin-Streptomycin (Pen/Strep, #15140122, ThermoFisher Scientific). The cell passaging was carried out every 2 days and up to 25 passages before thawing new cells. As the cells adhere to the 75 cm^2 flasks, a cell scraper was used to scrape the cells from the flask surface. The cells were transferred to a 15 mL falcon tube and centrifuged for 3 minutes at 300 *g* at room temperature. The supernatant was discarded, and the cell pellet was resuspended in culture medium and transferred to new 75 cm^2 flasks which were incubated under controlled conditions (5% CO_2, 37°C).

On the day of the experiment, the cell pellet was resuspended in 10 mL serum-free DMEM/F-12 medium, and seeded in 24-well plates with a cell density of 500.000 cells/mL. The cells were then treated with the cannabinoids at the concentrations described in the results section for 30 minutes before incubation with 100 ng/mL LPS for 24 hours.

Biochemical Assays

Griess Assay. 100 μL supernatant was transferred to 96-well plates containing 100 µL Griess reagent (Cat#328670500; Thermo Scientific). The plate was incubated in the dark at room temperature for 30 minutes and afterwards the absorbance was measured at 535 nm using the iMark Microplate Absorbance Reader (Bio-Rad Labs). The concentration was calculated from duplicates and standard corrected.

XTT Assay. The tetrazolium salt 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) was used to determine the viability of the SIM-A9 cells in the presence of LPS and cannabinoids (Scudiero et al., 1988), using the CyQUANT™ XTT Cell Viability Assay Kit from ThermoFisher Scientific. The working dilution was 6 mL XTT Reagent and mixed with 1 mL Electron Coupling Reagent, and 300 μL serum-free DMEM/F-12 was added to the wells followed by 200 μL working solution. The cells were incubated under controlled conditions (5% CO_2, 37°C) for 4 hours. After incubation 100 μL of the solution was transferred to a 96-well plate and the absorbance was measured at 450 nm and 655 nm as background using the iMark Microplate Absorbance Reader from Bio-Rad Laboratories.

Data collection, and statistical analysis. To ensure consistency and reliability of the inflammatory response, only experiments in which LPS produced more than 4-fold increase in nitrite levels relative to the untreated control were used.

The statistical analysis and the graphical presentation of data was conducted in GraphPad Prism (Version 10.3.1, San Diego, CA). Data are represented as mean ± SD. One-way ANOVA was utilized to determine the significant differences between the means of concentrations of test substances followed by an appropriate post-hoc test. A P-value below 0.05 was considered statistically significant.

The term synergism is generally used when the combined effect is markedly greater than the effect of each of the substances, but should be used with care (Calzetta and Koziol-White, 2021). Notably, the traditional t-test does not comply with the normal distribution assumption (Demidenko and Miller, 2019), so we determined the level of synergy as the level of additional effect produced by the compounds in combination as a single value. In all cases, the Bliss definition of independence was considered (Demidenko and Miller, 2019).

RESULTS

AEA, THC and CBD inhibit the effect of LPS in a concentration-dependent manner

The NO Griess assay was applied to determine the concentration-response effects of three cannabinoids (Fig. 1). Under basal conditions, the cells released detectable NO, but when incubated with 100 ng/ml LPS, this level increased more than 4-fold in all experiments. The pharmacological effects of AEA, THC,

and CBD on the LPS response were evaluated by adding increasing concentration of each compound to the cells 30 minutes prior to LPS.

Dexamethasone was used as a positive control and significantly inhibited LPS-induced NO release, reaching complete inhibition at 50 μM (not shown). Another inclusion criterion was that the compounds, either alone or in combination with LPS, had no effect on cellular metabolism as measured by the XTT assay. In accordance with the literature (Rodrigues et al., 2024), all three cannabinoids decreased metabolic viability at concentrations of 4 μM or higher when combined with LPS. Notably, THC and CBD had no effect on metabolic activity or NO release when administered in the absence of LPS (not shown).

When cells were treated with AEA prior to LPS stimulation, significant reductions in NO were seen at low AEA concentrations (0.125 μM) with maximal effects of approximately 20% reduction at 2 μM (Fig. 1A). Increasing concentrations of THC also inhibited LPS-induced NO release, but only at concentrations of 0.5 μM and higher (Fig. 1B). The maximal reduction in NO production was estimated to be approximately 20% at 2 μM THC (Fig. 1B). CBD led to a significant reduction in NO release at a concentration as low as 0.5 μM. The effect of CBD was concentration-dependent, ranging from a 12% reduction at 0.5 μM to more than 50% reduction relative to LPS at 2 μM CBD (Fig. 1C).

Combined effects of AEA and CBD.

The possible synergistic effects of AEA and CBD were tested by keeping the CBD concentration constant while increasing AEA concentrations from 0.25 μM to 2.0 μM (Figs. 2A-C). As expected, AEA reduced the LPS-induced NO response by up to approximately 20%. While the very low (0.25 μM) CBD concentrations had no significant effect alone (Figs. 2A), the combined effect with AEA and this low CBD concentration was positive at low concentrations. Even at concentrations where CBD alone had no significant effect, the combined response was nearly double that of AEA alone (Fig. 2A, D). At higher CBD concentrations (0.5 μM), where CBD by itself produced significant inhibition, additional effects were still detected when combining with AEA (Figs. 2B-C). The additional effect of CBD and AEA was the same at CBD concentrations at 0.5 μM and 1.0 μM (Figs. 2E, F).

Combined effects of THC and CBD were both synergistic and antagonistic.

The Griess assay was used to determine a potential combined effect between CBD and THC on the NO release of the SIM-A9 cells. In one set of experiments, increasing concentrations of CBD were tested with and without a fixed concentration of THC (Fig. 3A-C). Combinations of low THC concentrations (0.25 μM and 0.50 μM), which have no or minimal effects on their own, produced no prominent additional effect when combined with increasing concentrations of CBD (Figs. 3D, E). However, co-treatment with CBD and 1 μM THC resulted in a larger reduction in nitrite levels than either 1 μM THC or CBD alone, indicating a prominent and concentration-dependent synergistic effect (Figs. 3C, F).

By contrast, when the CBD concentration was kept constant, combining it with increasing concentrations of THC produced only limited additive effect (Fig. 4). At low concentrations, no interaction between the

two phytocannabinoids were observed (Figs. 4D, E). However, an antagonistic effect was observed at the high CBD concentration (Fig. 4F).

DISCUSSION

The three cannabinoids investigated here (AEA, CBD and THC) all significantly reduced LPS-induced NO release in SIM-A9 cells in a concentration-dependent manner. These are in overall agreement with previous work reported in BV-2 microglia cells (Rodrigues et al., 2024; Zaiachuk et al., 2023), RAW264.7 macrophage cell line, and in primary mouse macrophages (Coffey et al., 1996; Jeon et al., 1996).

In vitro studies using endocannabinoids, synthetic CBR agonists, or antagonists indicate that a CB2R-dependent mechanism is involved in the anti-inflammatory effects. For example, a CB2R antagonist significantly attenuated the effect of the cannabinoid agonist by reducing the NO release, but not with CB1R antagonist (Eljaschewitsch et al., 2006; Malek et al., 2015; Ross et al., 2000; Sugiura et al., 2000). The presence of CB2R mRNA and the absence of CB1R mRNA in microglia supports the pharmacological data (Bouaboula et al., 1993; Galiegue et al., 1995; Jeon et al., 1996). Since all three tested compounds are partial agonists of the CB2R (Pertwee, 2008; Tham et al., 2019), we suggest that they reduced inflammation via CB2R agonism in microglia. Microglia activation is involved in the development of neuropathic pain through the release of several cytokines known to produce neuronal desensitization (Clark et al., 2007) and it is suggested that this mechanism is responsible for the effect in vivo. The CB2R plays a crucial role in immune responses during neuropathic pain (Racz et al., 2008) and epilepsy (Terrone et al., 2018). The expression of CB2R is changing in cell lines in the presence of LPS (Grabon et al., 2024), and the receptor is involved in the effect of LPS (Reusch et al., 2022). Notably, CB2R is down-regulated by LPS in microglia (Grabon et al., 2024), which indicates that the effect of CB2R is dependent on the inflammation stage.

We further show that when two compounds are combined, the effect of the two is larger than each of the two together. Importantly, the eventual combined effect is dependent on the composition of cannabinoids, their concentrations, and the order of their application. CBD enhanced the effect of AEA at low AEA concentrations, and THC enhanced the effect of CBD at high concentrations. While these data support the concept of synergism or entourage effect of cannabis, it also raises speculation about the mechanisms behind this additional effect beyond the CB2R.

Although CBD and THC have been shown to reduce the NO release in BV-2 cells (Coffey et al., 1996; Jeon et al., 1996) as well as the SIM-A9 cells in this study, the evidence regarding their combined effect remains limited. Even earlier work has demonstrated that all three compounds are partial agonists of the CB2R (Pertwee, 2008; Tham et al., 2019) it would not be expected that two partial agonists would not produce additional effects on the same target at high concentrations. Actually, the antagonistic interaction between CBD and THC reported here, when CBD is applied to the cells after THC at high concentrations, is consistent with a shared receptor interaction.

When comparing similar effects of extracts of cannabis cultivars in the BV2 cells, LPS-induced NO was reduced more than 50%, indicating that the combinations produced a stronger effect consistent with the encourage effect (Fleisher-Berkovich et al., 2024). Studies have observed an enhanced potency of cannabis plant extracts in reducing pro-inflammatory cytokine release compared to pure CBD and THC (Zaiachuk et al., 2023). In that perspective, it is interesting that under some conditions, CBD and THC are producing stronger effects when administered together than when given individually.

Because CB2R is unlikely to be mediating the encourage effect alone, other receptors should be considered. CBD engages with other receptors likely involved in the immunological response, including G-protein coupled receptor-55 (GPR55), transient receptor potential vanilloid (TRPV), and peroxisome proliferator-activated receptor (PPAR) (Khosropoor et al., 2023; Landucci et al., 2022; Preteroti et al., 2023). In particular, GPR55 activation induces inflammatory responses (Kaplan et al., 2017; Saliba et al., 2021; Saliba et al., 2018), and CBD operates as an antagonist at this site (Kaplan et al., 2017; Ryberg et al., 2007). Further pharmacological studies are needed to elucidate the mechanism by which the different cannabinoids interact with these targets, and whether this interaction contributes to the synergistic effects of cannabis cultivators.

Declarations

Competing interest

The authors declare no competing interest

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declaration

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Author Contribution

Author contributionConceptualization: JDM, MA; Methodology: LP, HKS, EN, RNGO, AU, and POO; Formal analysis: LP, HKS, EN, RNGO, JDM; Resources: JDM, JB, MR, MA, Writing original draft: JDM; Supervision: JDM

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Figures

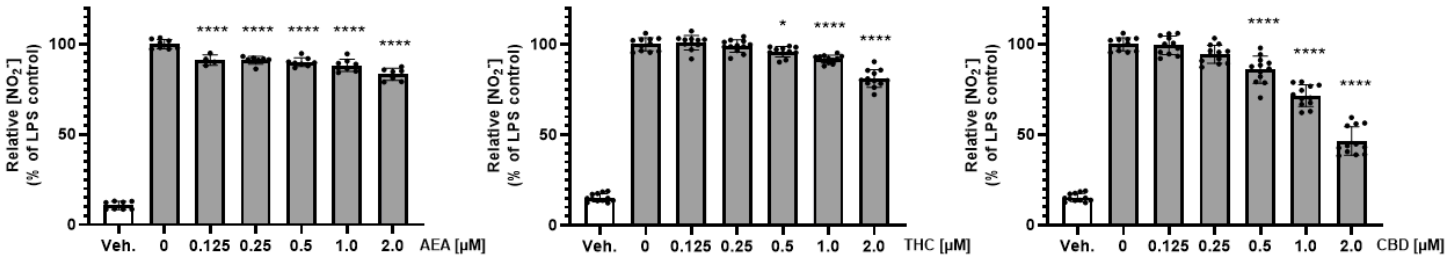

Figure 1

AEA, THC and CBD inhibit LPS-induced NO release in SIM-A9 cells. SIM-A9 cells were treated with multiple concentrations of AEA (A), THC (B) and CBD (C) 30 minutes prior to 100 ng/mL LPS stimulation for 24 hours. The amount of NO released by the SIM-A9 cells was measured by the Griess assay. AEA at low concentration produced a significant reduction in NO release (A). A significant reduction in the NO release is observed at 0.5 μM THC and a maximal response is observed at μM (B). A significant reduction in the NO release is observed at 0.5 μM CBD, and further reductions in the NO release are observed at 1 μM and 2 μM (C). Data are presented as a relative effect compared to the maximal response to LPS (100%) presented as mean ± SD pooled from 3 independent experiments. Statistical significance was determined by using ordinary one-way ANOVA followed by Dunnett's post-hoc test (*p < 0.05, ****p < 0.0001 compared to LPS).

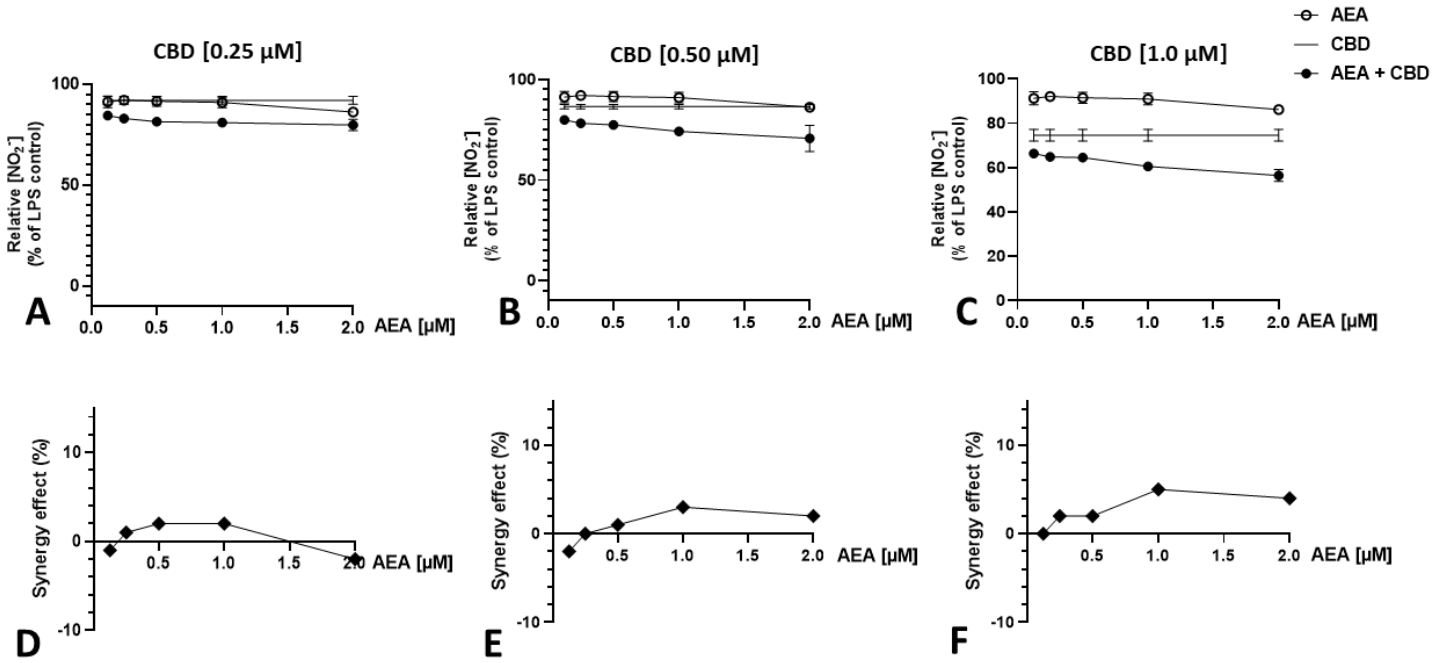

Figure 2

Synergistic effects of AEA and CBD

The upper row shows anti-inflammatory effects relative to the LPS group across increasing concentrations of AEA in the presence of 0.25 μM CBD (A), 0.5 μM CBD (B), and 1.0 μM CBD (C). The curves illustrate the effects of AEA alone (○), CBD alone (---), and the combination of the two (●). The lower row (D-F) illustrates the positive synergistic effect, determined as the combined effect subtracted the individual effects of AEA and CBD (D-F).

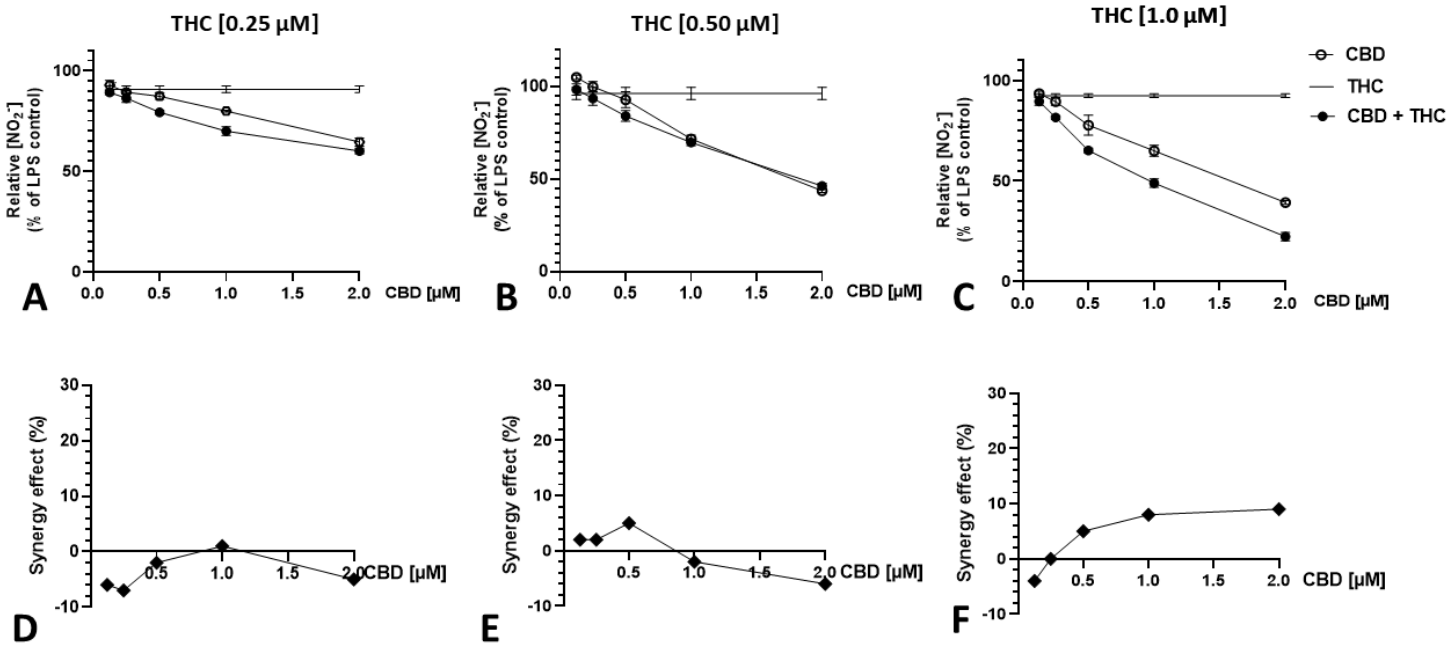

Figure 3

Synergistic effect between CBD and THC with fixed THC concentrations. LPS-stimulated SIM-A9 cells were treated with various concentrations of CBD (0.25 μM, 0.5 μM, 1.0 μM and 2.0 μM) in the presence of fixed THC concentrations (A-C). At low (D) and medium (E) concentrations, co-treatment produced no additional effect. However, at high THC concentration (F), a strong synergistic effect is observed.

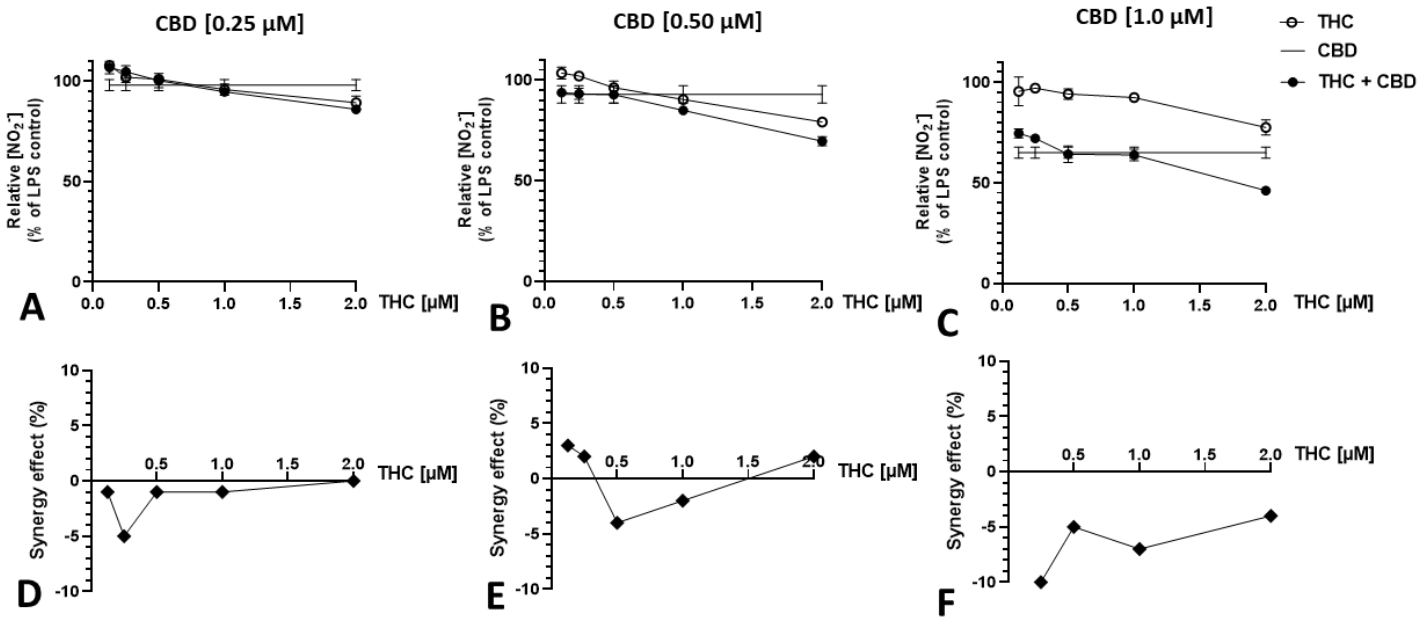

Figure 4

Antagonistic interaction between THC and CBD with fixed THC concentrations. The effect of THC at increasing concentrations with and without subsequent treatment with CBD at 0.25 µM (A), 0.50 µM (B), and 1.0 µM (C). Both compounds exert effects on their own, and the co-treatment did not produce combined effects. Instead, an antagonistic interaction was observed, particularly at the highest concentrations (E-F).