THE EFFECTS OF CANNABIS SATIVA EXTRACTS ON HUMAN MACROPHAGES *IN VITRO*

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DEDICATION

I dedicate this thesis to all people that supported me with love.

ABSTRACT

Inflammation is the immune system's response to an injury due to any agent. Currently, there is a wide array of anti-inflammatory agents available; however, they cause numerous side effects which compromise patients' quality of life. Therefore, new anti-inflammatory agents and strategies are required. Multiple research articles have demonstrated the strong potential of *Cannabis sativa* extracts and their active ingredients, including cannabinoids and terpenes, to be used in the treatment of inflammatory disorders. The current study explores the immunomodulatory potential of cannabis extracts *in vitro*. We investigated how the extracts of selected novel cannabis cultivars influence the secretion of cytokines in lipopolysaccharide (LPS)-induced macrophages. We discovered that studied cannabis extracts significantly reduced the levels of cytokines, such as IL-1β, TNF-α, IL-6, IL-8, MCP-1, and IL-10, while not being cytotoxic to macrophages. This study may serve as a roadmap for the future analysis of the anti-inflammatory effects of medical cannabis *in vivo*.

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LIST OF ABBREVIATIONS

2-AG: 2-arachidonoylglycerol
AEA: anandamide
ANOVA: one way analysis of variance
APCs: antigen-presenting cells
CB1 receptor: cannabinoid 1 receptor
CB2 receptor: cannabinoid 2 receptor
CBD: cannabidiol
CD4+: T helper cells
CD8+: cytotoxic T cells
CLRs: C-type lectin receptors
DAGL: diacylglycerol lipase
DAMPs: danger-associated molecular patterns
DMSO: dimethyl sulfoxide anhydrous
FAAH: Fatty acid amide hydrolase
GPR119: G protein-coupled receptors 119
GPR18: G protein-coupled receptors 18
GPR55: G protein-coupled receptors 55
HDF13: Human Cytokine Array Proinflammatory Focused 13-plex
HPLC: high performance liquid chromatography
IFN-γ: interferon-gamma
IL-1: interleukin 1
IL-10: interleukin 10
IL-12: interleukin 12
IL-13: interleukin 13
IL-2: interleukin 2
IL-4: interleukin 4
IL-5: interleukin 5
IL-6: interleukin 6
IL-8: interleukin 8
JAK: janus kinase
LPS: lipopolysaccharide
MAGL: monoacylglycerol lipase
MAPK: mitogen-activated protein kinases
MD2: myeloid differentiation factor 2
MDSCs: myeloid-derived suppressor cells
MHC: major histocompatibility complex
NAPE-PLD: NAPE-phospholipase D
NF-κB: nuclear factor kappa-B
NK: natural killer cells
NLRs: NOD-like receptors
NOD: nucleotide-binding oligomerization domains
NSAIDs: nonsteroidal anti-inflammatory drugs
PAMPs: pathogen-associated molecular patterns
PBMCs: peripheral blood mononuclear cells

PI3K: phosphatidylinositol 3-kinase
PRRs: pathogen recognition receptors
RLHs: RIG-like helicases
RPMI: Roswell Park Memorial Institute Medium
STAT: signal transducer and activator of transcription
Th1: T helper 1 cells
Th2: T helper 2 cells
THC: tetrahydrocannabinol
TLRs: Toll-like receptors
TNF-α: tumor necrosis factor alpha
TRPV1: transient receptor potential channels of the vanilloid subtype 1
TRPV2: transient receptor potential cation channel 2
TRPV2: transient receptor potential channels of the vanilloid subtype 2

1. Introduction

1.1 Immunity

Immunity is the capability of an organism to resist harmful microorganisms. There are two types of immunity: innate or native and adaptive or acquired. The main players of native immunity are neutrophils, macrophages, mast cells, dendritic cells, natural killer (NK) cells, myeloid-derived suppressor cells (MDSCs), and of adaptive immunity are immune cells such as T and B lymphocytes [1]. Inflammation is the major player in innate immunity. On the other hand, adaptive immunity can recognize several foreign substances in a specific way and is subdivided into humoral, mediated by B lymphocytes-produced antibodies and cellular, mediated by T lymphocytes. The successful immune response depends on the proper presentation of foreign material to T cells by antigen-presenting cells (APCs).The APCs, like macrophages and dendritic cells, display protein antigens to T cells and activate them. T cells can not recognize free antigens; instead, they recognize peptides displayed by major histocompatibility complex (MHC) molecules on other cells. Class I MHC molecules are present on all nucleated cells and cytotoxic T cells ($CD8^+$) respond to them, while Class II MHC molecules are expressed on APCs, and are recognized by T helper cells ($CD4^+$) [2].

T helper 1 cells (Th1) secrete interferon-gamma (IFN-γ), which induces classical macrophage activation and enhances cell-mediated toxicity (Figure 1). T helper 2 cells (Th2) cells produce interleukin 4 (IL-4), interleukin 5 (IL-5), which stimulate the alternative macrophage activation, as well as the production of antibodies. Classical macrophage activation (M1) is caused by microbial particles and cytokines produced by T cells. They secrete pro-inflammatory cytokines: tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), interleukin 12

(IL-12), interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 8 (IL-8) etc. These macrophages are actively microbicidal, participate in positive immune response and are able to stop the spread of cancer cells. Alternative macrophage (M2) activation is caused by IL-4, IL-5 and interleukin 13 (IL-13) produced by T cells. M2 secrete anti-inflammatory cytokines interleukin 10 (IL-10) and IL-4, have an immunosuppressive function, important in tissue repair and promote the growth and spread of cancer. Macrophages are considered one of the central cells that modulate the inflammatory responses via cytokine expression regulation. Interaction between T lymphocytes and macrophages is bidirectional (Figure 1). Macrophages present antigens to T cells, stimulate T cells, produce cytokines, and cause further stimulation of T lymphocytes. T cells make cytokines that cause macrophages activation, causing more cytokine production and antigen presentation [3],[4]. The released cytokines work in unison to achieve equilibrium where majority of them exhibit pro-inflammatory properties and few of them are anti-inflammatory in nature (Table 1).

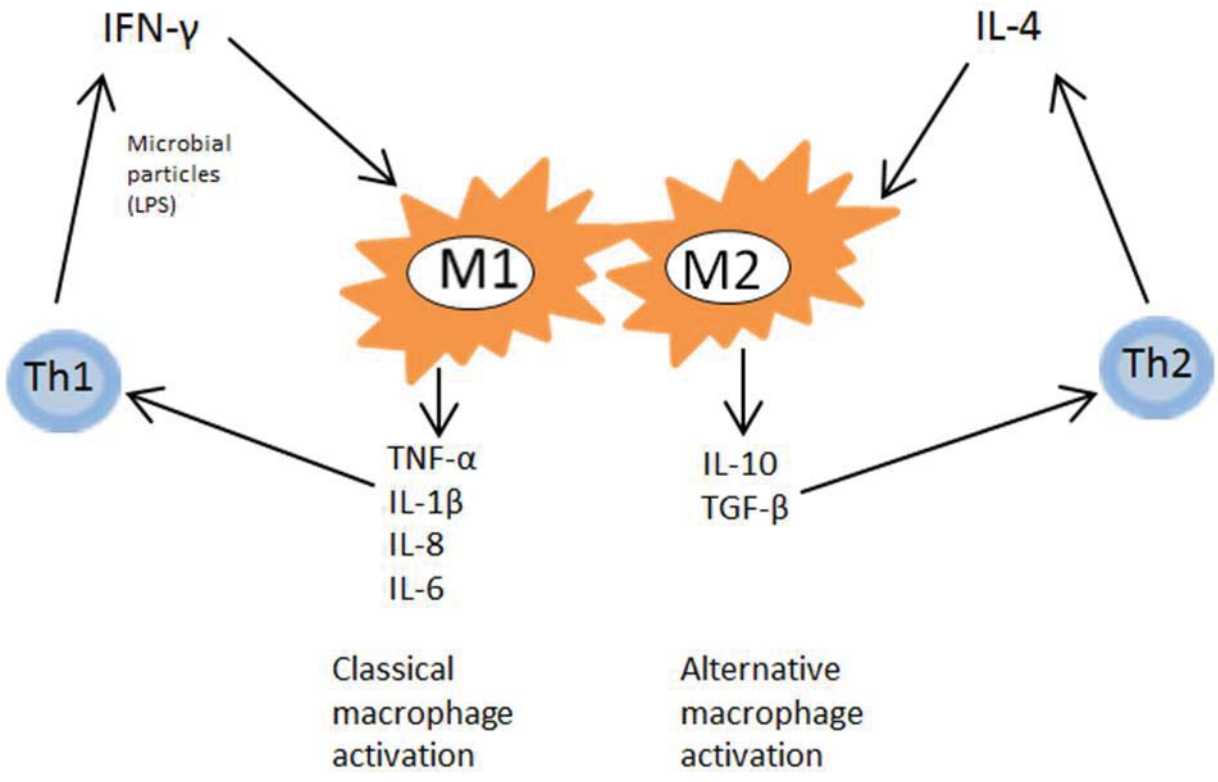


Figure 1. Interaction between T lymphocytes and macrophages

Table 1. The main effects of pro- and anti-inflammatory cytokines

Cytokines	**Production site**	**Effects**	Reference
TNF-α	Macrophages, T lymphocytes, NK cells, mast cells, eosinophils	Inflammation stimulation, resistance to infection and cancers.	[5]
IL-1β	Macrophages	Key mediator of inflammatory response; important in inflammasome signaling.	[6]
IL-8	Macrophages, lymphocytes, mast cells	Induce chemotaxis of granulocytes to the site of infection; phagocytosis stimulation.	[7]
MCP-1 (monocyte chemoattractant protein-1)	Macrophages, dendritic cells	Induce chemotaxis of neutrophils, monocytes, T ceels and dendritic cells to the site of infection and stimulate phagocytosis.	[8]
IL-6	T cells, macrophages	Stimulation of cellular differentiation, inflammation and the development of effector T cells; induces synthesis of acute phase proteins.	[9]
IL-12	Dendritic cells, macrophages	Encourages the advancement of the Th-1 response, enhances the cytotoxic activity of NK cells and CD8+ T cells, has anti-angiogenic effects.	[10]
IL-2	T cells, dendritic cells	A signal transducer and activator of transcription (STAT5), influences the differentiation of T helper cells, activates cytotoxic lymphocytes	[11]
IFN-γ	T helper cells (Th1), NK cells	Regulates the Th1/Th2 balance, promotes macrophage activation, enhances antigen presentation and leukocyte migration, activates STAT1.	[12]
Anti-inflammatory cytokines			
IL-10	Monocytes, lymphocytes, mast cells, macrophages, T helper cells (Th2),	Limiting a host immune response to pathogens, tissue homeostasis maintenance, the prevention of autoimmune conditions	[13]

	regulatory T cells	development; decreases antigen presentation and phagocytosis, enhances T reg cells	
IL-4	Mast cells, eosinophils, basophils, T cells	Regulates the Th1/Th2 balance, induces an alternative macrophage activation and immunoglobulin class switch to IgE and IgG	[14]
IL-5	T helper cells (Th2), mast cells	Stimulates the proliferation of B cells and their differentiation to Ig-secreting cells.	[15]
TGF-β	White blood cells	Controls cell proliferation, differentiation, wound healing; inhibition of B cells and activates macrophages; promotes T cells differentiation.	[16]
IFN-α	Plasmacytoid dendritic cells, macrophages	Chemokinesis and migration induction of T cells, anti-viral activity.	[17]

1.2 Cannabis plant and Endocannabinoid system

C. sativa plant has been known for centuries as a controversial plant. For many years, cannabis has been used in many cultures as alternative medicine. It contains phytocannabinoids, terpenoids, flavonoids, fatty acids, and other molecules. Cannabinoids act through the endocannabinoid system which is composed of receptors like cannabinoid 1 (CB1), cannabinoid 2 (CB2), transient receptor potential channels of the vanilloid subtype 1 and 2 (TRPV1, TRPV2), G protein-coupled receptors 18, 55, 119 (GPR18, GPR55, GPR119), endocannabinoids such as 2-arachidonoylglycerol and anandamide (2-AG, AEA), and enzymes responsible for their metabolism. The main biosynthetic enzymes are NAPE-phospholipase D (NAPE-PLD) and diacylglycerol lipase (DAGL); the main degradation enzymes are fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL). The main function of the endocannabinoid system is to maintain homeostasis [18]. The most well-known phytocannabinoids produced by the *C. sativa* are Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD). The endocannabinoids,

as well as phytocannabinoids, have high specificity to cannabinoid receptors. When these cannabinoids bind to receptors, they activate them and cause the inhibition of neurotransmitter release and, consequently, can affect the human organism in different ways. Other components of the plants, including minor cannabinoids, terpenoids and flavonoids, may act synergistically with cannabinoids, and their effect might be more potent as compared to these singular compounds alone. This phenomenon is known as "entourage effect" [19]. The CB1 receptor is mainly expressed in the central nervous system, and the CB2 receptor, being the most prevalent in the immune system, is mostly present in peripheral organs. Both receptors are G-protein-coupled cell surface receptors that are coupled to the adenylyl cyclase and cAMP-protein kinase A pathways and the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways [20]. With respect to the expression of cannabinoid receptors in immune cells, it has been demonstrated that these receptors are expressed in both adaptive and innate immune cells. For instance, CB1, CB2, and GPR55 receptors are expressed in the natural killer cells; CB1, CB2 receptors are present in the mast cells, T lymphocytes, B cells and macrophages. Therefore, phytocannabinoids can potentially influence the function of the immune system and regulate the process of inflammation [21].

1.3 Inflammation and its role in chronic disorders

Inflammation is body's defensive mechanism which is vital to health. Inflammation is an important part of both arms of the immune system and is crucial in regulating tissue homeostasis. The innate immune response is the first safeguard protecting our cells from harmful microorganisms. Microorganisms have pathogen-associated molecular patterns (PAMPs), while endogenous danger-associated molecular patterns (DAMPs) are released from damaged cells and cause a non-infectious inflammatory response. Lipopolysaccharides, which are the components

of gram-negative bacterial cell wall, proteins and glycolipids expressed in bacteria are the examples of PAMPs. The PAMPs and DAMPs are recognized by pathogen recognition receptors (PRRs), expressed on immune cells like macrophages and dendritic cells and non-immune cells, like fibroblasts, and stimulate the initial immune response, by activating PRRs [22]. The recognition of DAMPs and PAMPs by PRRs leads to formation of danger-sensing complex, called inflammasome. The innate immune system has five types of PRRs: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domains (NOD)-like receptors (NLRs), RIG-like helicases (RLHs), DNA sensors absent in melanoma 2 (AIM2) like receptors (ALRs) and DNA-dependent activator of IFN-regulatory factors (DAI)).

The inflammatory response process can be summarized as follows: 1) recognition of dangerous stimuli by PRRs; 2) activation of inflammatory pathways; 3) release of inflammatory markers; 4) recruitment of inflammatory cells [23]. There are three main pathways (Figure 2, 3) that play a major role in inflammation: nuclear factor kappa-B (NF-κB), Janus kinase (JAK)-signal transducer and activator of transcription (STAT), and MAPK pathways [24], [25], [26], [27].

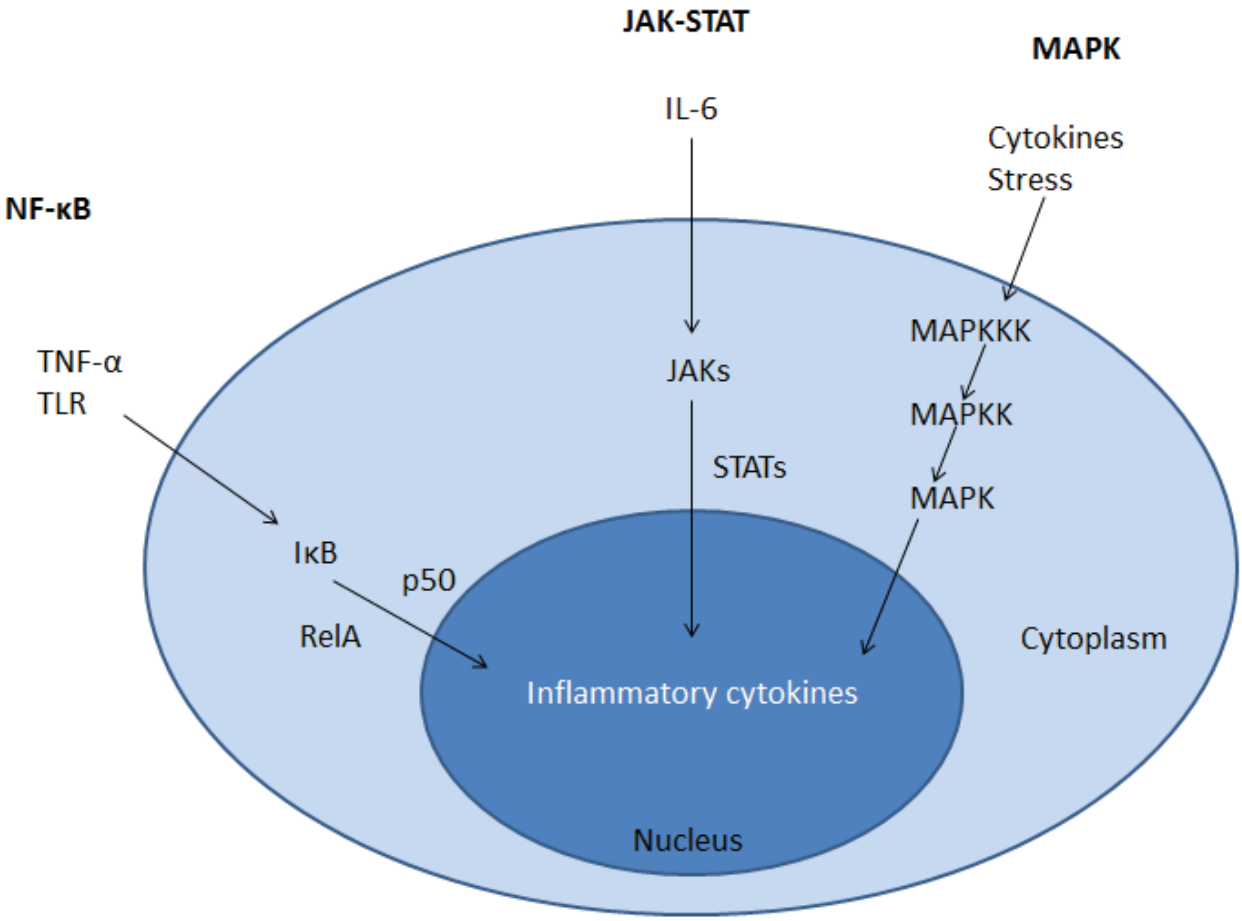


Figure 2. Inflammatory pathways. A. NFκB pathway. TLRs and pro-inflammatory cytokines (IL-1β, TNF-α) activate this pathway, followed by activation of RelA/p50 complexes, which actively regulate the expression of inflammatory cytokines. B. JAK-STAT pathway. This pathway is activated by IL-6 binding; then the signal is transduced to activate JAKs, and then -STATs. When STATs are phosphorylated, it leads to downstream activation of inflammatory cytokines. C. MAPK pathway. Extracellular stimuli, such as stress and cytokines, activate this pathway. MAPKKKs phosphorylate and activate MAPKKs, which in turn activate MAPKs. The activated MAPKs phosphorylate different proteins, like transcription factors that regulate inflammatory responses.

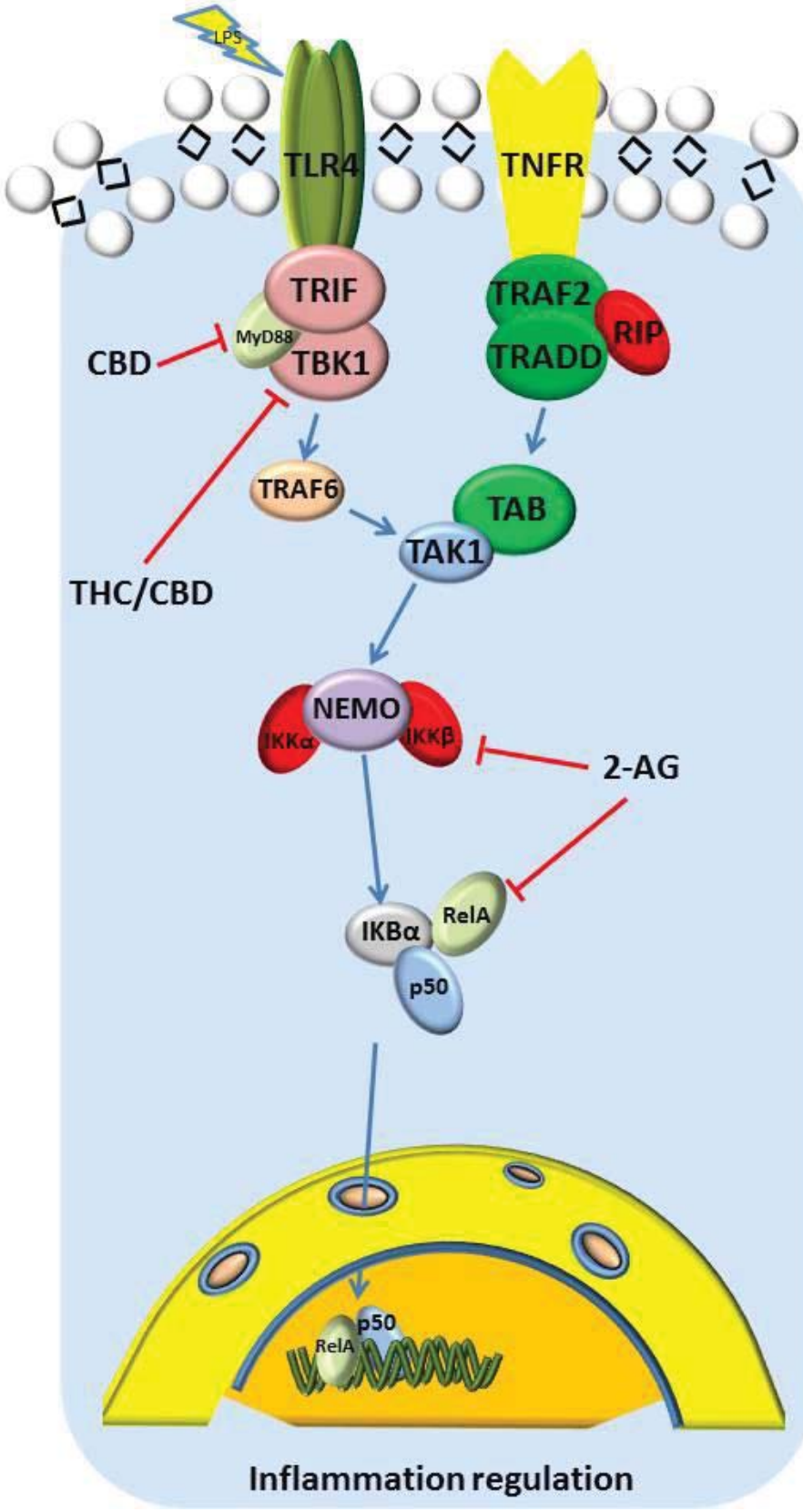

LPS
TLR4
TNFR
TRIF
MyD88
TBK1
CBD
TRAF2
RIP
TRADD
TRAF6
TAB
TAK1
THC/CBD
NEMO
IKKα
IKKβ
2-AG
IKBα
RelA
p50
p50
RelA
Inflammation regulation

Figure 3. The canonical NFκB signaling pathway in relation to TLR4/TNFR signaling pathway and possible sites for the anti-inflammatory activities of phyto- and endocannabinoids. TLR4 and TNFR downstream signaling pathways lead to activation of expression of pro-inflammatory cytokines. THC and CBD inhibit the important proteins of the TLR4 pathway, such as MyD88, TBK1, leading to downregulation of the NFκB pathway [28]. 2-AG inhibits the IKK and RelA proteins involved in the NFκB pathway [29].

Chronic inflammation plays a crucial role in the pathogenesis of many chronic conditions. The central nervous system disorders, like Parkinson's disease, Alzheimer's disease, multiple sclerosis, and epilepsy develop in the presence of chronic inflammation in the brain. Blood-brain barrier permeability is increased to different molecules, and neuronal excitability is enhanced due to inflammatory responses in the brain [30]. Excessive epithelial micro-injuries followed by acute lung inflammation causes pulmonary fibrosis [31]. Chronic lung inflammation is commonly observed in chronic obstructive pulmonary disease [32], cystic fibrosis [33], and asthma [34].

Cardiovascular diseases are the leading cause of death worldwide [35]. The mediators of inflammation play an important role in atherosclerosis development, which is the underlying pathology of cardiovascular disease [36]. Inflammatory bowel diseases, that include ulcerative colitis and Crohn's disease is characterized by an abnormal and excessive inflammatory response to intestinal microbial flora [37]. Recently, type II diabetes mellitus has been characterized as an inflammatory disease due to altered cytokines levels, tissue fibrosis, and changes in leukocytes' population number and activation state [38]. Excessive inflammation in the liver might cause loss of hepatocytes, ischemia-reperfusion injury, and permanent liver damage. Patients have an increased risk of developing the chronic liver disease due to destroyed hepatic parenchymal cells as a result of inflammation. Infectious hepatic inflammation is most commonly caused by hepatitis B and C virus, while sterile inflammation can lead to alcoholic or nonalcoholic

steatohepatitis [39]. All these conditions significantly contribute to the increase of general morbidity and mortality worldwide, and inflammation is a crucial element in the pathological progression of these diseases.

1.4 Cannabis and inflammation

According to previous reports, cannabinoids can be used as anti-inflammatory agents due to their potent immunomodulatory and anti-inflammatory properties [40], [41], [42], [43]. Currently, in medical practice, these substances have tolerable documented negative impact on patients in comparison with other commonly used anti-inflammatory drugs [44]. Therefore, herbal medicine, mainly cannabis derivatives, has gained a lot of attention due to its general efficacy and safety. Cannabinoids have other mechanisms of action on inflammation in comparison with nonsteroidal anti-inflammatory drugs (NSAIDs). NSAIDs inhibit the activity of cyclooxygenase enzymes, prostaglandins [45], while cannabinoids exert their anti-inflammatory activities mainly by reducing cytokine production, inducing apoptosis, inhibiting cell proliferation, and enhancing the function of T regulatory cells [43]. They influence the innate immune responses by suppressing the activity of NK cells, dendritic cells, migration of neutrophils and macrophages with their antigen presentation and phagocytosis processes [46], and by triggering the induction of MDSCs [47],[48]. The T cell immunity can be influenced by cannabinoids in different ways: they can affect the proliferation and the number of T cells by polarizing the cytokine response to either Th1 or Th2 (Figure 3) [49]. Cannabinoids have been shown to suppress the proliferation of T cells induce their apoptosis, and support the Th2 polarization [50],[51]. It has been shown that cannabinoids and cannabis extracts suppress the pro-inflammatory cytokines, like IL-2, IL-1β, TNF-α, IFN-γ, IL-12, IL-8, IL-6 and enhance the anti-inflammatory cytokines, like IL-10 in different cell lines and animal models [43]. CBD and

THC have been the most studied phytocannabinoids as anti-inflammatory compounds, while CBD has gained more attention due to the lack of psychoactive side effects. CBD can activate the immune response by elevating *TNF-α* and *IL-6* mRNA expression of, as was shown in mice in response to the LPS-induced pulmonary inflammation [52], while most studies demonstrated that CBD has an immunosuppressive potential by inhibiting pro-inflammatory cytokines like IL-6 and IL-8 in an *in vivo* mouse colon cancer CT26 cell model [53], by reducing the protein expression of IL-1β, IL-6, and TNF-α on microglia BV-2 cells [54], and by reducing inflammation in other models, like dinitrobenzene sulfonic acid (DNBS)-induced colitis [55], human ulcerative colitis [56] etc. Another study showed that CBD reduced the levels of TNF-α and IL-6 in murine models of chronic asthma [57]. CBD was shown to reduce the TNF-α levels in RAW264.4 macrophages, stimulated by LPS [58]. In another study, it was shown that CBD reduced IL-1β and TNF-α levels in mitogen-activated peripheral blood mononuclear cells [59]. Another paper reported that CBD significantly reduced TNF-α, IL-6, and IL-8 in an *in vitro* model of allergic contact dermatitis [60]. Recently, it was discovered that cannabis acts on mitochondrial homeostasis to counteract inflammatory dysregulation. CBD has been shown to alleviate cerebral ischemia in rats by reducing brain oedema, blood-brain barrier permeability, infarction size, and neurological deficit. This effect was due to increased protein expression of Na^{+}/Ca^{2+} exchangers [61]. When blood flow is restored in the ischemic area, it causes inflammation and oxidative-stress-related injury in the affected area. CBD has demonstrated a neuroprotective effect in oxygen–glucose-deprivation/reperfusion in *in vitro* model by reducing the oxidative stress, improving mitochondrial bioenergetics and being able to modulate the glucose metabolism [62]. THC treatment of the trophoblast cell line, HTR8/SVneo, showed a reduction in mitochondrial respiratory function and membrane potential. This data suggested that THC can cause dysfunction of mitochondria [63]. When THC effect was evaluated on

mitochondria extracted from the rat brain, similar results were obtained, that is, it enhanced oxidative stress and induced mitochondrial dysfunction in the brain [64]. Another study showed that THC was able to reduce the expression of pro-inflammatory transcription factors, like TNF-α, IFN-γ on murine delayed type hypersensitivity model [65]. THC was also demonstrated to be effective in combating intestinal inflammation on *in vivo* colitis model [66]. A study performed on LPS-stimulated J774 macrophages showed that THC significantly inhibited the levels of IL-6, prostaglandin E2, and nitric oxide production [67]. The experimental research conducted *in vivo* with SIV-infected macaques that were receiving THC for the period of seventeen months demonstrated an increase in T cells, a reduction in viral load, and an increase in the expression of Th2 cytokines [68]. Another study performed with HIV patients showed a higher concentration of $CD4^+$ and $CD8^+$ T cells in THC- positive patients versus THC-negative counterparts [69]. It was also shown that cannabis users living with HIV have lower neuroinflammation. This was confirmed by demonstrating that cannabis users had lower levels of $CD16^+$ monocytes and inducible protein 10 (IP-10) compared to HIV-infected patients that were not users. These data suggested that cannabis has potent anti-inflammatory effects [70].

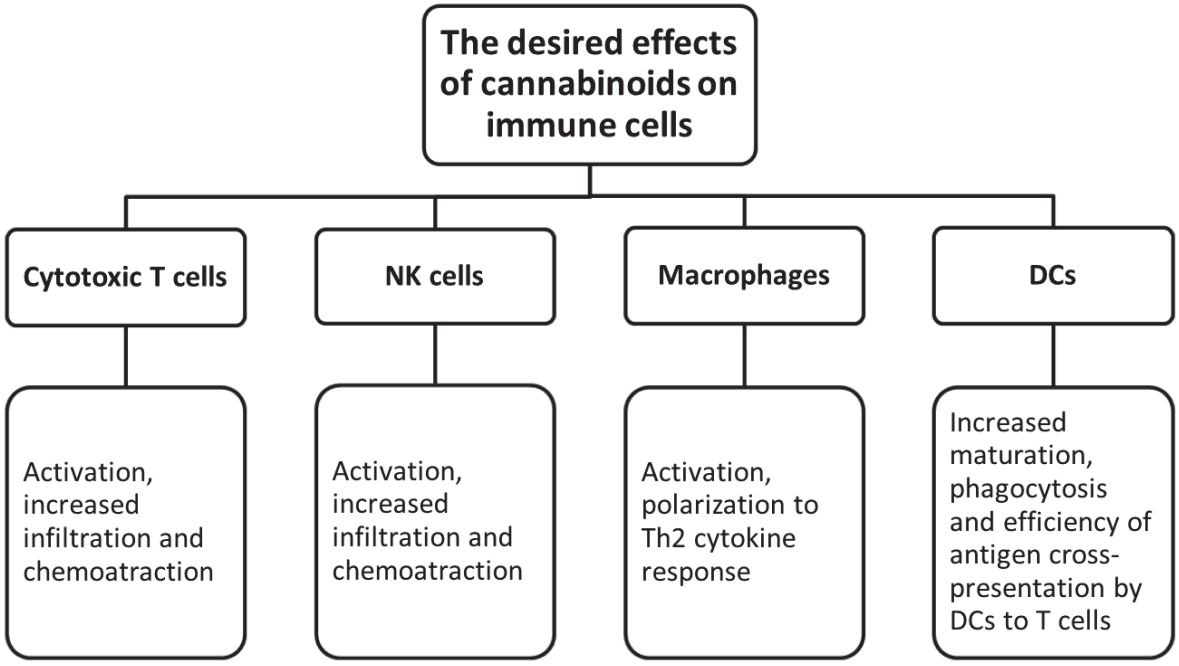


Figure 4. The desired effects of cannabinoids on immune cells

Terpenoids, apart from giving cannabis plant a characteristic fragrance and providing protection from insects, have been demonstrated as interesting pharmacological compounds for treating chronic inflammatory conditions, mainly by inhibiting NF-κB transcription factor and inflammasome activation. Triptolide terpenoid has been shown to reduce inflammation by inhibiting inflammasome NLRP3-ASC assembly in cardiac fibrosis mice model [71], downregulating TLR4 and NLRP3 expression in IgA nephropathy rats [72]. Limonene was demonstrated to inhibit the production of nitric oxide, prostaglandin E2, and pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 in RAW 264.7 macrophages, stimulated by LPS [73].

Beta-caryophyllene was demonstrated to suppress the expression of pro-inflammatory cytokines (TNF-α, IL-1β) in peripheral blood, stimulated by LPS [74].

The research was also performed to compare the anti-inflammatory properties of terpenoids versus CBD [75]. Essential oils were prepared from three non-psychoactive cannabis chemotypes, and terpenoids profile was evaluated, and their effect on inflammation was assessed both *in vitro* and *in vivo*. The *in vitro* results demonstrated that all three essential oils high in terpenoids partially inhibited the reactive oxygen species production in stimulated macrophages. However, when these oils were assessed *in vivo*, only moderate inhibition of inflammation, without an effect on TNF-α serum levels, was found. Interestingly, CBD showed prolonged immunosuppression, while terpenoids demonstrated transient immunosuppression. Such results indicate that terpenoids are more important for treatment of pathological acute inflammation rather than chronic inflammatory conditions [75].

The role of flavonoids in inflammation is still a new field of research. One research publication showed that cannflavins, isolated from *C. sativa* sprouts inhibited the production of prostaglandin E2 and 5-lipooxygenase [76]. Apigenin, another type of flavonoid found in cannabis, was shown to inhibit NLRP3 activation in THP-1 cells and reduce peritoneal infiltration of inflammatory cells in the monosodium urate-induced peritonitis mice model [77]. In addition, apigenin dietary supplementation on murine experimental colitis model was able to inhibit the NLRP3 inflammasome activation by suppressing the expression of IL-1β and IL-18, and decreased the micro-and macroscopic signs of colitis [78].

1.5 Human macrophages – an *in vitro* inflammation model

As discussed above in the section on immunity, macrophages play an important role in

innate immunity, hence, we have decided to use these cells as our experimental model. Macrophages recognize foreign substances with the help of PRRs on their surface, produce pro-inflammatory and anti-inflammatory cytokines, perform phagocytosis to digest and eliminate pathogens, and interact bidirectionally with adaptive immune compartment.

The suspension monocytic cell line, called THP-1, isolated from the peripheral blood of a childhood case of acute monocytic leukemia, was chosen for my study due to several reasons. Firstly, it is an immortal cell line with a high growing rate. Secondly, we had an established protocol of differentiation and inflammation induction, and when differentiated, it becomes adherent cell line, which makes it easier to work with. Thirdly, this cell line is commonly used in inflammation study and is an accurate and valid cell model for LPS response evaluation in macrophages. Lastly, it is reliable, suitable, and simplified model to study the functions of macrophages [79]. Moreover, some studies demonstrated that these cells behave as primary macrophages in view of morphology and functional properties [80], [81]. THP-1 macrophages after being stimulated with LPS, express important genes for LPS signaling, such as myeloid differentiation factor 2 (MD2), CD14, a cell surface-localized glycosylphosphatidylinositol-anchored monocyte differentiation antigen and others [82], [83]. Phorbol-12-myristate-13-acetate (PMA), 50 ng/ml, was used for terminal differentiation of monocytes into macrophages, making them no longer proliferate after the transformation [84]. The successful transformation was assessed under the light microscope.

2. Rationale and Hypothesis

Research in our laboratory has demonstrated a potential of cannabinoids and cannabis extracts as immunomodulators [85]. It was shown that cannabis extracts are superior to CBD or THC in decreasing the expression of pro-inflammatory cytokines in intestine, oral and lung epithelial cells, and 3D tissue models.

Here, we hypothesized that previously tested novel cannabis cultivars also exhibit immuno-modulatory potential in *in vitro* inflammation model of THP-1 human macrophages.

3. Materials and methods

3.1. Main reagents

The pure cannabinoids were purchased from Sigma: Δ9-THC (Cat#T4764), CBD (Cat#C-045). The cannabinoids were dissolved in methanol to make 1 mg/ml stock solutions and then were stored at -20℃. Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 was purchased from Sigma-Aldrich (Cat#L4391). The LPS was dissolved in sterile PBS according to the manufacturer's protocol to make 1 mg/ml stock solution. Phorbol-12-myristate-13-acetate (PMA) - CAS 16561-29-8 – Calbiochem, was purchased from Sigma-Aldrich (Cat#524400). The DMSO (Dimethyl sulfoxide anhydrous, Life Technologies) was used to make 1 mg/ml stock solution. The sterile PBS was used to make 5 μg/ml stock solution. Trypan Blue Solution, 0.4%, was purchased from ThermoFisher Scientific (Cat#15250061).

3.2. Plant growth and extract preparation

The cannabis plants were grown in the licensed facility at the University of Lethbridge. *Cannabis sativa* cultivars #14, #131, #178 were used for the experiments. The selection of extracts was done based on internal lab data. Four plants were grown per cultivar at 22° C, 18 h light 6 h dark for 4 weeks. After this, plants were grown in the chambers with 12 h light/12 h dark regime to promote flowering. The flowers were harvested and then dried. Flowers from four plants were combined and subsequently used for the extraction. In total, three grams of powdered plant tissue per each cultivar was used for further extraction. The prepared material was placed into a 250 mL Erlenmeyer flask, then 100 mL of ethyl acetate was added into each flask. The flasks were incubated overnight at 21°C on shaker at 120 rpm, covered in tin foil. The filtration of extracts was done, then they were concentrated with help of rotary vacuum evaporator and

transferred to a tared 3-dram vial. The elimination of solvent was performed by evaporating to dryness in an oven overnight at 50°C. The crude extracts (3-6 mg) were dissolved in DMSO (Dimethyl sulfoxide anhydrous, Life Technologies) to obtain 60 mg/mL concentration and were stored at -20°C. The complete culture media (RPMI + 10% FBS) was used for diluting the stock concentration of extracts to the desired concentration (7 µg/ml). After this, the extracts were filtered with 0.22 µm filter.

3.3. High performance liquid chromatography (HPLC)

The levels of cannabinoids (CBD and THC) was analyzed with the help of Agilent Technologies 1200 Series HPLC system which has G1315C DAD, G1316B column compartment, autosampler (G1367D), and binary pump (G1312B). The Phenomenex Kinetex EVO C18 column with a Phenomenex SecurityGuard ULTRA guard column was used for the separation. The data acquisition, control of the instrument, and integration were performed with help of software, ChemStation LC 3D Rev B.04.02 (Agilent Technologies). For calibrating the standards and analysis of samples, the injection volume of 2 µL was used. The detection of compound peaks was done for 230 nm and 280 nm. On the A side, the mobile phases included 50 mM ammonium formate (Sigma-Aldrich) in HPLC grade water (Fisher Chemical), and 100% methanol on the B side. The flow rate was 0.3 ml/min. Per each cultivar, two samples were analyzed, with two technical repeats for each sample.

3.4. Terpene analysis

The analysis of terpenes was done on dry flowers with help of 8610C GC coupled with a flame ionization detector (SRI Instruments at Canvas Labs, Vancouver, BC, Canada). Two

samples per cultivar were analyzed.

3.5. Cell cultures and treatments

The THP-1 cells (TIB-202), human leukemia monocytic cell line, were purchased from American Type Culture Collection ((ATCC, (Rockville, MD, USA)) and were used in this study [79]. The cell line was grown as suspension culture in 100 mm petri dish, in the Roswell Park Memorial Institute Medium ((RPMI-1640 (Cat# 350-000-CL, WISENT INC., Quebec, Canada)) supplemented with a final concentration of 10% heat-inactivated Premium Grade Fetal Bovine Serum (Cat# 97068-085, VWR International LLC, Radnor, USA), according to the ATCC recommendation. The cell line was incubated in a humidified atmosphere of 5% CO_2 at 37°C. The cells were differentiated into macrophages by administering PMA, 50 ng/ml for two days. Then, cells were incubated in fresh RPMI-1640 medium, but without PMA, for one day. The cell differentiation was verified under the light microscope by evaluating the cell adhesion and spreading [86]. Inflammation was induced by adding 0.5 μg/ml of LPS for 3 hours (Figure 4) [87]. Cells were then harvested for the analysis.

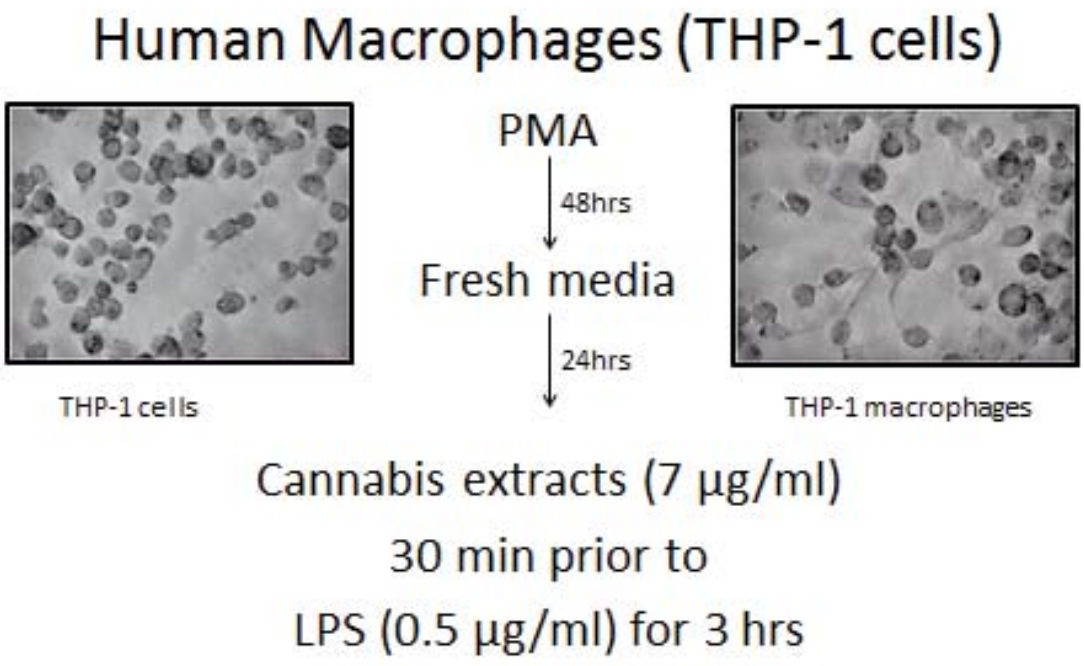


Figure 5. Schematic representation of the experimental plan. THP-1 suspension cells were terminally differentiated into THP-1 macrophages with the help of PMA (50ng/ml) for two days. Next, cells were supplemented with fresh media for one day. The successful differentiation was verified under the light microscope. Cannabis extracts (7µg/ml) or single cannabinoids (5 µM) were added 30 min prior to LPS (0.5 µg/ml).

The cells were categorized into groups:

A. Untreated cells

B. Vehicle

C. Cannabis extracts (#14, #131, #178)

D. CBD/THC

E. LPS

F. LPS + Cannabis extracts (#14, #131, #178)

G. LPS + CBD/THC

3.6. Cell viability assay using trypan blue

The determination of THP-1 macrophages' viability and morphology was performed by trypan blue assay. The cells were washed with PBS, then trypsinized. The fresh media was added to the trypsinized cells followed by centrifugation at 1,500 rpm for 5 min at 20°C. The supernatant was removed, and the pellet was resuspended by fresh media. An aliquot of the cells was mixed with the trypan blue solution in 1:1 ratio. The cells were counted using the LUNA I, automated cell counter (Logos Biosystems). The number of viable cells was determined by trypan blue exclusion. The results were shown as a percent of viability [88],[89].

3.7. Immunoblotting

After treatments, cells from each experimental group were harvested by adding cell lysis buffer (Cell Signaling Technology), followed by sonication and centrifugation (10,000 g, 2 min). Bradford assay was used for protein concentration estimation. Approximately 50 µg of proteins were loaded per each well for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The polyvinylidene difluoride (PVDF) membranes (Amersham Hybond® P, GE Healthcare) were used for transferring the resolved proteins for 2 hrs on ice. Then membranes were blocked with 5% non-fat dry milk for 2hrs. Membranes were incubated with primary antibodies at 4 ℃ overnight. Total NFκB (NFκB) expression levels were detected using 1:500 dilution of mouse monoclonal NFκB p65 antibody (Cat#sc-8008, Santa Cruz Biotechnology, Inc., Texas, United States). The phosphorylated NFκB (p-NFκB) expression levels were detected using 1:500 dilution of rabbit polyclonal p-NFκB p65 antibody (Cat#sc-33039, Santa Cruz Biotechnology, Inc., Texas, United States). The mouse monoclonal GAPDH antibody (Cat#sc-

47724, Santa Cruz Biotechnology, Inc., Texas, United States) was used for detecting the expression of GAPDH. Next, membranes were washed three times in 0.1% Tween-20 in PBS (PBS-T) and incubated with secondary antibodies 1:2000 for 2hrs at room temperature. Immunoreactivity was visualized with help of FroggaBio substrate (CCH345-B). Protein expression (four independent replicates) was analyzed using ImageJ software.

3.8. Multiplex ELISA

Multiplex ELISA was done for cytokine assessment [87]. Cell culture supernatant from each experimental group was centrifuged at 3,000 x g at 4°C for 3 min prior to aliquoting to remove debris. Then, the supernatants were transferred to new tubes and stored at -70°C. The prepared samples were shipped to Eve Technologies (Calgary, Alberta, Canada) on dry ice. The cytokines levels were measured using Human Cytokine Array Pro-inflammatory Focused 13-plex (HDF13). The measurement of all samples was done at the same time upon the first thaw (Table 2). Multiplex immunoassay results were analyzed with a BioPlex 200 [90],[91]. The following cytokines were tested: TNF-α, IL-6, IL-1β, IL-10, MCP-1, IL-8. It was expected cannabis extracts or cannabinoids would decrease TNF-α, IL-6, IL-1β, IL-8, MCP-1 but increase IL-10 [92].

Table 2. Summary of groups and number of samples used for Multiplex ELISA

Groups	Number of samples
Vehicle	**4**
LPS	**4**
Extracts 14, 131, 178 only (7µg/ml)	**12**
LPS + Extract 14, 131, 178 (7µg/ml)	**12**
CBD	**4**
CBD + LPS	**4**
THC	**4**
THC + LPS	**4**
Total:	**48**

For the simplicity of data analyses, we grouped our data into two main groups: CBD and THC groups (Table 3).

Table 3. CBD and THC groups

Experimental Groups			
CBD Group		THC Group	
Vehicle	LPS	Vehicle	LPS
CBD only	LPS + CBD	THC only	LPS + THC
Extract 131 only	LPS + Extract 131	Extract 14 only	LPS + Extract 14
		Extract 178 only	LPS + Extract 178

3.9. Statistical analysis

Statistical significance was analyzed using one way analysis of variance (ANOVA), after which Tukey post-hoc multiple comparison test was performed in GraphPad Prism 6.0 software (La Jolla, CA, USA) [93]. A p value < 0.05 was considered statistically significant [94].

4. RESULTS

4.1. Analysis of cannabinoids content using HPLC

In order to separate extracts into the categories of high CBD or high THC extracts, the cannabinoids content was analyzed by HPLC (Table 4).

Table 4. Levels and concentrations of single cannabinoids in extracts of selected *C. sativa* cultivars

	Total THC, %	Total CBD, %	THC (µM)	CBD (µM)
14 (high THC)	33.35	2.81	**7.42**	0.63
131 (high CBD)	2.11	19.65	0.47	**4.37**
178 (high THC)	33.98	1.01	**7.56**	0.22
THC	-	-	5	-
CBD	-	-	-	5

Table 4 also shows the molarity of THC and CBD in each studied extract. For comparison purposes, it also shows the concentration of pure cannabinoids, THC and CBD used in our study.

The HPLC analysis revealed the cannabinoids composition of studied extracts. The results demonstrated that Extract #14 is high THC type, Extract 131 is high CBD, and Extract #178 is high THC. The total % of THC in Extract#14 was 33.35%, in Extract #178 – 33.98%, in Extract 131 – 2.11%. The total % of CBD in Extract #131 was 19.65, in Extract #14 – 2.81, in Extract #178 – 1.01%. Based on these results, the molarity of cannabinoids (THC, CBD) was calculated

in each extract. The molarity of THC in both high THC extracts, #14 and #178, was very similar, 7.42 and 7.46 μM, respectively. The molarity of THC in extract #131 was 0.47 μM. The molarity of CBD in high CBD extract, #131 was 4.37; the molarity of CBD in extract # 14 was 0.63 μM, and in extract #178 was 0.22. The concentration of THC and CBD in extracts was compared to the concentration of pure cannabinoids used in this study as additional positive controls. The molarity of both pure cannabinoids (THC and CBD) used was 5 μM.

4.2. Analysis of terpenes

In order to evaluate potential impact of terpenes on anti-inflammatory properties of extracts, terpene analysis was performed (Table 5).

Table 5. Composition of terpene and their concentrations in each extract

Terps in mg/g	#131	#178	#14
α-Pinene	0.295	0.649	0.048
β-Pinene	0.212	0.245	0.068
β-Myrcene	ND	0.361	0.124
Limonene	0.262	0.003	0.263
Terpinolene	0.025	0.008	0.004
Linalool	0.058	0.029	0.193
α-Bisabolol	0.003	0.244	0.061
trans-Caryophyllene	0.04	0.076	0.545
α-Humulene	ND	0.04	0.136
trans-Nerolidol	ND	0.008	0.187
cis-Nerolidol	0.001	0.003	ND
Camphene	0.022	0.025	0.015
β-Ocimene	ND	0.089	ND
Fenchone isomers	ND	0.003	0.004
δ-3-Carene	0.506	0.001	ND
α-Terpinene	0.006	0.573	ND
Eucalyptol	0.002	ND	ND
γ-Terpinene	ND	0.001	ND
p-Cymene	ND	0.048	ND
Camphor isomers	ND	0.118	ND
Isopulegol	0.01	0.016	ND
Caryophyllene oxide	0.017	0.098	ND
Valencene	ND	0.01	ND
Geraniol	0.005	0.004	ND
Guaiol	0.029	0.116	ND
trans-β-Ocimene	0.016	ND	ND
a-Humulene	0.025	ND	0.136
Fenchyl Alcohol	ND	ND	0.036
Borneol isomers	ND	ND	0.012
α-Terpineol	ND	ND	0.052
Total Terpene content	1.534	2.768	1.75

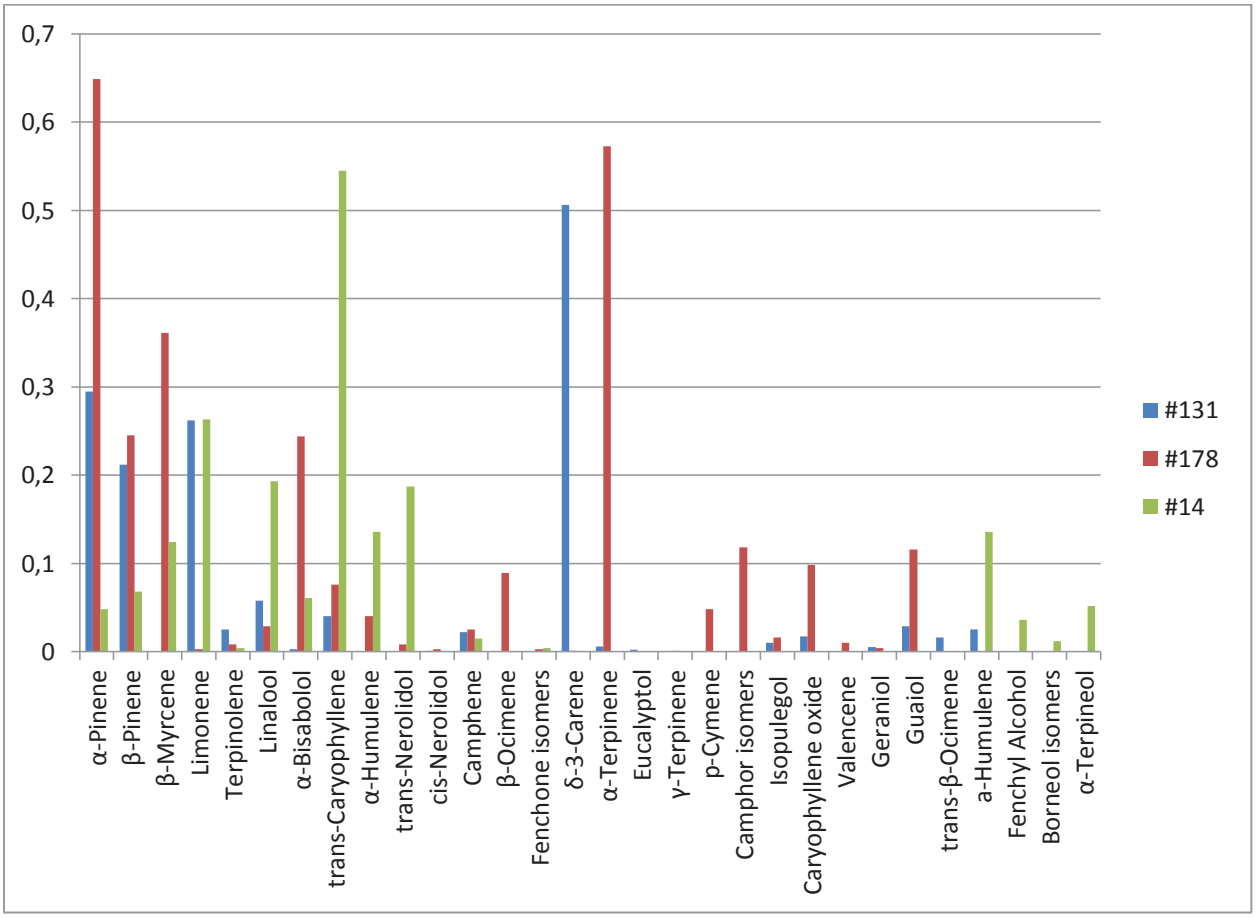


Figure 6. Level of terpenes in extracts #131, #178 and #14.

The terpenes analysis revealed that Extract #14 was the highest in trans-caryophyllene, limonene, linalool, and trans-nerolidol content. Extract #131 was the most prominent in having δ-3-carene, α-pinene, limonene, and β-pinene. Extract #178 had high percentage of α-pinene, α-terpinene, β-myrcene, β-pinene, and α-bisabolol. The comparison analysis demonstrated that all three studied extracts had high amount of α-pinene, β-pinene, linalool, and trans-caryophyllene. Interestingly, Extract #14 was the only one containing fenchyl alcohol, borneol isomers, and α-

terpineol. Valencene, camphor isomers, p-cymene, γ-terpinene, and β-ocimene were only present in Extract #178. Extract #131 was the only one prominent in eucalyptol and trans-β-ocimene (Figure 5).

4.3 Pilot experiment for establishing the appropriate duration of LPS stimulation and finding the appropriate samples for Multiplex ELISA

For establishing the appropriate duration of LPS stimulation, we performed a Western blot analysis for the expression of NFκB and p-NFκB. We used two time points for LPS stimulation: 3 and 6 hrs. Our aim was to achieve the upregulation in p-NFκB expression and downregulation of NFκB expression to conclude that our inflammation model is working. The quantification of the p-NFκB/NFκB ratio showed that the desired inflammatory response was achieved at 3 hrs post-LPS stimulation (Figure 7). Based on these results, we proceeded with 3 hrs duration for LPS stimulation for our actual experiment.

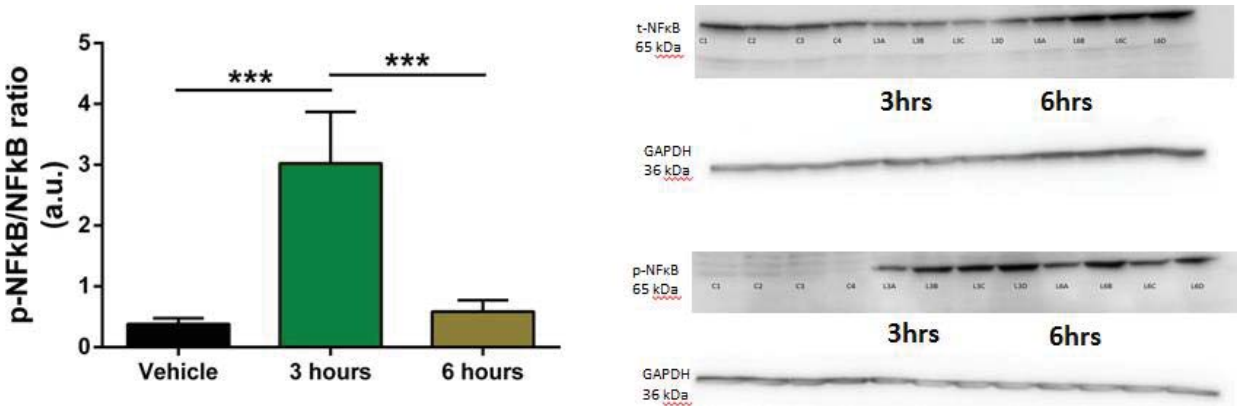
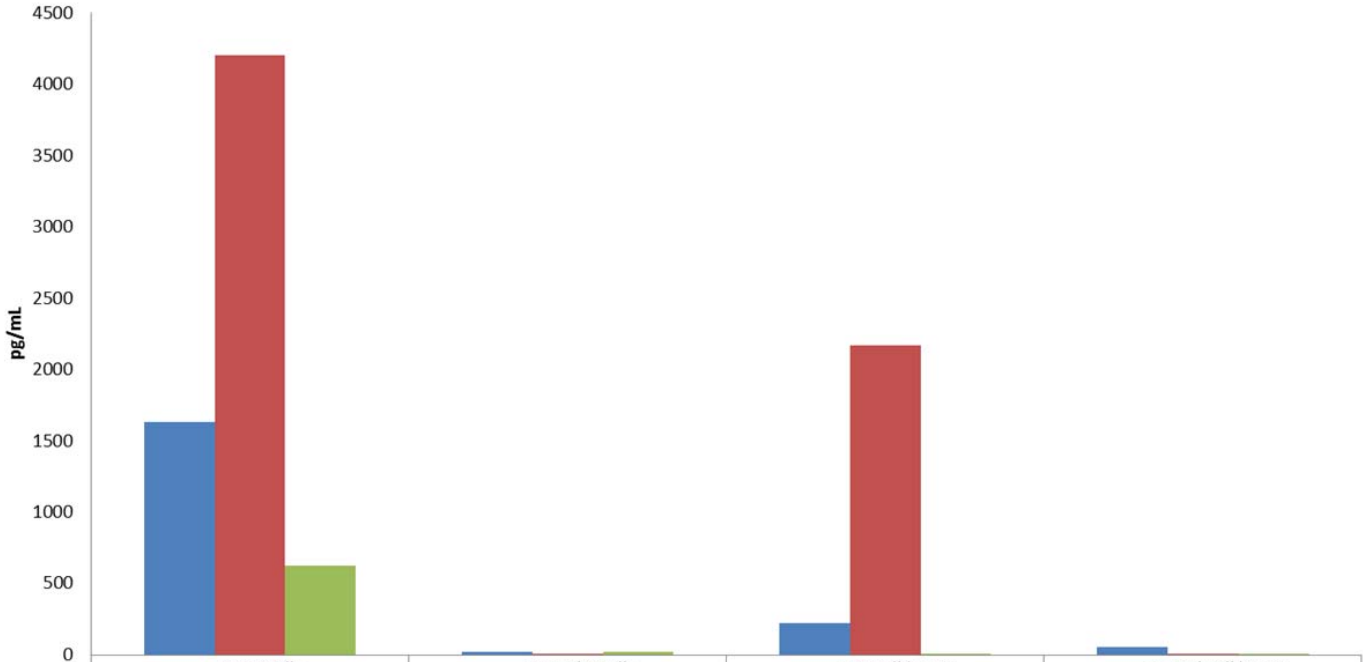


Figure 7. Quantification of p-NFκB/ NFκB. Statistical analysis was performed by ANOVA followed by Tukey post-hoc multiple comparison test. A value of $P < 0.05$ was considered to be significant. Significant differences between groups are marked with: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

After finalizing the LPS stimulation time, we performed a pilot Multiplex ELISA experiment to find out the best type of samples for cytokine assessment. There are two possible ways to check for cytokines *in vitro,* either in the cell culture supernatant (media) or cell lysate supernatant. The pilot experiment was performed on samples with expected low cytokines levels (control) and expected high cytokines levels (LPS). We found that LPS media samples had much higher levels of cytokines in comparison to LPS cell lysate samples (Figure 8). It was concluded, that media samples are much more efficient for studying the cytokines, that is why for our actual experiment, we proceeded with cell culture supernatant samples to perform a Multiplex ELISA.

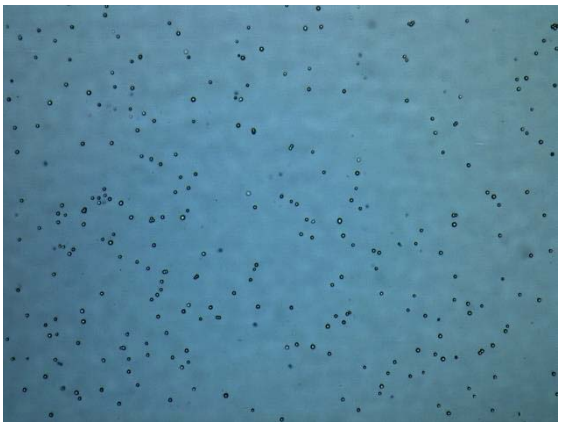


	LPS media	control media	LPS cell lysate	control cell lysate
IL-1β	1635,39	21,51	224,67	54,11
TNFα	4202,42	6,46	2168,15	7,93
IL-10	625,18	20,37	3,57	5,65

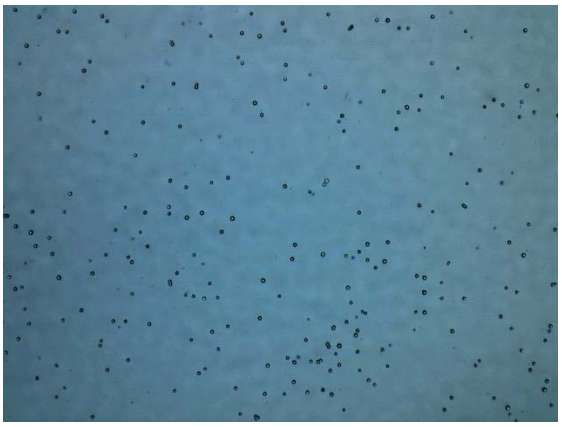
Figure 8. Comparison of the cytokines levels in cell culture supernatant (media) versus cell lysate supernatant.

4.4. Selected concentrations of *Cannabis sativa* extracts do not reduce the viability of macrophages

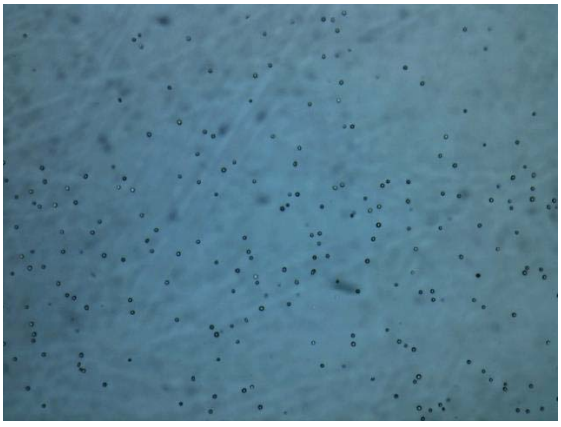
Cell viability assay results



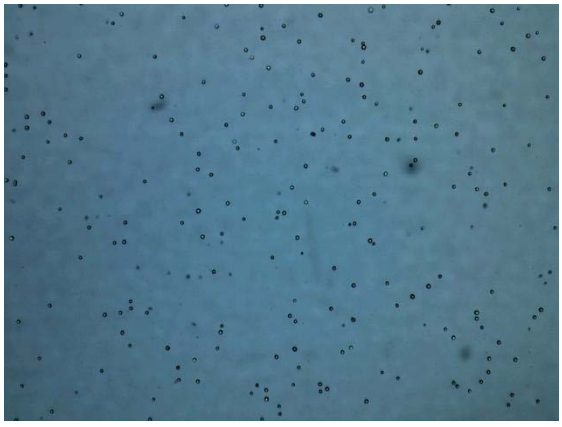
A. untreated



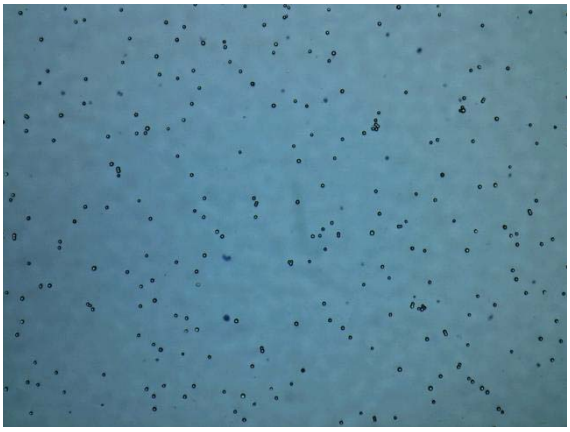
B. vehicle



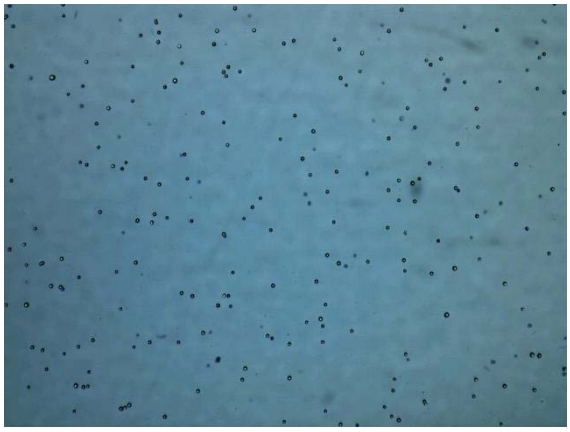
C. CBD only



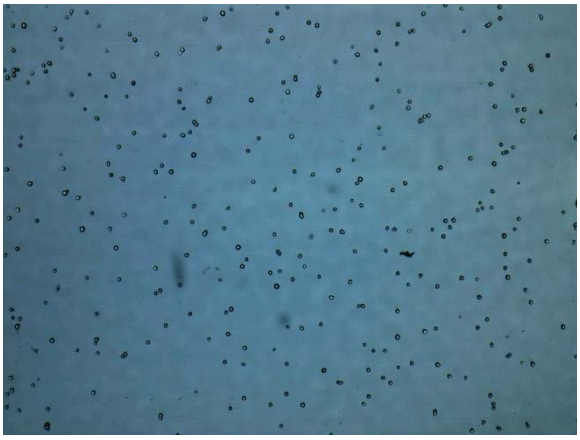
D. THC only



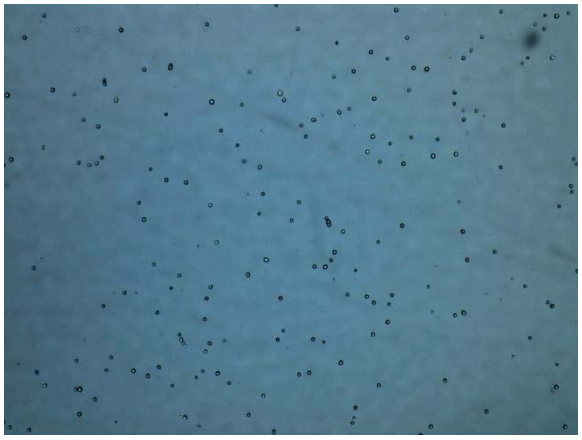
E. extract #14 only



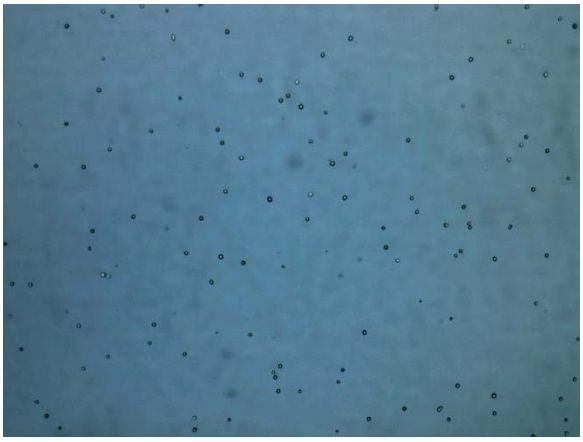
F. extract #131 only



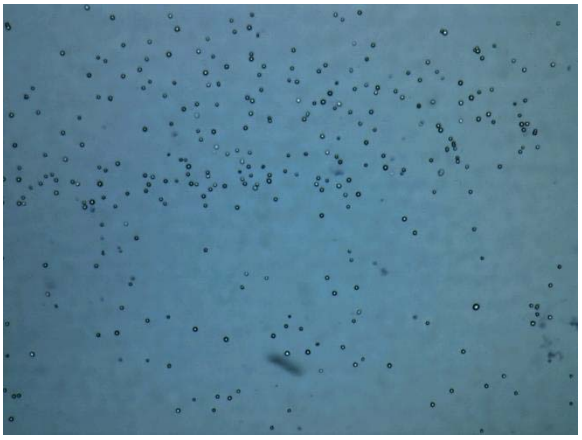
G. extract #178 only



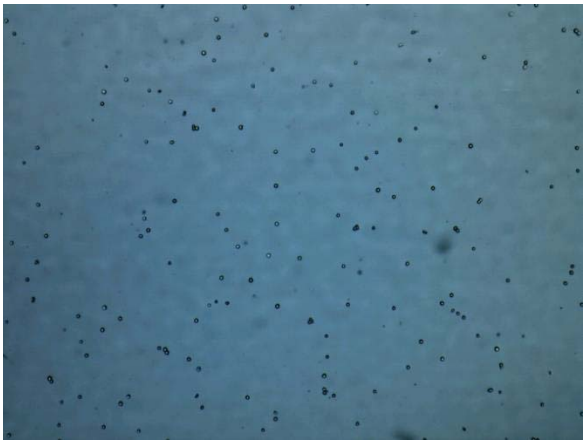
H. LPS only



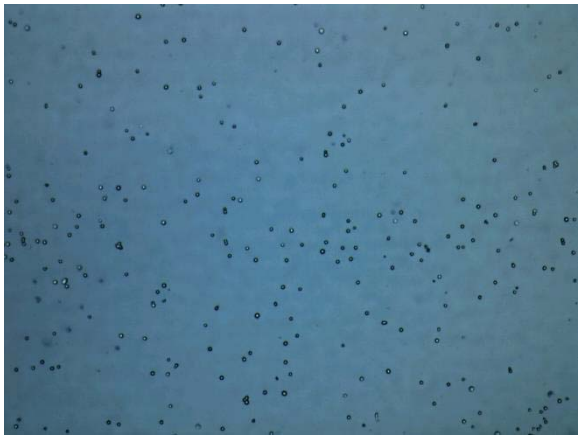
I. LPS + CBD



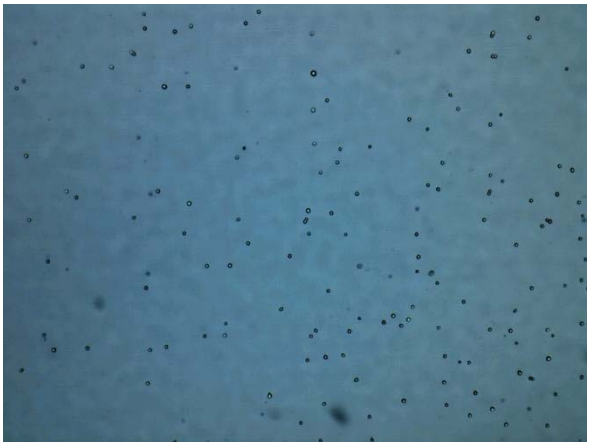
J. LPS + THC



K. LPS + Extract #14



L. LPS + Extract#131



M. LPS + Extract #178

Figure 9. The trypan blue cell viability assay photos were taken 3 minutes after staining with trypan blue using LUNA automated cell counter. The THP-1 macrophages from each experimental group were subjected to trypan blue assay. Approximately 1,5x10^6 of THP-1 cells were plated in each well of the 6-well culture plate. Next, they were transformed, with help of PMA (50ng/ml) for 48 hrs as shown in the Figure 3. For treatments, 5 μM of CBD and THC was used; in extract #14 the concentration of THC was 7.42 μM, in extract #178 -7.56 μM; the concentration of CBD in extract #131 was 4.37 μM. Cannabinoids and extracts were given 30 min prior to LPS (0.5 μg/ml). Cells were harvested 3 hrs after being treated with LPS. An aliquot of the cells was mixed with the trypan blue solution in 1:1 ratio. The cells were counted using the LUNA I, automated cell counter (Logos Biosystems) as per the manufacturer's instructions.

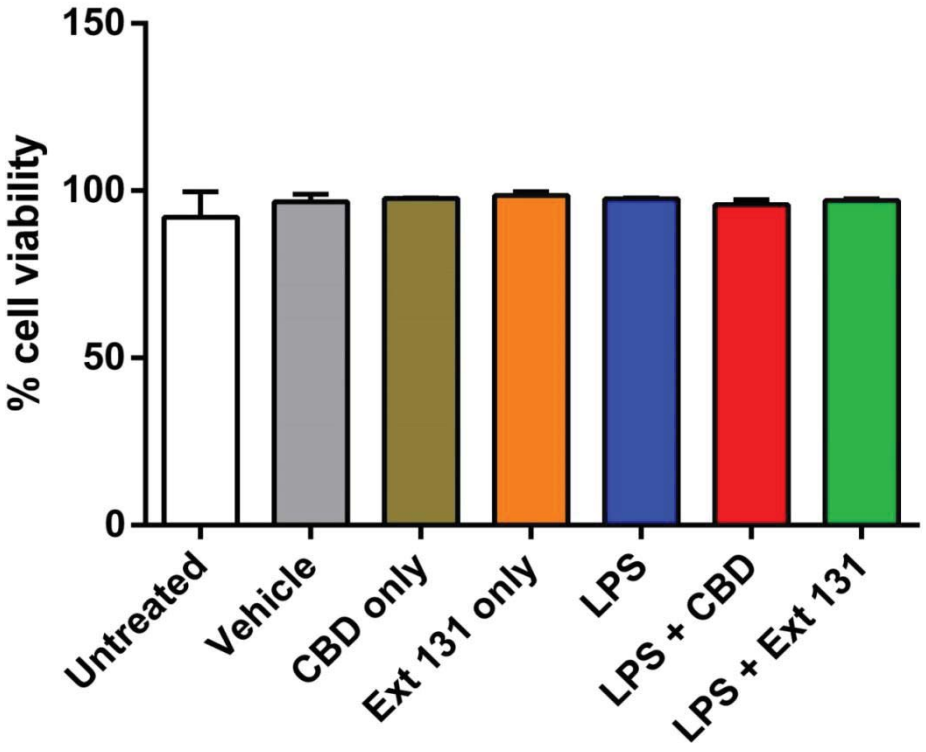


Figure 10. Analysis of cell viability using trypan blue assay in CBD group in THP-1 macrophages. Results are expressed as means ± standard deviations of each group in triplicate.

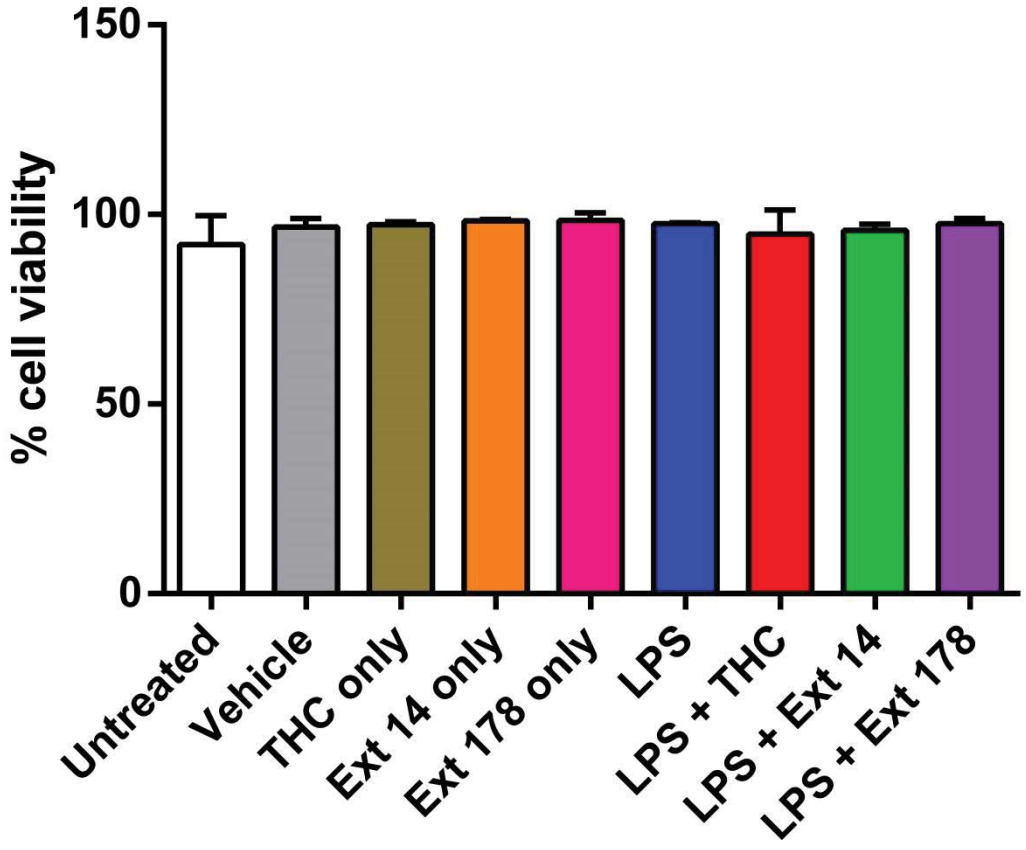


Figure 11. Analysis of cell viability using trypan blue assay in THC group in THP-1 macrophages. Results are expressed as means ± standard deviations of each group in triplicate.

The trypan blue exclusion assay did not show any reduction in cell viability among all groups, and cell morphology was not affected. This is important, because the level of inflammation can change due to the impact of active ingredients on cell viability. Since the cell viability was not compromised at studied concentrations of CBD/THC in any of the studied groups, we proceeded with Multiplex ELISA assay.

4.5. *Cannabis sativa* extracts attenuate TNF-α, IL-1β, IL-8, MCP-1, IL-10, and IL-6 production in LPS-stimulated THP-1 macrophages

As explained in the methods, after differentiation, macrophages were pretreated with cannabinoids or cannabis extracts, and after 30 min, LPS was added, and 3 hours later, cell supernatants were evaluated for the levels of cytokines. For simplicity and better comparison, each cytokine is explained below with respect to CBD and THC groups separately.

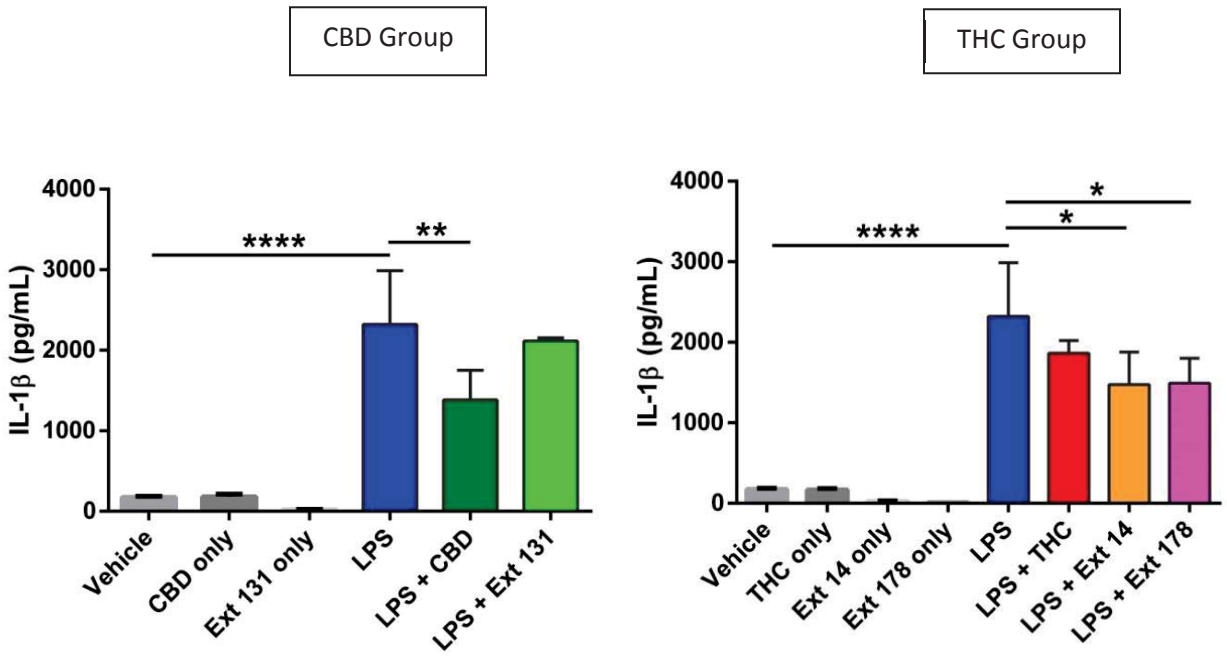


Figure 12. Analysis of IL-1β cytokine. Results are presented as mean of calculated concentration [pg/ml] ± SD. Statistical analysis was performed by ANOVA followed by Tukey post-hoc multiple comparison test. A value of $P < 0.05$ was considered to be significant. Significant differences between groups are marked with: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$.

In the CBD group as shown above, the LPS-treated cells showed significantly higher levels of IL-1β release in comparison to vehicle. CBD treatment after LPS stimulation was able to reduce the levels of IL-1β significantly in comparison to LPS group, while Extract #131 (high CBD) had no significant effect (Figure 9).

In the THC group as shown above, the LPS treatment significantly upregulated the levels of

IL-1β as compared to the vehicle. Both extracts high in THC (#14 and #178) were very efficient in downregulating the levels of this cytokine significantly in comparison to LPS group, while THC treatment alone was not effective in reducing the IL-1β levels.

The next studied cytokine was IL-6.

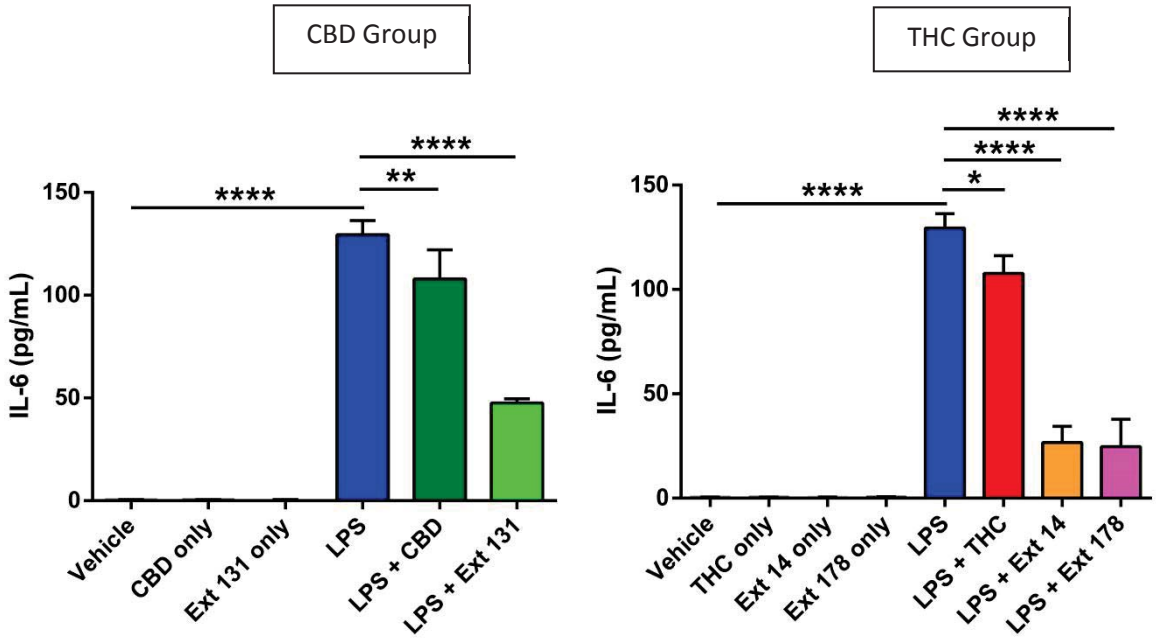


Figure 13. Analysis of IL-6 cytokine. Results are presented as mean of calculated concentration [pg/ml] ± SD. Statistical analysis was performed by ANOVA followed by Tukey post-hoc multiple comparison test. A value of $P < 0.05$ was considered to be significant. Significant differences between groups are marked with: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$.

Concerning the levels of IL-6, LPS was able to increase it significantly in comparison to vehicle. High CBD extract #131, as well as CBD, after LPS stimulation, significantly suppressed the levels of this cytokine in comparison to LPS (Figure 10). The extracts, high in THC (#14 and #178) and pure THC, effectively inhibited IL-6 cytokine levels as compared to LPS group.

Then we studied the levels of IL-8.

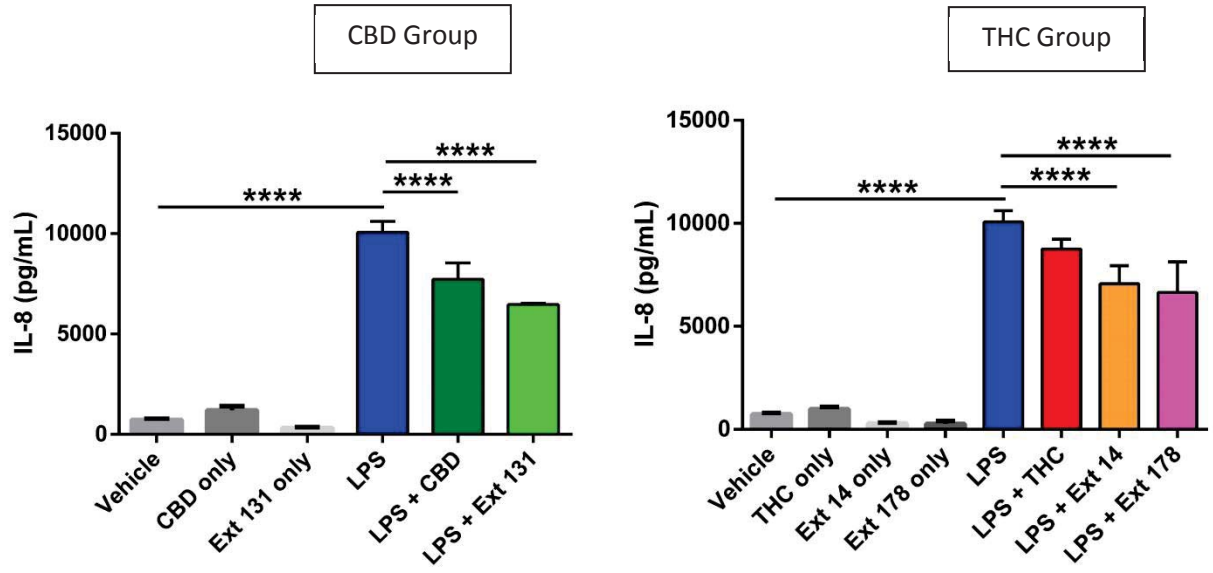


Figure 14. Analysis of IL-8 cytokine. Results are presented as mean of calculated concentration [pg/ml] ± SD. Statistical analysis was performed by ANOVA followed by Tukey post-hoc test. A value of $P < 0.05$ was considered to be significant. Significant differences between groups are marked with: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$.

The LPS was also efficient in increasing the levels of this pro-inflammatory cytokine significantly in comparison to vehicle as shown above. Pure CBD, as well as, all studied extracts, significantly reduced the levels of this cytokine after LPS stimulation in comparison to LPS only group (Figure 11). Interestingly, THC + LPS group did not affect the levels of IL-8 significantly in comparison to LPS.

Then, we examined the levels of MCP-1 cytokine.

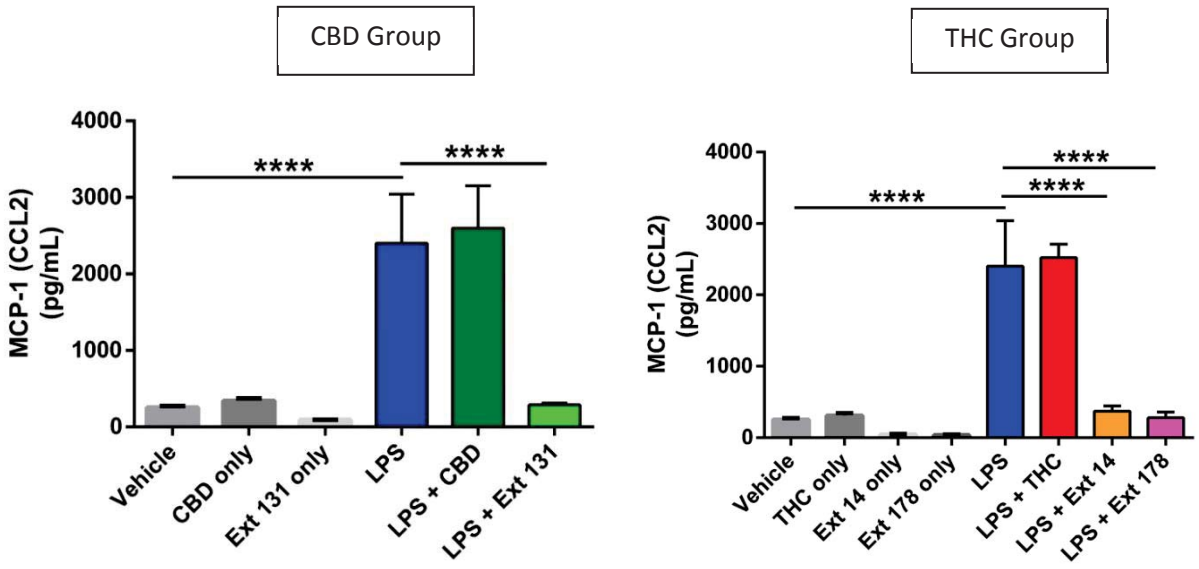


Figure 15. Analysis of MCP-1 cytokine. Results are presented as mean of calculated concentration [pg/ml] ± SD. Statistical analysis was performed by ANOVA followed by Tukey post-hoc test. A value of $P < 0.05$ was considered to be significant. Significant differences between groups are marked with: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$.

LPS treatment significantly elevated the levels of MCP-1 in comparison to vehicle group. The high CBD extract, #131, significantly downregulated the levels of this cytokine after LPS stimulation, while LPS + CBD group did not show any significant change as compared to the LPS group (Figure 12).

Both high THC extracts, extracts #14 and #178, significantly inhibited the levels of MCP-1 cytokine in comparison to LPS group, while THC + LPS did not affect the levels of this cytokine significantly.

Next, we studied the levels of TNF-α.

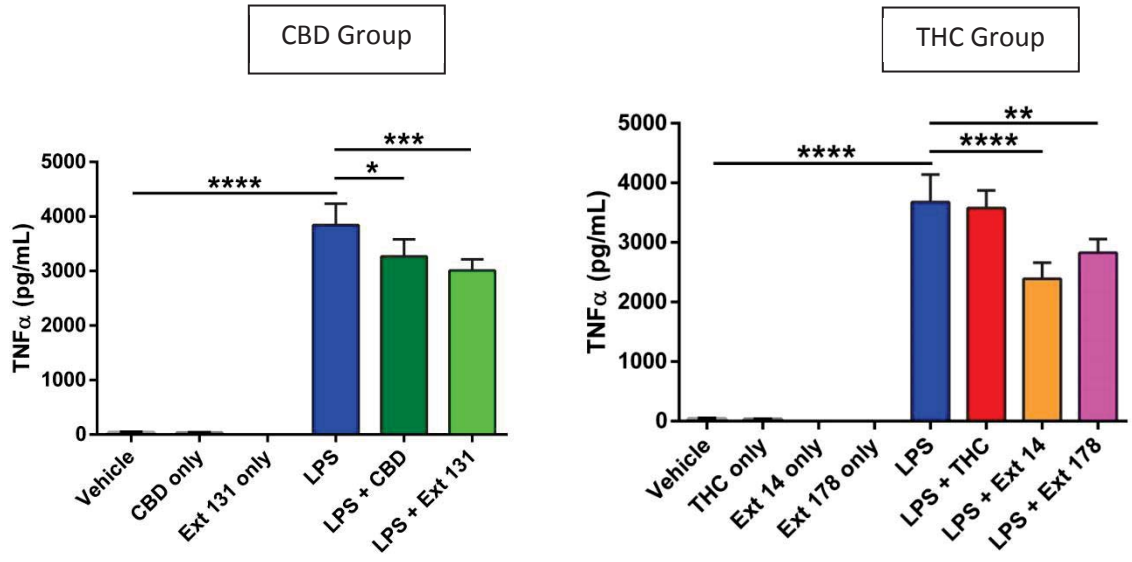


Figure 16. Analysis of TNF-α cytokine. Results are presented as mean of calculated concentration [pg/ml] ± SD. Statistical analysis was performed by ANOVA followed by Tukey post-hoc test. A value of $P < 0.05$ was considered to be significant. Significant differences between groups are marked with: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$.

The levels of TNF-α pro-inflammatory cytokine were significantly upregulated in LPS group in comparison to vehicle group (Figure 13). LPS + Extract #131 and LPS + CBD groups significantly downregulated the levels of this cytokine as to LPS group. LPS + THC group did not change the levels of TNF-α significantly in comparison to LPS, while both extracts high in THC, #14 and #178, significantly inhibited the levels of this cytokine after LPS stimulation.

The last cytokine we examined is IL-10, and this is the only anti-inflammatory cytokine we studied.

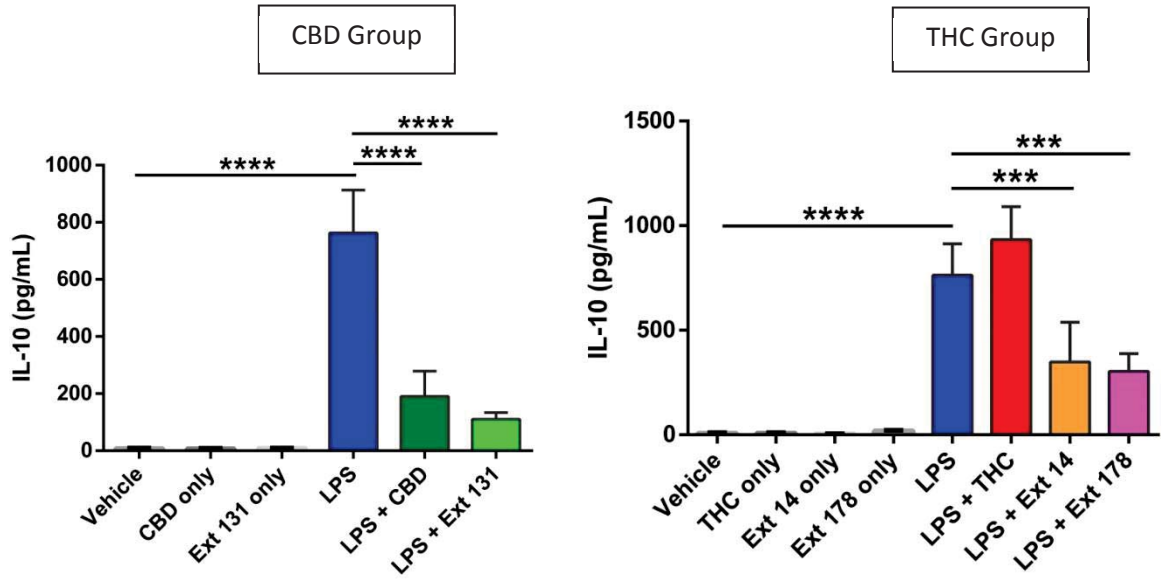


Figure 17. Analysis of IL-10 cytokine. Results are presented as mean of calculated concentration [pg/ml] ± SD. Statistical analysis was performed by ANOVA followed by Tukey post-hoc test. A value of $P < 0.05$ was considered to be significant. Significant differences between groups are marked with: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$.

LPS significantly increased the levels of IL-10 cytokine in comparison to vehicle group (Figure 14). CBD and high CBD extract, #131, as well as high THC extracts, #14 and #178 after LPS stimulation significantly suppressed its levels as compared to the LPS group. In contrast, the THC after LPS stimulation did not show any significant change in comparison to LPS, although there was a tendency to an increase.

We have noted that in nearly all cases, the effect of cannabis extracts was more significant than the effect of single cannabinoids (Table 6). We have not found, however any significant difference between performance of extracts #131, #14 or #178.

	CBD	THC	Extract 131 (high CBD)	Extract 14 (high THC)	Extract 178 (high THC)
IL-1β	↓	No change	No change	↓	↓
TNF-α	↓	No change	↓	↓	↓
IL-6	↓	↓	↓	↓	↓
MCP-1	No change	No change	↓	↓	↓
IL-8	↓	No change	↓	↓	↓
IL-10	↓	No change	↓	↓	↓

Table 6. Summary of the effects of extracts versus CBD/THC on studied cytokines

4.6. High THC extracts were more efficient reducing studied cytokines production than high CBD extract in LPS-stimulated THP-1 macrophages

To find out if there is any significant difference between the performance of all studied extracts a one-way ANOVA analysis followed by Posthoc Tukey's Multiple Comparison test was performed. We found that high THC extracts (#14 and #178) were more efficient in reducing the

levels of IL-1β after LPS stimulation as compared to LPS than high CBD extract (#131). Also, both high THC extracts (#14 and #178) were more effective in suppressing the levels of IL-6 as compared to high CBD extract (#131). There was no difference in the performance of all studied extracts with respect to IL-8 and MCP-1 levels. The most effective extract in view of inhibiting TNF-α levels was high THC extract (#14). Interestingly, the high CBD extract (#131) was the most effective in suppressing the levels of IL-10. Overall, high THC extracts performed better in view of reducing studied cytokines (Figure 18).

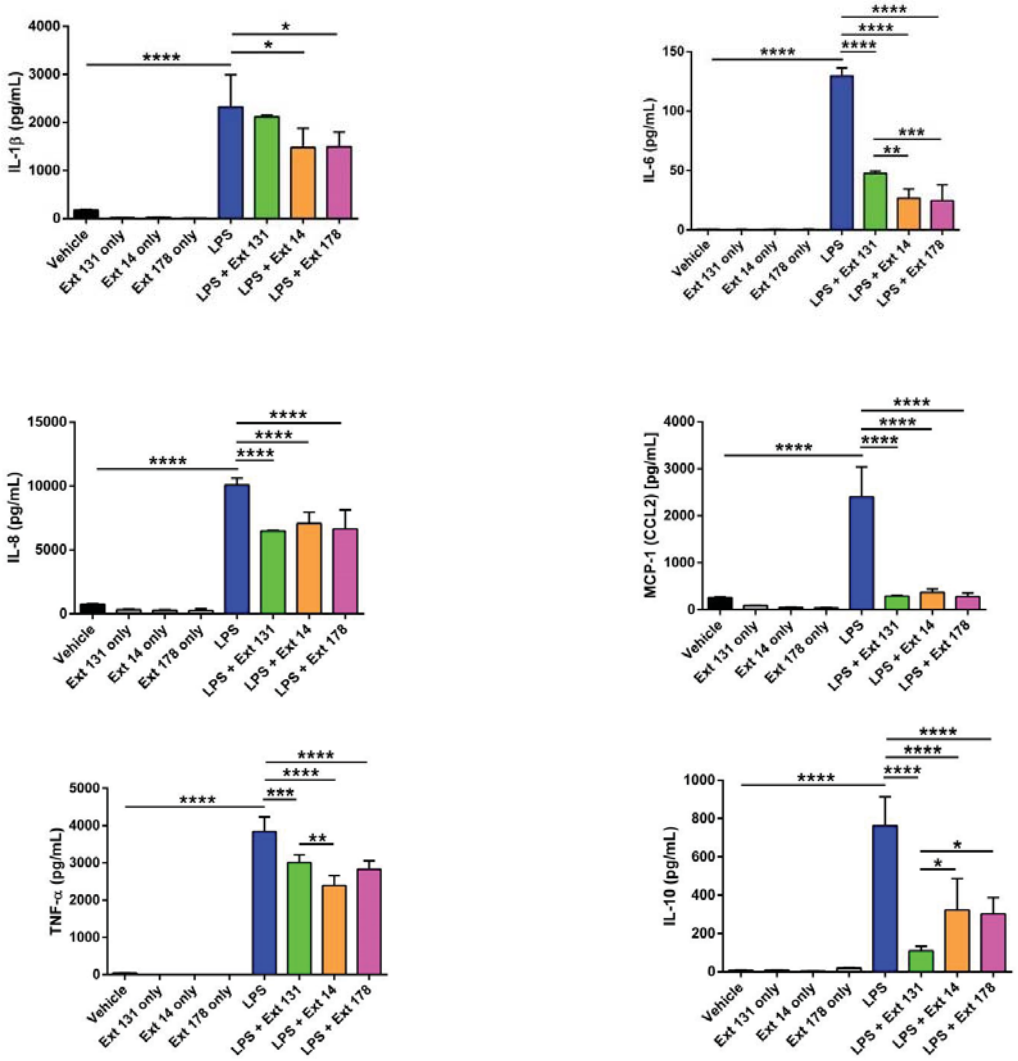


Figure 18. Comparison of studied cytokines between the extracts. Results are presented as mean of calculated concentration [pg/ml] ± SD. Statistical analysis was performed by ANOVA followed by Tukey post-hoc test. A value of $P < 0.05$ was considered to be significant. Significant differences between groups are marked with: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$.

5. DISCUSSION

It is crucial to compare our cell model of inflammation to other models available nowadays. Cell lines, such as U937 [95], and Mono Mac 6 [96], are commonly used in inflammation research. U937 cells are monocytes derived from tissue origin of human histiocytic lymphoma. This type of cell line can also be transformed into macrophage-like phenotype and be stimulated with LPS for inflammation induction [97], [98]. However, U937 cell line in contrast to THP-1 cell line, showed a slight response to LPS by upregulating the expression of only 7 inflammatory genes out of 34 genes by real time polymerase chain reaction data, making this cell line inferior in studying LPS-response *in vitro* [99]. Mono Mac 6 cell line, a human monocytic line, is also a common model in biomedical research, but it was only shown to produce TNF-α, IL-1α/β, and IL-6 upon stimulation with different inflammatory triggers, narrowing its application in the inflammation field in general [100]. Another type of cells, that can be used in inflammation is human peripheral blood mononuclear cell (PBMC) derived macrophages. These primary cells are considered superior to cell lines, as they more resemble *in vivo* settings and are not genetically modified. The main disadvantages of primary cells are their short lifespan and heterogenic response between donors which arises during their differentiation from progenitors [101]. A study, using a real time PCR to compare the response to LPS in THP-1 cells versus PBMC derived macrophages, revealed a close correlation in inflammatory gene expression between these two studied groups, making a THP-1 cell line a good model for studying LPS-induced changes [99]. Another possibility to study the inflammatory response *in vitro,* is by using primary macrophages from mouse bone marrow, because they represent a homogeneous and non-transformed population of macrophages [102]. The only disadvantage is that those macrophages are not from human origin, making it difficult for translating their results into clinical trials [103].

The immunomodulatory effects of cannabis are well documented [104], [105], [106] . Nowadays, there are many well-known cannabis cultivars, and each one has a unique composition of different compounds [107], [108]. Many studies have demonstrated the effects of single cannabinoids, such as THC and CBD, on inflammation [109]. Other components of the plant (such as minor cannabinoids, terpenes, terpenoids, flavonoids, and others) may act synergistically with cannabinoids and can be useful from a therapeutic point of view [110]. The modulating effect of these compounds is known as "an entourage effect"; such modulation is typically positive which means that the medicinal effect of the whole plant extract is more significant than the effect of isolated compounds [111], [19]. Like with any other drug, the effects significantly depend on the concentration. This study showed that *Cannabis sativa* extracts are very potent anti-inflammatory compounds by reducing the expression of pro-inflammatory cytokines (IL-1β, TNF-α, MCP-1, IL-8, IL-6) in LPS-stimulated THP-1 macrophages. The production of pro-inflammatory cytokines by macrophages that have been exposed to LPS is well established, and the concentration of LPS used also corresponds to other similar studies [112], [113], [114]. IL-1β is the most prominent pro-inflammatory cytokine which plays a crucial role in inflammasome signaling [115]. IL-6, a pro-inflammatory cytokine, also plays an important role in the inflammatory response of many chronic inflammatory conditions [116]. TNF-α cytokine stimulates inflammation and is one of the most studied and most important pro-inflammatory cytokines [117]. IL-8 and MCP-1 induce chemotaxis of granulocytes to the site of infection and stimulate phagocytosis [7], [118]. Our cannabis extracts significantly downregulated the levels of all these cytokines. The results of this study were similar to other studies, where it was shown that cannabinoids and cannabis extracts efficiently inhibit inflammation by suppressing the levels of pro-inflammatory cytokines. For example, cannabis users that suffered from multiple sclerosis had significantly lower levels of many pro-inflammatory cytokines, like TNF-α, IL-1, IL-6, IL-

12, IFN-γ, while higher levels of the anti-inflammatory cytokine, IL-10, in comparison to non-cannabis users [119]. Another study reported that cannabis extract high in CBD was superior to pure CBD in reducing the IL-6 and IL-8 levels effectively in an alveolar epithelial cell line, A549, while cannabis extract high in THC on the same cell line showed only minor anti-inflammatory activity and was more cytotoxic. In this study, the concentration of extracts was 5 μg/mL, while in our study, it was 7 μg/mL [120]. In a 3D EpiDermFT tissue inflammation model, it was shown that the extracts high in CBD and THC were effective in reducing the expression of IL-6, IL-1β, MCP-1, and TNF-α [121]. Another study demonstrated that cannabis extract high in CBD was more effective than pure CBD in zymosan-induced inflammation mice model. The extract effectively reduced pain and paw swelling, prevented TNF-α production, and overcame the bell-shaped dose response of CBD [122]. High CBD and high THC extracts were shown to be superior over the pure CBD and THC in reducing inflammation in graft versus host disease in allogeneic bone marrow transplantation model in view of reducing the severity of disease and improving survival rate [123].

The cannabis extracts and cannabinoids in this study were given 30 min prior to LPS stimulation, which corresponds to other studies performed in a similar field of research [124], [114]. The reason for this is that cannabinoid receptor activation includes three waves, with the first two being receptor binding and receptor internalization, and they take approximately 20 min which is sufficient to trigger all the molecular responses [125]. The trypan blue data confirmed that the canabis extracts used in our study were not cytotoxic and did not change cells' morphology.

The data we obtained on IL-1β levels was quite interesting. This was the only cytokine that decreased in response to CBD more efficiently than in response to the high CBD extract or

THC/high THC extracts. It remains to be shown whether this is a specific reaction to our extracts or the nature of the reaction of this cytokine to cannabinoids.

Another interesting result was the effect of cannabinoids on IL-10 levels. Many *in vivo* and *in vitro* studies demonstrated that cannabinoids and cannabis extracts enhance the levels of anti-inflammatory cytokines. For example, on murine model of bone marrow transplantation, it was demonstrated that pure THC and CBD, as well as cannabis extracts high in CBD and THC, reduced inflammation, by reducing IL-17 secretion and enhancing IL-10 secretion [123]. Another study performed on mouse primary bone marrow-derived macrophages stimulated by LPS showed that flavonoids, luteolin and quercetin, increased the levels of IL-10 secretion [102]. Next, it was also shown that THC reduced inflammation on endotoxemic mice model by significantly upregulating the plasma level of anti-inflammatory cytokine, IL-10, while suppressing the pro-inflammatory cytokine, MCP-1 [126]. Along this line, it was reported that CBD and THC suppressed the secretion of IL-17 but elevated the secretion of IL-10 by mouse-derived encephalitogenic T cell line [127]. CBD was reported to significantly reduce the plasma levels of pro-inflammatory cytokines (IFN-γ, TNF-α), along with the levels of anti-inflammatory cytokines (IL-10, IL-4) on diabetic mice model [128]. In our study we found, that LPS stimulation causes significant increase in IL-10 levels, while cannabis extracts, as well as pure cannabidiol given before LPS stimulation reduce its levels. This results correspond to some studies reported in the literature. For instance, it was reported that CBD suppressed levels of IL-10 in LPS-stimulated macrophages,obtained either from naive animals or from treated animals [129]. IL-10 is an anti-inflammatory cytokine, which protects cells against profound inflammation. CBD and THC were shown to inhibit the levels of IL-10 in HUT-78 T cells [130]. Some studies reported the similar effect of LPS on IL-10. LPS was demonstrated to increase

levels of IL-10 on Raw 264.7 murine macrophages *in vitro* [131] and murine bone-marrow-derived macrophages [132].

Since LPS was used to induce the inflammatory response, it was expected that it would also induce anti-inflammatory cytokines to counteract high levles of pro-inflammatory cytokines, which is required for cell survival. For effective clearance of pathogens, the pro-inflammatory response is crucial, while excessive inflammatory response causes tissue damage. Hence, human body always maintains equilibrium by activating counteractive pathways to bring down pro-inflammatory response. Since levels of pro-inflammatory cytokines were elevated by LPS in our study, we observed the corresponding increase in the IL-10 levels in LPS group to counteract the pro-inflammatory cytokine storm. Similarly, our extracts and CBD were able to significantly downregulate the levels of several pro-inflammatory cytokines and hence, they did reduce the levels of IL-10, again, to maintain the equilibrium. On the other hand, THC was not able to reduce the levels of pro-inflammatory cytokines and hence, did not change the high levels of IL-10 after LPS stimulation. It is also possible that cannabis extracts inhibit all cytokines, regardless of their nature.

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6. Limitations and future studies

We focused on the anti-inflammatory effects of cannabis extracts using the THP-1 macrophages model. Specifically, we analyzed the effects of the extracts of selected novel cannabis cultivars on the secretion of cytokines in LPS-induced macrophages. We found that studied cannabis extracts significantly reduced the levels of pro-inflammatory cytokines and were not cytotoxic to macrophages. While these studies will lay a foundation for the future analysis of anti-inflammatory potential of cannabis extracts, they are not without limitations.

The biggest limitation of this study is that it was not performed *in vivo*, since the effects in *in vivo*, where all cells and tissues communicate with each other and contribute to inflammation, are more clinically relevant. The other potential concern is the very type of cells used in this study. The THP-1 cells are not primary cells and hence may behave differently than freshly isolated primary monocytes/macrophages. The primary human monocytes isolated from bone marrow of healthy donors or primary macrophages from mouse bone marrow are likely much more suitable alternatives to such inflammation studies. However, these *ex vivo* cells are much more difficult to work with and need highly specialized media to culture. In the future, the validation of our results should be performed on some of above-mentioned freshly isolated monocytic cells.

The next limiting aspect of this study is the use of PMA, which may be considered as not physiological. The better option to be tried in the future is interferon-gamma with LPS stimulation to achieve M1 polarization. Stimulation with PMA and LPS is very stressful to the cells, and allows for much stronger induction of inflammation, thus allowing to observe a more prominent effect of extracts. It remains to be shown whether milder induction with LPS (lower

concentrations) or with other compounds would also to see the effect of extracts. Milder stimulation with LPS can be considered for future studies to try to simulate more physiological response *in vitro*. When we use very strong stimulation, we could miss many subtle things, like some extracts might be more immunomodulatory rather than extremely immunosuppressive.

The other limitation of this study is that we have not analyzed the CB1/CB2 receptor expression. Low expression of CB1/CB2 oin this model may explain why CBD and THC alone were not very efficient in decreasing the expression of cytokines. It would also be interesting to study the CB1/CB2 expression profile in response to LPS.

Also, in our work, we have pretreated cells with extracts before treating with LPS. This approach mimics the situation where cannabis users have lower levels of cytokines. It would be more natural to treat cells with extracts after the induction of inflammation, since, in general, there is a need to treat inflammation rather than to prevent it. We may also want to compare the effect of extracts to well-known anti-inflammatory agents such as dexamethasone.

Another limiting aspect is that so far, we performed only one technical replicate, but we are currently working on performing one more technical replicate for the publication purpose.

For better understanding of our results, it would be very beneficial to look at key transcription factors and target genes by performing RNA sequencing, transcriptome profiling, followed by the analyses of relevant protein expression. The most important consideration in future is to perform this study *in vivo* and to confirm the immunomodulatory activity of our extracts. In conclusion, our data show strong potential for tested cannabis extracts as anti-inflammatory agents for various chronic inflammatory conditions.

In the future, with more research being done, we might gain more insight into the potential immunomodulatory or even immunostimulatory effects of individual cannabinoids or cannabis extracts. This knowledge can help medical professionals to integrate cannabis extracts into different medical fields, potentially as adjunct therapy.

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