



Phytocannabinoids as anti-inflammatory agents: Synergistic effects when combined with *Cannabis sativa* L. matrices

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ABSTRACT

Ethnopharmacological relevance: *Cannabis sativa* L. has a long history of traditional use for the treatment of pain and inflammatory disorders in both Asian and European medical systems, which supports its investigation as a source of bioactive compounds with potential relevance for chronic immune-mediated diseases.

Aim of the study: This study evaluated the ten major non-psychotropic phytocannabinoids for their anti-inflammatory and antioxidant activities, and for synergistic interactions with non-cannabinoid matrices derived from the same plant (polar, non-polar and terpenoid).

Material and methods: Anti-inflammatory effects were assessed in macrophage-differentiated THP-1 cells by measuring pro-inflammatory cytokine production (ELISA) and nuclear factor kappa B (NF-κB) pathway activation (luciferase reporter assay).

Results: All tested phytocannabinoids demonstrated anti-inflammatory effects, in particular, cannabidivarin (CBDV) reduced IL-6, TNF-α production and also inhibited NF-κB activation. Several phytocannabinoids, especially their acidic forms, exhibited high oxygen radical absorption capacity (ORAC), but none showed significant cellular antioxidant activity (CAA), possibly due to limited bioavailability. Importantly, several phytocannabinoid-matrix mixtures displayed synergistic anti-inflammatory effects, with combinations containing cannabigerol (CBG) or cannabinal (CBN) being particularly potent.

Conclusion: These findings highlight the potential of lesser-known phytocannabinoids, especially in combination with specific *C. sativa* L. matrix components, to modulate inflammatory pathway supporting their development as functional ingredients for managing chronic gut-associated inflammation.

1. Introduction

The inflammatory response is defined as a natural defence mechanism that protects the body from infection or injury by eliminating the cause and initiating the healing process (Pahwa et al., 2025). If inflammation becomes chronic, it can persist for years and lead to serious diseases such as autoimmune, gastrointestinal, and cardiovascular disorders (Murakami and Hirano, 2012). Nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are commonly used for treatment of such inflammation. However, their prolonged

administration can result in serious adverse side effects (Panchal and Prince, 2023). Consequently, there is a growing interest in natural substances, such as those found in the *Cannabis sativa* L. plant, which have potential to offer broad biological activity along with reduced toxicity and fewer unwanted reactions (Zhou et al., 2023). These compounds may also serve as functional ingredients in foods and dietary supplements aimed at managing chronic intestinal inflammation. Inflammatory bowel diseases (IBD), such as Crohn's disease and ulcerative colitis, are characterised by recurrent inflammation of the gut, and natural bioactive compounds capable of modulating immune responses may help alleviate symptom (Gasparrini and Mazzoni, 2024).

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List of abbreviations

5-HT1A – serotonin 5-hydroxytryptamine receptor 1A
 AAPH – 2,2'-azo-bis-(2-methylpropionamide) dihydrochloride
 CAA – cellular antioxidant activity
 CB1 – cannabinoid receptor 1
 CB2 – cannabinoid receptor 2
 CBC – cannabichromene
 CBCA – cannabichromenic acid
 CBD – cannabidiol
 CBDA – cannabidiolic acid
 CBDV – cannabidivarin
 CBDVA – cannabidivarinic acid
 CBG – cannabigerol
 CBGA – cannabigerolic acid
 CBN – cannabinol
 DCFH-DA – 2',7'-dichlorodihydrofluorescein diacetate

DMSO – dimethylsulfoxide
 FBS – fetal bovine serum
 IBD – inflammatory bowel diseases
 IL-6 – interleukin-6
 LPS – lipopolysaccharide
 MD2 – myeloid differentiation factor 2
 NC – negative control
 NF- κ B – nuclear factor kappa B
 NSAIDs – nonsteroidal anti-inflammatory drugs
 ORAC – oxygen radical absorption capacity
 PC – positive control
 PMA – phorbol 12-myristate 13-acetate
 RPMI – Roswell Park Memorial Institute 1640 medium
 Δ^9 -THC – tetrahydrocannabinol
 Δ^9 -THCA-A – tetrahydrocannabinolic acid
 TNF- α – tumor necrosis factor alpha
 TRPV1 – transient receptor potential vanilloid 1

The traditional medicinal use of *C. sativa* L. for inflammation treatment has been documented for millennia, particularly in traditional systems across Asia for pain and inflammatory disorders (Zuardi, 2006). Certain ethnomedical practices, including those still present in parts of South Asia, continue to utilise cannabis-based preparations for the management of various somatic and immune-related conditions (Shakil et al., 2021). In Europe, tinctures and aqueous extracts of *C. sativa* L. were incorporated into official pharmacopoeias during the 18th and 19th centuries as treatments for inflammatory and spastic disorders (Ryz et al., 2017; Zuardi, 2006). This longstanding history underlines the relevance of *C. sativa* L. as a potential source of bioactive compounds for the modulation of inflammatory processes.

The *C. sativa* L. plant is the primary source of phytocannabinoids, a group of compounds that have the potential to interact with the endocannabinoid system to modulate inflammation in chronic diseases. This system can modulate immune cell behaviour and influence signaling through inflammatory factors, primarily via two receptors: cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) (Barrie and Manolios, 2017). CB1 is predominantly located within the central nervous system and has been linked to the regulation of stress and pain relief. In contrast CB2, which is found in immune cells, has the potential to modulate inflammatory processes (Marzo et al., 2004).

One of the most well-known phytocannabinoids is tetrahydrocannabinol (Δ^9 -THC), which is able to bind to both of these receptors. By binding to CB1 it causes psychoactive effects and by binding to CB2 it causes anti-inflammatory effects (Pellati et al., 2018). Another highly studied phytocannabinoid is CBD, for which anti-inflammatory and antioxidant effects have been found. However, CBD has only weak affinity for CB1 and CB2 receptors, and its anti-inflammatory activity is instead thought to involve other molecular targets, including, among others, the transient receptor potential vanilloid 1 (TRPV1) channel and the serotonin 5-hydroxytryptamine receptor 1A (5-HT1A) (Burstein, 2015). Nevertheless, no CBD-containing dietary supplements have been authorised in the European Union, due to persisting safety concerns related to potential hepatotoxicity and drug interactions (Engeli et al., 2025). Conversely, CBD has been approved as a pharmaceutical drug (Epidiolex) for the treatment of rare forms of epilepsy, underscoring both its therapeutic potential and the necessity for rigorous safety evaluation (Engeli et al., 2025).

Due to ongoing safety concerns associated with CBD, research has increasingly focused on other non-psychoactive phytocannabinoids with similar therapeutic potential but fewer adverse effects. Cannabigerol (CBG), cannabichromene (CBC), and cannabinol (CBN) have shown anti-inflammatory and antioxidant properties in preclinical studies (Gojani et al., 2023; Lowin et al., 2023). Acidic phytocannabinoids, such

as cannabidiolic acid (CBDA) and tetrahydrocannabinolic acid (Δ^9 -THCA-A), have also demonstrated biological activity, including anti-inflammatory potential (Formato et al., 2020). However, several other acidic phytocannabinoids present in *C. sativa* L. remain largely unexplored, despite their structural similarity and possible relevance to inflammatory processes.

In addition to nearly one hundred phytocannabinoids, *C. sativa* L. also produces a variety of other bioactive compounds, including terpenes, flavonoids, and alkaloids (Khalsa et al., 2022). These non-cannabinoid components may act together with phytocannabinoids in a synergistic manner, a phenomenon known as the “entourage effect”, where the combined activity of many molecules presented exceeds the sum of their individual effects (Bautista et al., 2021). This concept was first demonstrated within the endocannabinoid system, when Ben-Shabat et al. (1998) showed that the endocannabinoid 2-arachidonoyl-glycerol exhibited enhanced activity in the presence of related fatty acid esters. Despite this early evidence, studies specifically addressing the “entourage effect” between phytocannabinoids and other *C. sativa*-derived compounds remain limited (Christensen et al., 2023).

We investigated the anti-inflammatory activity of ten major non-psychoactive phytocannabinoids found in *C. sativa* L. In addition, we examined whether other active compounds present in the same plant exhibit biological activity and whether their combination with individual phytocannabinoids could lead to synergistic effects. The evaluation focused on the ability of these compounds and their mixtures to modulate the production of pro-inflammatory cytokines and influence the NF- κ B signalling pathway. Our findings suggest that phytocannabinoids have potential as anti-inflammatory agents and that combining them with other plant-derived compounds may enhance their anti-inflammatory effects. These findings support the potential of non-psychoactive phytocannabinoids, alone or in combination with other *C. sativa*-derived compounds, as active ingredients in the development of novel dietary supplements and functional food products targeting inflammation-related conditions.

2. Material and methods

2.1. Material

Roswell Park Memorial Institute 1640 medium (RPMI, Sigma-Aldrich, St. Louis, Missouri, USA); fetal bovine serum (FBS, Sigma-Aldrich); 100 $\times$ antibiotic antimycotic solution (Sigma-Aldrich); resazurin sodium salt (Sigma-Aldrich); phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich); dimethylsulfoxide (DMSO, Sigma-Aldrich); lipopolysaccharides from *Escherichia coli* O127:B8 (Sigma-Aldrich); TNF

alpha Monoclonal Antibody (MAB1), eBioscience (Thermo Fisher Scientific, Waltham, Massachusetts, USA); TNF alpha Monoclonal Antibody (MAB11), Biotin, eBioscience (Thermo Fisher Scientific); IL-6 Monoclonal Antibody (MQ2-13A5), eBioscience (Thermo Fisher Scientific); IL-6 Monoclonal Antibody (MQ2-39C3), Biotin, eBioscience (Thermo Fisher Scientific); Avidin, HRP conjugate (Thermo Fisher Scientific); 3,3',5,5'-Tetramethylbenzidine (Sigma-Aldrich); Blocker Casein (Thermo Fisher Scientific); QUANTI-Luc 4 Lucia/Gaussia (BARIA, Prague, Czech Republic); 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich); 2,2'-azo-bis-(2-Methylpropionamide) dihydrochloride (AAPH, Sigma-Aldrich); fluorescein (Sigma-Aldrich); ascorbic acid (Sigma-Aldrich); lipopolysaccharides from *Escherichia coli* O127:B8 (LPS, Sigma-Aldrich). Ethanol (LC-MS-grade) was purchased from Merck (Darmstadt, Germany).

2.2. Phytocannabinoids

Certified standards of CBC, cannabichromenic acid (CBCA), CBD, CBDA, CBG, cannabigerolic acid (CBGA), CBDV, cannabidivarinic acid (CBDVA), CBN and Δ^9 -THCA-A were purchased from Cerilliant Corporation (USA), all in a range of purity of 95.0–99.8 %. Phytocannabinoid standards were obtained as methanolic stock solutions at a concentration of 1 mg/mL.

2.3. Non-cannabinoid matrices

The experiments were performed using the CBD Tatanka variety of *C. sativa* L., and the cannabis-based matrices were prepared according to Binova et al. (2025). Briefly, the primary ethanolic extract was prepared by repeatedly extracting 142 g of dried, homogenized cannabis flowers through intensive shaking in 500 mL of ethanol. After extraction, the sample was filtered, the ethanolic extracts from two consecutive steps were combined, and the solvent was removed using a Rotavapor R-300 vacuum rotary evaporator (Büchi, Switzerland).

To gain the non-cannabinoid matrices, 0.5 g of the evaporated cannabis extract was dissolved in 5 mL of ethanol and injected into an AutoPure Prep LC-MS preparative chromatography system (Waters, USA) equipped with an XBridge Prep C18 OBD chromatographic column (100 × 19 mm; 5 µm) from Waters (USA). The mobile phases consisted of (A) water–methanol (95:5, v/v) with 5 mM ammonium formate, and (B) isopropyl alcohol–methanol–water (65:30:5, v/v/v) with 5 mM ammonium formate. The flow rate was set at 17 mL/min, with an injection volume of 1000 µL. To obtain polar and non-polar cannabis fractions, a multistep gradient programme was applied to achieve separation: starting at 0 % B, increasing linearly from 1 to 5 min to 55 % B, then to 60 % B within 2 min, followed by a transition to 100 % B within 1 min, which was held for 5 min before returning to the initial conditions within 2 min. The polar cannabis fraction (**'polar matrix'**) was collected between 1 and 6 min, stopping before the elution of the first phytocannabinoid. The non-polar cannabis fraction (**'non-polar matrix'**) was collected between 11 and 20 min, starting after the elution of the last phytocannabinoid. The polarity of the matrices is therefore relatively related to the cannabinoids. The polar matrix is isolated from the front of the chromatogram, followed by the cannabinoids and then the non-polar matrix.

The essential oil for getting the 'terpenoid matrix' was extracted using hydrodistillation with a Clevenger-type apparatus. Approximately 100 g of dried plant material was placed in a 1 L round-bottom flask containing distilled water. The mixture was heated to boiling (~100 °C) for 2 h, allowing volatile compounds to evaporate, condense, and collect in the graduated receiver of the apparatus as the **'terpenic matrix'**.

2.4. Cell lines and cell cultures

Monocytic THP-1 cells (Sigma Aldrich, 88081201-1VL) and THP-1-Lucia NF-κB (InvivoGen, thpl-nfkbv2) were cultivated in RPMI

supplemented with (10 % FBS and 1 % antibiotic mixture (penicillin, 0.1 IU/l, streptomycin, 10 g/l and amphotericin B, 25 mg/l) and incubated at standard conditions (37 °C, 5 % CO₂, humidified atmosphere).

2.5. Cytotoxicity assay

THP-1 monocytes were seeded into a 96-well plate at a concentration of 1×10^5 cells/mL, along with a concentration range of the tested substances. The cells were incubated for either 24 or 72 h in a 37 °C incubator with 5 % CO₂. After incubation, a resazurin assay was performed according to Sancha et al. (2024). Briefly, the medium was replaced with 100 µL of resazurin solution (0.03 g/l in PBS), and after 1 h, the fluorescence was measured using SpectraMax i3x multi-mode microplate reader (Molecular Devices, San Jose, California, USA) at 560/590 nm (ex./em.).

The viability assay for monocytes differentiated into macrophages (further referred to as macrophages) was conducted similarly to that of monocytes. THP-1 monocytes were seeded into a 96-well plate at a concentration of 3×10^5 cells/mL, followed by the addition of 100 µg/L PMA for 24 h to induce differentiation. The medium was then replaced with PMA-free medium for an additional 24 h. After this period, the medium was replaced again, and a concentration series of the tested sample was added. Following 24 or 72 h of incubation, the resazurin assay was performed as described above.

2.6. Release of pro-inflammatory cytokines

The anti-inflammatory effects were evaluated by measuring the levels of released cytokines (TNF-α and IL-6) from macrophages, following a previously described method (Tran et al., 2023). In this experiment, the samples were added to the cells in combination with LPS (500 µg/L). The positive control represented untreated cells exposed to LPS in the presence of DMSO at a concentration matching that in the test samples. The negative control represented untreated cells without LPS.

2.7. Effect on NF-κB pathway

NF-κB pathway activation was assessed using THP-1-Lucia NF-κB cells. The cells were seeded in a 96-well plate at a density of 3×10^5 cells/mL and treated with 100 µg/L PMA for 24 h. Following this, the PMA-containing medium was replaced with PMA-free medium for another 24 h. The medium was then replaced again, and non-toxic sample concentrations were added. After 24 h of incubation, luciferase activity was measured using the QUANTI-Luc 4 Lucia/Gaussia assay, following the manufacturer's instructions.

For the inhibition assay, cells were treated similarly to the activation assay, with following variations. Firstly, the cells were incubated with both the test samples and LPS (500 µg/L) for 24 h. Secondly, the cells were pre-incubated with the samples for 24 h, followed by a further 24 h incubation with both the samples and LPS (500 µg/L).

2.8. Oxygen radical absorption capacity (ORAC)

ORAC assay was performed according to Hurtová et al. (2024). Briefly, 75 µL of fluorescein solution (1.6 µg/mL in PBS, pH 7.4) was pipetted into a 96-well plate with the concentration series of the sample. The plate was incubated for 15 min at 37 °C and then 25 µL of AAPH solution (60 mg/mL) was added to all wells except controls, where PBS was pipetted instead. Fluorescence (485/535 nm, ex./em.) was measured by a microplate reader immediately after addition at 5 min intervals for 2 h.

2.9. Cellular antioxidant activity (CAA)

The CAA assay was measured as described by Viktorová et al. (2019)

on human monocyte cells differentiated into macrophages (THP-1). Briefly, cells (3×10^4 cells/well) were seeded into a 96-well plate and differentiated over 24 h by the addition of PMA (100 μ g/L). After that, the medium was replaced and the cells were further incubated for 24 h. Subsequently, the medium was replaced again with a new one containing the fluorescent probe DCFH-DA (12.5 mg/L) and the sample concentration series. After 1 h of incubation at 37 °C and 5 % CO₂, the medium was replaced with AAPH solution (0.16 mg/mL) except for the control, where only PBS was added. Immediately thereafter, fluorescence (485/540 nm, ex./em.) was recorded at 5 min intervals for 2 h.

2.10. Statistical analysis

All data are presented as mean $\pm$ SEM from at least three independent experiments. Statistical analysis was performed using GraphPad Prism (version 7.05; GraphPad Software, San Diego, CA, USA). Differences between groups were evaluated using one-way ANOVA followed by Dunnett's or Tukey's *post hoc* test, as appropriate. A *p*-value < 0.05 was considered statistically significant.

Synergistic, additive, or antagonistic interactions between phytocannabinoids and plant-derived matrices were evaluated using the Bliss independence model (Zhao et al., 2014). Details of the calculation and evaluation criteria are provided in the Supplementary Materials.

3. Results

To evaluate the anti-inflammatory and antioxidant properties of *C. sativa*-derived compounds, we tested the ten major non-psychoactive phytocannabinoids, three plant-derived non-cannabinoid matrices (polar, non-polar, and terpenoid), and their combinations. The evaluation included cytotoxicity assessment, analysis of cytokine production (IL-6 and TNF- α), NF- κ B signalling pathway modulation, and antioxidant activity using both chemical (ORAC) and cellular (CAA) assays. These experiments were designed not only to characterize the biological activity of individual compounds and mixtures, but also to assess their potential relevance as candidates for functional foods, dietary supplements, or therapeutic applications targeting inflammation.

3.1. Effects of phytocannabinoids

Cytotoxicity was assessed using the resazurin assay on the THP-1 cell line, tested in both its monocytic form and after differentiation into macrophages. Measurements were taken after 24 h (Table 1) and 72 h (Table S1). The results showed no major differences in cytotoxicity

Table 1

Cytotoxic activity of phytocannabinoids in THP-1 monocytes and macrophages represented as concentration halving the metabolic activity of the cells (IC₅₀) in resazurin assay after 24h incubation. Data are presented as mean $\pm$ SEM (n = 3 or 4). Different letters (a–k) indicate groups with statistically significant differences between the effects of phytocannabinoids, analyzed collectively across monocytes and macrophages (one-way ANOVA, Tukey's *post hoc* test, *p* < 0.05). If individual cannabinoids share the same letter, there is no statistically significant difference and the cannabinoids showed comparable activity.

Compound	IC ₅₀ (mg/L)	
	Monocytes	Macrophages
CBC	6.9 $\pm$ 0.1 ^b	12.3 $\pm$ 0.1 ^{f,g}
CBGA	16.1 $\pm$ 0.4 ⁱ	18.6 $\pm$ 0.2 ^j
CBD	5.1 $\pm$ 0.1 ^a	6.5 $\pm$ 0.1 ^{a,b}
CBDa	14.8 $\pm$ 0.4 ^{h,i}	11.4 $\pm$ 0.2 ^{d,e,f}
CBG	5.8 $\pm$ 0.2 ^{a,b}	11.1 $\pm$ 0.1 ^{d,e,f}
CBGA	13.6 $\pm$ 0.2 ^{g,h}	11.5 $\pm$ 0.3 ^{e,f}
CBDV	9.5 $\pm$ 0.1 ^c	10.8 $\pm$ 0.1 ^{c,d,e,f}
CBDVA	18.8 $\pm$ 0.5 ^j	22.3 $\pm$ 0.5 ^k
CBN	6.1 $\pm$ 0.2 ^{a,b}	10.1 $\pm$ 0.1 ^{c,d,e}
Δ^9 -THCA-A	10.1 $\pm$ 0.3 ^{c,d}	10.6 $\pm$ 0.1 ^{c,d,e}

between the two time points. Toxicity ranged from 5.1 $\pm$ 0.1 mg/L for CBD to 18.8 $\pm$ 0.5 mg/L for CBDVA. In six cases, the phytocannabinoids were significantly less toxic to macrophages than to monocytes. In contrast, CBDA showed the opposite trend, exhibiting higher toxicity toward macrophages.

To evaluate anti-inflammatory activity, we measured the effects of phytocannabinoids on pro-inflammatory cytokine production (TNF- α and IL-6; Fig. 1A) and on the NF- κ B signalling pathway (Fig. 1B–S1) at the highest non-toxic concentration for each compound. Macrophages were stimulated with LPS to induce inflammation and treated with phytocannabinoids. Cytotoxicity was excluded by performing a resazurin viability assay after the culture medium was collected for cytokine analysis. Results are expressed as a percentage relative to the positive control (LPS-treated cells), as shown in Fig. 1.

Eight of ten tested phytocannabinoids significantly reduced IL-6 production, only CBG and CBN showed no significant effect. The strongest reductions were observed for CBC (57 $\pm$ 6 %) and CBDVA (59 $\pm$ 4 %). In the case of TNF- α , only three phytocannabinoids exerted a significant inhibitory effect: CBG, CBGA, and CBDV, with CBDV showing the most pronounced reduction (22 $\pm$ 5 %).

The influence on NF- κ B signalling, a key transcription factor regulating the expression of pro-inflammatory cytokines during inflammation, was assessed under three experimental conditions: (1) treatment with phytocannabinoids alone, without LPS, for 24 h (Fig. S1), to test whether phytocannabinoids by themselves could activate NF- κ B in resting cells; (2) co-treatment with phytocannabinoids and LPS for 24 h (Fig. 1B), simulating a possible therapeutic effect by reducing an ongoing inflammatory response; and (3) a 24 h pre-incubation with phytocannabinoids, followed by a 24 h co-treatment with phytocannabinoids and LPS (Fig. 1B), simulating a preventive scenario where phytocannabinoids might protect cells from subsequent induction of inflammation.

In the LPS-stimulated conditions, all phytocannabinoids except CBGA significantly reduced NF- κ B activation in at least one of the setups, compared to the positive control (cells treated only with LPS). The most effective compound was Δ^9 -THCA-A, which reduced activation by 42 $\pm$ 2 %. In three cases, pre-incubation led to notable differences. For Δ^9 -THCA-A, pre-incubation resulted in a weaker reduction of NF- κ B activation. For CBGA, the reduction observed without pre-incubation was no longer statistically significant after pre-incubation. In contrast, CBD appeared to enhance LPS-induced activation of NF- κ B when added without pre-incubation, but significantly reduced activation when pre-incubation was included. None of the phytocannabinoids induced NF- κ B activation in the absence of LPS (Fig. S1).

Taken together, these results show that several non-psychoactive phytocannabinoids can significantly suppress the production of pro-inflammatory cytokines and reduce NF- κ B activation in macrophages. This supports their potential role as modulators of key inflammatory pathways.

Antioxidant activity was evaluated using both ORAC and CAA assays at the highest non-cytotoxic concentration for each compound (Table 2). Seven out of ten tested phytocannabinoids showed activity in the ORAC assay, and in all cases, the acidic forms were equally or more active than their corresponding neutral forms. No antioxidant activity was observed in the CAA assay for any of the tested phytocannabinoids. These findings indicate that phytocannabinoids possess antioxidant potential in solution but are likely unable to sufficiently penetrate cell membranes to mitigate oxidative stress inside cells.

3.2. Activity of plant matrices

In addition to individual phytocannabinoids, we also tested three non-cannabinoid matrices from *C. sativa* L. for their potential to reduce inflammation and oxidative stress. The same matrices as described by Binova et al. (2025) were used in this study: the polar matrix, obtained in the early chromatographic window and containing polar

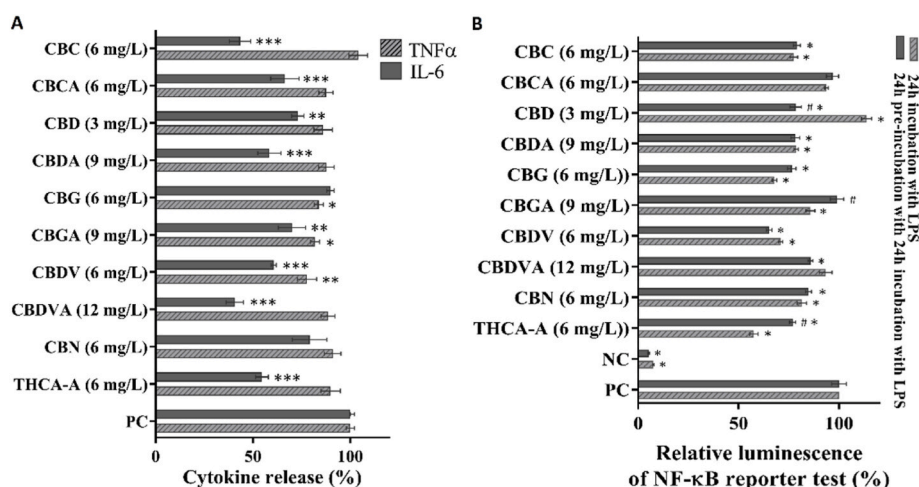


Fig. 1. Anti-inflammatory effects of phytocannabinoids on THP-1-derived macrophages. (A) Levels of pro-inflammatory cytokines TNF- α and IL-6 released after 24h incubation with phytocannabinoids in the presence of LPS (500 μ g/L). Cytokine concentrations were measured using sandwich ELISA. (B) NF- κ B pathway activation measured by luminescence reporter assay under two experimental conditions: 24h co-incubation with phytocannabinoids and LPS, and 24h pre-incubation with phytocannabinoids followed by 24h incubation with LPS. The negative control (NC) represents untreated cells. The positive control (PC) corresponds to LPS-treated cells without samples but with the same concentration of DMSO used in treatments. Data are presented as mean $\pm$ SEM (n = 4). Statistical significance was determined by one-way ANOVA followed by Dunnett's *post-hoc* test (* p < 0.05, ** p < 0.01, *** p < 0.001 vs. PC; # p > 0.05 compared to co-incubation without pre-incubation).

Table 2

Antioxidant properties of phytocannabinoids evaluated using chemical – oxygen radical absorption capacity (ORAC), and cellular antioxidant activity (CAA) assay. Data are presented as concentrations halving the oxygen radicals (IC₅₀, mg/mL). IC₅₀ values are presented as mean $\pm$ SEM (n = 3 or 4). For the CAA assay, THP-1 cells were used. For the ORAC assay, different letters indicate statistically significant differences between the antioxidant effects of individual phytocannabinoids (one-way ANOVA followed by Tukey's *post-hoc* test, p < 0.05). The highest tested concentration is limited by the cytotoxicity of individual phytocannabinoids.

Compound	IC ₅₀ (mg/L)	
	ORAC	CAA
CBC	>6	>6
CBCA	2.2 $\pm$ 0.1 ^a	>6
CBD	>3	>3
CBDA	3.3 $\pm$ 0.1 ^{a,b}	>9
CBG	>6	>6
CBGA	3.5 $\pm$ 0.1 ^{a,b}	>9
CBDV	5.7 $\pm$ 0.3 ^b	>6
CBDVA	4.0 $\pm$ 0.1 ^{a,b}	>12
CBN	>6	>6
Δ^9 -THCA-A	4.0 $\pm$ 0.2 ^{a,b}	>6
Ascorbic acid	132.9 $\pm$ 1.3 ^c	305 $\pm$ 4

non-cannabinoid components (Table S7), was followed by the cannabinoid fraction, followed by the non-polar matrix obtained in the late chromatographic window and composed of more lipophilic components of plant origin (Table S6); and a terpenoid matrix, prepared by hydro-distillation of dried *Cannabis* inflorescence, representing the fraction rich in volatile terpenes. The composition of individual matrices is described in Tables S5–S7. None of the matrices contained detectable phytocannabinoids, as confirmed by UHPLC-HRMS/MS analysis in the original study. The extraction yield was 0.53 % in the case of a polar matrix, 1.14 % in the case of a non-polar matrix and 0.01 % in the case of a terpenoid matrix. The maximum tested concentrations of the polar (104 mg/L) and non-polar (230 mg/L) matrices were determined by solubility and were non-toxic at these levels. In contrast, the terpenoid matrix exhibited measurable cytotoxicity, with an IC₅₀ of 0.03 μ L/L.

Their anti-inflammatory activity was evaluated in the same way as for phytocannabinoids, based on their ability to reduce the production of

pro-inflammatory cytokines (Fig. 2A) and to decrease activation of the NF- κ B signalling pathway (Fig. 2B–S1). Among these matrices, only the polar matrix reduced TNF- α production (by 24 $\pm$ 7 %). In contrast, IL-6 production was reduced by the non-polar matrix (47 $\pm$ 4 %) and the terpenoid matrix (43 $\pm$ 12 %). Regarding the effects on NF- κ B activation, both the polar and non-polar matrices significantly reduced activation in at least one LPS-stimulated condition, compared to the positive control (LPS-only). For the non-polar matrix, pre-incubation led to a substantially stronger effect, increasing the reduction in NF- κ B activation from 31 $\pm$ 5 % to 87 $\pm$ 1 %. None of the matrices activated NF- κ B signalling in the absence of LPS (Fig. S1).

Antioxidant properties of the matrices were assessed, again following the same approach as for phytocannabinoids, using both ORAC and CAA assays (Table 3). The polar and terpenoid matrices showed measurable activity in the ORAC assay, while the non-polar matrix was inactive. None of the matrices showed activity in the CAA assay.

Overall, the results show that non-cannabinoid plant matrices are also capable of modulating inflammatory responses and displaying antioxidant activity, with effects varying between matrices, reflecting their distinct chemical composition.

3.3. Cannabinoid–matrix interactions

To evaluate potential interactions, the combined impact of phytocannabinoids and matrices on cytokine production was assessed. The ratios between phytocannabinoid and matrix concentrations were set to reflect their natural proportions in the original plant material (see Supplement for details). Synergy analysis was performed using the Bliss independence model (Fig. 3), which compares the observed effect of a combination with the effect expected under independent action. Interactions were classified as synergistic when the observed/expected ratio was significantly greater than 1, additive when it did not differ from 1, and antagonistic when it was significantly lower than 1.

Out of all tested combinations, five showed a synergistic effect. CBN, when combined with the polar or terpenoid matrix, and CBG combined with polar matrix synergistically reduced IL-6 production. For TNF- α , synergistic effects were observed with CBD and CBG in combination with the non-polar matrix. Although these five combinations demonstrated clear synergistic effects, highlighting the potential value of specific phytocannabinoid–matrix interactions, many others were

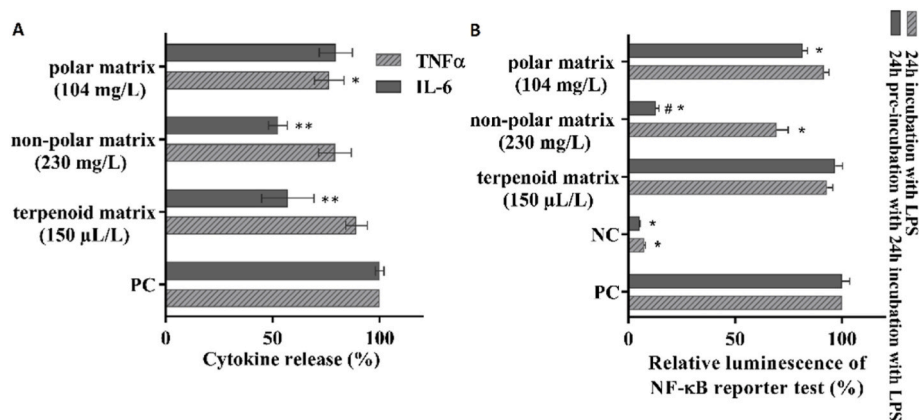


Fig. 2. Anti-inflammatory effects of *Cannabis sativa* L. matrices on THP-1-derived macrophages. (A) Levels of pro-inflammatory cytokines TNF- α and IL-6 released after 24h incubation with polar, non-polar, or terpenoid matrices in the presence of LPS (500 μ g/L). Cytokine concentrations were measured using sandwich ELISA. (B) NF- κ B pathway activation measured by luminescence reporter assay under two experimental conditions: 24h co-incubation with matrices and LPS, and 24h pre-incubation with matrices followed by 24h incubation with LPS. The negative control (NC) represents untreated cells. The positive control (PC) corresponds to LPS-treated cells without matrices but with the same concentration of DMSO used in treatments. Data are presented as mean $\pm$ SEM (n = 4). Statistical significance was determined by one-way ANOVA followed by Dunnett's *post-hoc* test (* p < 0.05, ** p < 0.01, *** p < 0.001 vs. PC, # p > 0.05 compared to co-incubation without pre-incubation).

Table 3

Antioxidant activity of *Cannabis*-derived matrices measured by ORAC and CAA assays. IC₅₀ values presented as mean $\pm$ SEM (n = 3). For the CAA assay, THP-1 cells were used.

Compound	IC ₅₀	
	ORAC	CAA
Polar matrix (mg/L)	66.1 $\pm$ 6.8	>104
Non-polar matrix (mg/L)	>230	>230
Terpenoid matrix (μL/L)	0.34 $\pm$ 0.01	>0.03
Ascorbic acid (mg/L)	132.9 $\pm$ 1.3	305.1 $\pm$ 3.8

additive, indicating that whole-plant preparations provide enhanced anti-inflammatory activity, highlighting encourage effect of *Cannabis* extracts.

4. Discussion

While the anti-inflammatory activities of major phytocannabinoids like CBD and CBG are well-documented (De Petrocellis et al., 2011; Jastrzab et al., 2022; Kim et al., 2023; Pagano et al., 2019), their acidic precursors remain comparatively understudied. Our study addresses this knowledge gap by providing a comprehensive analysis of the ten major phytocannabinoids, including less characterized acidic forms such as CBCA and CBDVA. We demonstrate that all tested compounds possess significant anti-inflammatory properties - including up to a 59 $\pm$ 4 % reduction in the pro-inflammatory cytokine IL-6, up to a 22 $\pm$ 5 % reduction in TNF- α , and up to a 42 $\pm$ 2 % reduction in NF- κ B activation - highlighting the untapped potential of the full phytocannabinoid spectrum.

These findings highlight the relevance of non-psychoactive phytocannabinoids as potential bioactive compounds for future dietary applications.

The strongest anti-inflammatory effects in our study were observed for IL-6 and NF- κ B, consistent with previous reports. TNF- α , in contrast, responded only weakly to phytocannabinoid treatment, although earlier studies have reported stronger effects. For example, CBD has been shown to reduce TNF- α levels both *in vitro* and *in vivo* (Carrier et al., 2006; Fouda et al., 2022), but we did not observe this under our experimental conditions. Methodological differences may partly explain this discrepancy. In particular, variations in pre-treatment protocols and cell culture preparation, such as the use of PMA and resting periods, can

influence THP-1 cell responsiveness and cytokine release kinetics (Lund et al., 2016).

This limited response of TNF- α to phytocannabinoids may also be explained by its different regulatory pattern. TNF- α is a rapid-response cytokine, and although its expression is partly NF- κ B-dependent, its early induction after LPS stimulation likely occurs before phytocannabinoid-mediated inhibition of NF- κ B takes effect (Falvo et al., 2010). By contrast, IL-6 expression requires sustained NF- κ B activation together with additional transcription factors, making it more sensitive to later-stage regulatory effects (Inoue et al., 2006).

To test whether longer phytocannabinoid exposure might enhance NF- κ B suppression, we included a pre-incubation step in our assays. However, in most cases this did not have a significant effect, suggesting that extended exposure alone is insufficient to improve NF- κ B inhibition in our THP-1 model.

Among the tested phytocannabinoids, CBDV was a notable exception. Unlike most others, it significantly reduced all three markers—TNF- α , IL-6, and NF- κ B. This wider effect may be linked to a specific mechanism of action: CBDV has been shown to interact directly with myeloid differentiation factor 2 (MD2), a co-receptor in the TLR4 complex, thereby interfering with LPS binding and blocking the inflammatory cascade at an earlier stage (Wang et al., 2022). Such upstream interference could explain why CBDV was able to suppress TNF- α production despite its rapid induction, setting it apart from the other phytocannabinoids.

Beyond their anti-inflammatory effects, we also sought to characterize the antioxidant potential of these compounds. The antioxidant potential of phytocannabinoids is generally expected because most of them contain phenolic hydroxyl groups (Borges et al., 2013). Despite this structural potential, our ORAC assay showed no measurable antioxidant activity for CBC, CBD, CBDV, or CBN at the tested concentrations. This outcome was consistent with previous reports showing antioxidant effects for some phytocannabinoids, such as CBD and CBDV, only at much higher concentrations (Russo et al., 2021). Furthermore, study Dawidowicz et al. (2021) demonstrated that measured antioxidant activity is highly dependent on the assay method. Specifically, they found acidic phytocannabinoids to be more effective in the ORAC assay than their neutral counterparts. Our results support this finding: in all cases, the acidic forms were equal to or more active than their neutral analogues.

In the cellular CAA assay, none of the tested phytocannabinoids showed measurable activity. This may be due to poor cellular uptake or

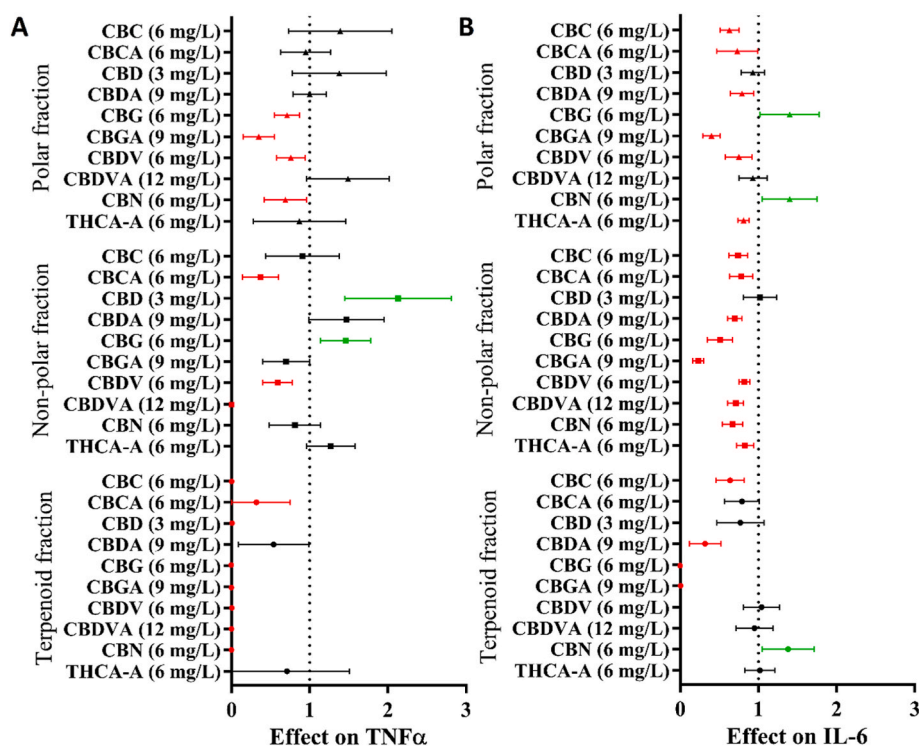


Fig. 3. Synergistic effects of phytocannabinoid and non-cannabinoid *Cannabis*-derived matrix combinations on pro-inflammatory cytokine production. The graphs show the results of Bliss independence analysis evaluating the interactions between individual phytocannabinoids and three *cannabis*-derived matrices: polar (rich in polyphenols and flavonoids), non-polar (containing sterols and fatty acids), and terpenoid. The effects were measured on the production of (A) TNF- α and (B) IL-6 in LPS-stimulated THP-1-derived macrophages. Bliss values greater than 1 indicate a synergistic effect (green), values less than 1 indicate an antagonistic effect (red), and values close to 1 suggest an additive interaction (black). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

a need for longer exposure to trigger transcriptional responses. For example, CBD is known to activate the Nrf2 pathway and increase antioxidant enzyme levels, but such effects likely require extended incubation times (Atalay et al., 2019). These results underline the importance of using both chemical and cellular assays to capture the full antioxidant potential of phytocannabinoids.

In this work, we not only tested the anti-inflammatory effects of pure phytocannabinoids but also examined their interactions with plant-derived matrices containing other constituents of *Cannabis sativa* L. The first evidence of such interactions, often referred to as the “entourage effect”, was described for the endocannabinoid 2-arachidonoylglycerol, which acted synergistically with 2-acyl-glycerol esters (Ben-Shabat et al., 1998). Although 2-arachidonoylglycerol is an endocannabinoid, it engages the endocannabinoid system in a way similar to phytocannabinoids, suggesting that comparable synergy could also occur with plant-derived cannabinoids. In line with this, we observed a significant synergistic effect in reducing IL-6 production when CBD or CBG were combined with the non-polar matrix. We hypothesize that fatty acids present in this matrix are the primary drivers of this enhanced activity. According to the composition, at least 4 different fatty acids are found in the non-polar fraction. In addition, the presence of glycolipids, carotenoids, phytosterols and triterpenoids is also worth noting. While the first three groups of substances mentioned can explain the additive effect of the non-polar fraction through its own anti-inflammatory activity (Doumar et al., 2024; Paes-Colli et al., 2022; Pacher et al., 2020), epifriedelanol, a triterpenoid found primarily in the cannabis root, contributes to the overall therapeutic potential of the plant through the “entourage effect” (Hanuš and Hod, 2020; Ryz et al., 2017). This effect posits that the various compounds in cannabis work better together than in isolation.

Two major groups of *Cannabis sativa* L. constituents that can interact synergistically with phytocannabinoids are flavonoids and terpenoids

(Nahler and Jones, 2018). For example, the flavonoid cannflavin A has been shown to exert a synergistic anticancer effect when combined with CBD (Tomko et al., 2022). In our study, flavonoids present in the polar matrix may account for the observed synergy between CBG or CBN and the polar matrix.

A synergistic antitumor effect has been reported for combinations of terpenoids and phytocannabinoids (Namdar et al., 2019). In contrast, in our study, the terpenoid matrix combined with phytocannabinoids mostly resulted in additive or, in many cases, antagonistic effects. Thus, such combinations did not show clear benefits for anti-inflammatory activity. This lack of synergy may reflect overlapping mechanisms of action between terpenoids and phytocannabinoids.

While CBD has attracted the most attention among phytocannabinoids, safety concerns have limited its approval as a dietary supplement (Engeli et al., 2025). Our findings demonstrate that other non-psychoactive phytocannabinoids also possess anti-inflammatory activity, sometimes comparable to, or even broader than CBD, and may represent promising alternatives with potentially fewer adverse effects. At the same time, both residual matrix components (even in highly purified preparations) and whole-plant extracts need to be carefully considered, as we observed that matrices can interact with phytocannabinoids in synergistic as well as antagonistic ways. Such interactions are critical for the development of functional foods and supplements, where the overall composition of the preparation will determine its efficacy. Further characterization of matrix constituents driving these interactions could support the design of more effective formulations.

5. Conclusions

Our results demonstrate that phytocannabinoids can act as anti-inflammatory agents. Several phytocannabinoids were shown to

reduce the production of pro-inflammatory cytokines and to suppress activation of the NF- κ B signalling pathway. These effects were observed not only for well-known phytocannabinoids but also for less-studied acidic forms, highlighting the potential of exploring a broader range of phytocannabinoids naturally present in *Cannabis sativa* L. In addition to phytocannabinoids, other plant-derived compounds, tested here in the form of isolated matrices, also exhibited anti-inflammatory activity. Notably, some combinations of phytocannabinoids and matrices produced synergistic effects. This suggests that the full therapeutic potential of the plant may lie in its complex chemical composition rather than in single isolated compounds. It is important to note that the matrices used in this study were derived from a single plant source, and their composition may vary across different cultivars. Therefore, further combinatorial testing is needed, along with efforts to identify the specific compounds responsible for the enhanced activity. Overall, phytocannabinoids offer considerable potential due to their chemical diversity and range of effects. From a nutritional perspective, these findings support the idea that non-psychoactive phytocannabinoids, alone or in combination with other plant constituents, could be developed into functional food ingredients or dietary supplements aimed at managing chronic inflammation. Transitioning these findings into disease-relevant cell models and ultimately *in vivo* systems would be the critical next step in translating the therapeutic promise of these complex plant extracts into tangible benefits for managing chronic inflammation. By demonstrating matrix-dependent synergistic anti-inflammatory effects among non-psychoactive phytocannabinoids and co-occurring *Cannabis sativa* L. constituents, this study provides experimental support for traditional multi-component use of the plant and establishes a rational basis for developing complex, plant-derived preparations targeting chronic inflammation.

CRedit authorship contribution statement

Ondřej Strnad: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Data curation. **Tomáš Nejedlý:** Writing – review & editing, Methodology, Investigation, Data curation. **Petr Svoboda:** Writing – review & editing, Supervision. **Helena Mertlíková-Kaiserová:** Writing – review & editing, Methodology, Conceptualization. **Matěj Malý:** Writing – review & editing, Methodology. **Milena Stránská:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Jitka Viktorová:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Milena Stranska reports financial support was provided by Czech Science Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jep.2025.121134>.

Data availability

The data supporting this article have been included as part of the Supplementary Information.

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