

Fighting the storm: novel anti- TNF α and anti-IL-6 *C. sativa* lines to tame cytokine storm in COVID-19

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Abstract

The main aspects of severe COVID-19 disease pathogenesis include the increasing hyper-induction of proinflammatory cytokines, also known as 'cytokine storm', that precedes acute respiratory distress syndrome (ARDS) and often leads to death. COVID-19 patients often suffer from lung fibrosis, a serious and untreatable condition. There remains no effective treatment for these complications. Out of the cytokines, TNF α and IL-6 play crucial roles in cytokine storm pathogenesis and are likely responsible for the escalation in disease severity. These cytokines also partake in the molecular pathogenesis of fibrosis. Therefore, new approaches are urgently needed that can efficiently and swiftly block TNF α , IL-6, and the inflammatory cytokine cascade in order to curb inflammation and prevent fibrosis, and lead to disease remission.

Cannabis sativa has been proposed to modulate gene expression and inflammation and is under investigation for several potential therapeutic applications against autoinflammatory diseases and cancer. Here, we hypothesized that the extracts of our novel *C. sativa* lines may be used to modulate the expression of pro-inflammatory cytokines and pathways involved in inflammation and fibrosis.

To analyze the anti-inflammatory effects of novel *C. sativa* lines, we used a well-established full thickness human 3D skin artificial EpiDermFT™ tissue model, whereby tissues were exposed to UV to induce inflammation and then treated with extracts of seven new cannabis lines.

We noted that out of seven studied extracts of novel *C. sativa* lines, three (#4, #8 and #14) were the most effective, causing profound and concerted down-regulation of TNF α , IL-6, CCL2, and other cytokines and pathways related to inflammation and fibrosis. Most importantly, one of the tested extracts had no effects at all, and one exerted effects that may be deleterious, signifying that cannabis is not generic and cultivar selection must be based on thorough pre-clinical studies.

The observed pronounced inhibition of TNF α and IL-6 is the most important finding, as these molecules are currently considered to be the main actionable targets in COVID-19 cytokine storm and ARDS pathogenesis.

Many currently trialed agents, such as anti-TNF α and anti-IL-6 biologics are expensive and cause an arrays of side effects. On the other hand, anti-TNF α and anti-IL-6 cannabis extracts that are generally regarded as safe (GRAS) modalities can be a useful addition to the current anti-inflammatory regimens to treat COVID-19, as well as various rheumatological diseases and conditions, and 'inflammaging' - the inflammatory underpinning of aging and frailty.

Introduction

To date, raging pandemic of COVID-19 disease caused by the SARS-CoV2 virus has affected over 4.7 million people and claimed over 310,000 lives worldwide. SARS-CoV2 has human-human transmission and spreads easily via airborne and contact routes; its R_0 is currently estimated to be 2-2.5¹. COVID-19 has a rather broad spectrum of clinical manifestations, ranging from asymptomatic, to mild flu-like disease, to pneumonia, that in some cases can further progress to acute respiratory distress syndrome (ARDS), major organ failure and death. Approximately 20% of COVID-19 cases are serious or severe, and death rate is currently estimated to be around 10%. While elderly and individuals with pre-existing conditions are among the most affected, it has recently become apparent that COVID-19 affects all age groups.

The key aspects of the severe COVID-19 disease pathogenesis include increasing hyper-induction of proinflammatory cytokines, which is also known as 'cytokine storm' that precedes acute respiratory distress syndrome (ARDS)^{2,3}. Overall, various plasma cytokines and chemokines were reported to be deregulated in COVID-19 patients; these include TNF- α , interleukins (IL-1, IL-2, IL-4, IL-7, IL-10, IL-12, IL-13, IL-17), macrophage colony-stimulating factor (M-CSF), IP-10, MCP-1 (C-C motif chemokine 2, CCL2), MIP-1 α , hepatocyte growth factor (HGF), IFN- γ , CCL3, CCL5 and many others⁴. Cytokine levels correlate with disease severity⁵. Patients with moderate COVID-19 disease had elevated levels of TNF α and IL-6, and in severe COVID-19 cases the production of IL-6 and TNF- α and other cytokines was profoundly increased⁵. Moreover, patients requiring ICU admission had higher levels of IL-6, IL-2, IL-7, IL-10, G-CSF, IP10, CCL2, MIP1A, and TNF α than did those not requiring ICU admission, suggesting that the cytokine storm was important in COVID-19 pathogenesis^{6,7}.

Of the cytokine milieu, TNF α and IL-6 play key roles in cytokine storm and are likely to be responsible for the escalation in disease severity⁸⁻¹⁰. TNF alpha is an inflammatory cytokine that stimulates and maintains cellular activation and migration of leukocytes to inflammatory sites. TNF acts through binding to its receptors (TNFR) that are located throughout the body. Interaction of TNF with receptors causes increased expression of other cytokines (IL-1 and IL-6) and chemokines, which, in turn, activate leukocytes, suppresses

regulatory T cells, causes production of MMP proteins which degrade tissues and induces apoptosis¹¹. IL-6 is another important player in the acute host response to infection whereby it promotes inflammation, immune reactions, and hematopoiesis. Long-term elevation of IL-6 levels maintains chronic inflammation and autoimmunity, making IL-6 one of the main druggable targets in autoinflammatory and autoimmune disorders¹².

Even though TNF α and IL-6-mediated cytokine storm and ARDS has been previously well-documented in SARS, MERS, as well as in severe cases of influenza^{3,13}, there still is no effective treatment for this grievous complication. Therefore, new approaches are urgently needed that can efficiently and swiftly block TNF α , IL-6 and inflammatory cytokine cascades and thus curb inflammation and lead to disease remission.

Furthermore, COVID-19 convalescents face a long recovery and may be at risk of developing pulmonary fibrosis (PF), a debilitating complication that is very hard to treat¹⁴. Mechanisms of PF are not fully understood, albeit it has been established that inflammatory cytokines and chemokines, such as IL-1, IL-6, TNF α , C-C motif chemokines are important in its etiology^{15,16}. New therapies are much needed to prevent and mitigate pulmonary fibrosis complications in COVID-19 patients. Since COVID-19, and especially ARDS patients are extremely weak and vulnerable, it would be crucial that novel anti-cytokine storm and anti-fibrosis therapies have minimal side effects.

Cannabis sativa has been proposed to modulate gene expression and inflammation and is under investigation for several potential therapeutic applications against autoinflammatory diseases and cancer. Here, we hypothesized that extracts of novel *C. sativa* lines may be used to modulate expression of pro-inflammatory cytokines and pathways involved in inflammation and fibrosis.

Results And Discussion

To analyze the anti-inflammatory effects of novel *C. sativa* lines, we used a well-established full thickness human 3D skin artificial EpiDermFT™ tissue model, whereby tissues were exposed to UV to induce inflammation and then treated with extracts of seven new cannabis lines.

Global gene expression profiling revealed that several new extracts strongly down-regulated expression of interleukins, pro-inflammatory cytokines, C-C motif chemokines and C-X-C subfamily cytokines involved in ARDS and other autoinflammatory conditions ($p_{adj} < 0.05$) (Fig. 1 and Table 1).

Upon original screening of over 200 extracts, seven - extracts #4, #6, #8, #12, #13, #14, #15, were identified for further analysis. Application of the extracts # 4, #8, #6 and #14 down-regulated both TNF α and IL-6. Extract #13 downregulated TNF α but not IL-6. Interestingly, extract #12 upregulated the expression of IL-6 and IL-23A, pro-inflammatory chemokines, and down-regulated the expression of anti-inflammatory IL-37. Application of extract #15 did not result in any statistically significant gene expression changes (Fig. 1, Table 1).

We further explored the effects of *C. sativa* extracts on the levels of IL-6 protein using western immunoblotting, and found that all extracts, except #15, downregulated IL-6 (Fig. 2). Interestingly, application of extract #12 down-regulated IL-6 on the protein level, but not on the level of the transcript. This is an interesting finding that may suggest the presence of post-transcriptional regulation of IL-6 expression via small interfering RNAs and the potential effects of cannabis extracts on these processes. All extracts down-regulated an important inflammation marker, COX-2 (Fig. 2).

Along with two key regulators of cytokine storm – TNF α and IL-6, *C. sativa* extracts also affected the levels of other key pro-inflammatory interleukins – IL-1, IL-17, IL-23 (Fig. 1, Table 1). IL-1 family of interleukins is important in innate inflammation and autoimmunity¹⁷. IL-1 α was shown to be constitutively present in numerous epithelial and mesenchymal cell types of healthy individuals, whereas IL-1 β is mainly induced under disease conditions¹⁷. Both pro-inflammatory interleukins are upregulated in numerous inflammatory and autoinflammatory diseases and are important druggable targets. Recent studies show that levels of IL-1 were strongly elevated in individuals with COVID-19, and IL-1 levels correlated with disease severity¹⁸. Here, we noted that extracts #4 and #8 down-regulated both IL-1 α and IL-1 β (Fig. 1, Table 1).

Extracts #4, #6, #8, #13 and #14 down-regulated, while extract #12 upregulated IL-23A, a member of the IL-12 family of cytokines with pro-inflammatory properties¹⁹. Extracts #8 and #13 downregulated IL-17C, a pro-inflammatory cytokine and a member of IL-17 family, that, together with IL-23 mediates inflammation in psoriasis, psoriatic arthritis, and ankylosing spondylitis²⁰. Increased expression of IL-23/IL-17 pathway was previously correlated with pulmonary inflammation in polymicrobial sepsis²¹. While on the one hand, the IL-17 family confers protection from a variety of extracellular pathogens and was shown to drive leukocyte infiltration to facilitate clearance of infectious pathogens, aberrant IL-17 signaling can lead to excess inflammation and tissue damage and fibrosis²², and has been implicated in ARDS, cystic fibrosis, and pulmonary fibrosis and other pathological conditions (reviewed in ²²).

Three extracts, #4, #8 and #14 downregulated the levels of Toll-like receptor 2 (TLR2). Toll-like receptor 2 (TLR2) signaling has been implicated in numerous inflammatory diseases²³, including pulmonary diseases and ARDS²⁴.

In addition, extracts #4, #8, #13 and #14 significantly down-regulated the expression of NFKB2 gene. NF- κ B pathway has been often referred to as a prototypical proinflammatory signaling pathway. NF- κ B is usually upregulated by IL-1 and TNF α , and play important roles in the expression of other proinflammatory genes²⁵.

Further analysis revealed that extracts #4, #8, #13 and #14 down-regulated CCL2, also known as MCP-1 (Fig. 1 and Table 1). Along with inflammation and ARDS, CCL2 expression is an important hallmark of fibrosis, and CCL2 has been explored as a potential druggable anti-fibrotic target²⁶. In previous studies, CCL2 was shown to promote fibroblast differentiation and facilitate their recruitment to the alveolar space, thus leading to excessive collagen deposition²⁷. Besides, CCL2 promoted fibroblast survival and stimulated IL-6 production²⁸.

Importantly, along with CCL2, IL-1, IL-6 and TNF α also regulate fibrosis²⁶, and their down-regulation may be viewed as a potential anti-fibrotic effect. Together with IL-1, IL-6 and TNF α genes, novel cannabis extracts regulated the expression of various other genes involved in fibrosis, including pulmonary fibrosis (PF) (Table 1). Among those were metalloproteinases (MMPs), key proteases involved in ECM remodelling²⁶. MMP1, MMP2, MMP7, and MMP9 were previously reported to be upregulated in PF. In our study, several extracts down-regulated MMPs (Table1).

Extracts #4, #6, #8, #14 and #13 also down-regulated WNT2 and WNT5a. WNT signaling alterations have been linked to pathogenesis of a variety of diseases and conditions, including pulmonary fibrosis^{29,30}. Previous studies have shown that *in vivo* inhibition of WNT-5A attenuated tissue destruction, improved lung function and restoration of alveolar epithelial cell markers expression in two animal models of COPD^{29,31}. Furthermore, extracts also affected the levels of iCAM1 and iCAM5 genes. Levels of iCAM1 were shown to be elevated in sera of PF patients³², and recent studies showed that iCAM-1 inhibition reduced exacerbations of lung inflammation³³.

One more important pro-fibrotic protein is CXCL12, and its down-regulation was shown to dampen fibrocyte recruitment and collagen deposition³⁴. In our study, extracts #6 and #13, along with down-regulation of numerous pro-inflammatory cytokines, upregulated CXCL12. The role of CXCL12 upregulation in PF still needs to be fully established, but, based on the current knowledge, CXCL12 upregulation can be viewed as a potential PF contributor, and thus its upregulation may negate the potential benefits of cytokine down-regulation by extracts #6 and #13.

Having seen cannabis extract-induced changes in pro-inflammatory and pro-fibrotic genes, we further conducted an in-depth analysis of the effects of the extracts on global signalome using Pathview Bioconductor platform. We found that extracts # 4, #8, #14 significantly down-regulated cytokine-cytokine receptor interaction pathway, rheumatoid arthritis pathway, chemokine signalling, Toll-like receptor signalling, JAK-STAT signalling and other pathways involved in inflammation, immunity and autoimmunity, as well as tissue remodeling and fibrosis. Contrarily, extract #12 upregulated these pathways (Table 2, Fig. 3).

Overall, our study revealed that cannabis extracts exerted different effects on the 3D tissue inflammation model - some profoundly down-regulated pro-inflammatory cytokines and pro-fibrotic molecules, some affected only several key cytokines, some did not cause any significant changes at all (extract #15), while extract #12 promoted expression of pro-inflammatory genes. This is a very important finding that shows that cannabis is non-generic, and each *C. sativa* line has to be thoroughly evaluated for its medicinal properties.

Taken together, our results suggest that out of 7 studied extracts of novel *C. sativa* lines three were most effective down-regulating pro-inflammatory pathways and key cytokines implicated in the cytokine storm and ARDS in COVID-19. Extracts #4, #8 and #14 were the most effective, causing profound and concerted down-regulation of TNF α , IL-6 and CCL2. Pronounced inhibition of TNF α and IL-6 is

the most important finding, as these molecules are currently considered to be the key actionable targets in COVID-19 cytokine storm and ARDS. Anti-cytokine therapies are thought to be important for prevention of COVID-19 pneumonia³⁵, as currently there is a race to develop novel anti-cytokine storm regimens. To that effect several anti-cytokine therapies have been proposed and are now in clinical trials. These include anti-IL-6 receptor antibody tocilizumab^{9,10,36}, colchicine, an agent that can potentially influence levels of IL-6 and other cytokines³⁷, chloroquine¹³, metronidazole³⁸, and statins³⁹, as well as melatonin as an anti-inflammatory adjuvant therapy⁴. Chloroquine has some immunomodulatory effects, potentially suppressing the production and release of TNF- α and IL-6¹³. Colchicine has been shown to effectively suppress interleukin IL-1b, IL-18 and IL-6 in patients with acute coronary syndrome^{40,41} and is now being trialed in COVID-19 ARDS, albeit it also has very significant side effects³⁷.

Numerous rheumatological drugs are now being evaluated for therapeutic potential to tame COVID-19 pneumonia, ARDS, and prevent further complications such as PF¹⁸. Suppression of pro-inflammatory IL-1 family members and IL-6 have been shown to have a therapeutic effect in many inflammatory diseases, including viral infections, and has been explored as a potential therapeutic avenue in COVID-19⁴². Tocilizumab, a humanized monoclonal antibody against the interleukin-6 receptor, is showing some promise, albeit it carries a hefty price tag and a lot of side effects^{10,43}.

TNF α not only is the main cytokine storm driver, it also was shown to mediate the transition from pulmonary inflammation to fibrosis⁴⁴. Surprisingly, up to now, no TNF α inhibitors have been trialed for COVID-19. The recent expert commentary in Lancet stated that "*trials of anti-tumour necrosis factor therapy for COVID-19 are urgently needed*"⁴⁵. While potentially effective, anti-TNF α and anti-IL-6 biologics are very expensive and cause an array of side effects, including malignancies.

On the other hand, anti-TNF α and anti-IL-6 cannabis extracts that are generally regarded as safe (GRAS) modalities can be a useful addition to the current anti-inflammatory regimens to treat COVID-19, as well as various rheumatological diseases and conditions such as rheumatoid arthritis (RA), psoriasis and psoriatic arthritis, osteoarthritis, fibromyalgia and others. Indeed, lines targeting TNF α , IL-6, IL-1 β and causing concerted and significant down-regulation of the rheumatoid arthritis pathway, pending thorough verification and clinical validation, may present a novel and promising natural resource for RA treatments and management of other TNF α , IL-6, IL-1 β -mediated diseases.

Conclusions

Overall, we are the first to show that application of *C. sativa* extracts profoundly decreases the level of pro-inflammatory cytokines in human 3D tissues. Still, our study has several pitfalls. Here, we used human 3D full-thickness skin model to analyze the effects of cannabis extracts on inflammation and fibrosis. While it would be important to replicate the data in an airway epithelial and alveolar tissue models, our data can be used as a roadmap for the future analysis. Moreover, key fundamental mechanisms of inflammation and fibrosis are similar in various tissues, and key roles of TNF α , IL-6 and other interleukins, chemokines, and MMPs have been well-established in an array of fibroproliferative diseases¹⁵. Pending further validation in lung tissue models, our novel extracts need to be studied in a clinical trial aimed to prevent or mitigate COVID-19 pneumonia and ARDS. To do so, the extracts have to be administered early upon positive diagnosis has been made to allow sufficient time for modulation of cytokine levels.

Most importantly, out of 7 selected extracts, only 3 performed best, one had no effects at all, and one exerted effects that may in turn be deleterious, signifying that cannabis is not generic and cultivar selection must be based on thorough pre-clinical studies. Furthermore, the current study was developed to analyze the effects of medical cannabis applications rather than smoking.

In the future, anti-TNF α and anti-IL-6 extracts need to be analyzed for their potential to mitigate inflammation in rheumatoid arthritis, ankylosing spondylitis, and other rheumatologic conditions, especially given the fact that extracts profoundly downregulate the RA pathway and target TNF α and IL-6. Also, the effects of novel extracts also need to be analyzed for their potential to combat 'inflammaging' - the inflammatory underpinning of aging and frailty⁴⁶.

Materials And Methods

Plant growth, extract preparation: All cannabis plants were grown in the licensed facility at the University of Lethbridge (license number LIC-62AHHG0R77-2019). *C. sativa* lines #4, #6, #8, #12, #13, #14, #15 were used for the experiments. Four plants per line were grown at 22°C 18 h light 6 h dark for 4 weeks and then transferred to the chambers with 12 h light/ 12 h dark regime to promote flowering. Plants were grown to maturity and flowers were harvested and dried. Flower samples from four plants per variety were combined and used for

extraction. Three grams of the powdered plant tissue per each line were used for extraction. Plant material was placed inside a 250 mL Erlenmeyer flask, 100 mL of Ethyl Acetate was poured into each flask. The flasks were covered with tin foil and incubated overnight in the dark at 21°C with continuous shaking at 120 rpm. Extracts were filtered, concentrated using a rotary vacuum evaporator and transferred to a tared 3-dram vial. The leftover solvent was evaporated to dryness in an oven overnight at 50°C to eliminate the solvent completely. Levels of cannabinoids was analysed using Agilent Technologies 1200 Series HPLC system. The extract stocks were prepared from the crude extracts whereby 3-6 mg of crude extract were dissolved in DMSO (Dimethyl sulfoxide anhydrous, Life Technologies) to reach 60 mg/mL final concentration and stored at -20°C. Appropriate cell culture media (RPMI + 10% FBS or EMEM + 10% FBS) were used to dilute the 60 mg/mL stock to make working medium containing 0.01 mg/ml. Extracts were sterilized using 0.22 µm filter.

Tissue models and treatments

Tissue models: EpiDermFT™ tissues were purchased from Mattek Life Sciences (Ashland, MA), equilibrated and cultured according to manufacturer's instructions. Three tissues were used per extract.

EpiDermFT recreates normal skin tissue structure with differentiated dermis and epidermis. It consists of human-derived epidermal keratinocytes and dermal fibroblasts that are mitotically and metabolically active. The tissues were cultured according to the manufacturer's protocol, using an air-liquid interface tissue culture technique. To induce inflammation, tissues were exposed to UV. The extracts or vehicle (DMSO) were dissolved in media and applied to the media surrounding the tissues (n=3 for each condition). Tissues were incubated with extracts for 24 hours and flash frozen for RNA and protein analysis.

Gene expression analysis

RNA extraction: Three tissues per group were used for the analysis of gene expression profiles. RNA was extracted from tissues using TRIzol® Reagent (Invitrogen, Carlsbad, CA), further purified using an RNAeasy kit (Qiagen), and quantified using Nanodrop2000c (ThermoScientific). Afterwards, RNA integrity and concentration were established using 2100 BioAnalyzer (Agilent).

Library construction and sequencing: In all cases, the sequencing libraries were prepared using NEBNext Ultra II mRNA library preparation kit for Illumina (NEB) following the manufacturer's instructions. The samples were processed by the same technician at the same time to avoid the introduction of technical batch effects. The cDNA fragment libraries were sequenced using NextSeq500 sequencing analyzer (Illumina). The samples were balanced evenly across the lanes of the sequencing flowcell.

Bioinformatics analysis: Base-calling and demultiplexing were done with Illumina CASAVA v.1.9 bioinformatics pipeline. The base qualities were examined using FastQC v.0.11.8. The adapters and low-quality bases were trimmed using Trim Galore! v.0.6.4 https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/. Trimmed reads were mapped to the human genome version GRCh37 using HISAT2 version 2.0.5⁴⁷. Counts of reads mapping to the gene as a meta-feature were obtained using featureCounts v.1.6.1⁴⁸ taking to account the directionality of the sequencing libraries. Counts of reads mapping to features were loaded into R v.3.6.1 and normalized using DESeq2 v.1.24.0 Bioconductor package as described in the manual⁴⁹. The differences between all experimental groups were examined using the likelihood ratio test (LRT) test implemented in DESeq2. The reduced model included the intercept and the full model was the experimental group (Cannabis extracts and controls). Multiple comparisons adjustment of p-values was done using Benjamini-Hochberg procedure⁵⁰. Specific comparisons between groups were extracted using *results()* function with *contrast* argument specified. Genes with adjusted p-values below 0.05 were considered significant.

Western blot analysis

After treatment with cannabis extracts for the indicated time, whole cellular lysates of 3D tissues were prepared in radioimmunoprecipitation assay buffer using 2.0 mm ZR BashingBead beads (Zymo Research). Proteins (30-100 µg per sample) were electrophoresed in 10% sodium dodecyl sulfate polyacrylamide gel and electrophoretically transferred to polyvinylidene difluoride membranes (Amersham Hybond™-P, GE Healthcare) at 4°C for 1.5 h. The blots were incubated for 1 h with 5% nonfat dry milk to block nonspecific binding sites and subsequently incubated at 4°C overnight with 1:1000 dilution of polyclonal antibody against IL-6 and COX-2 (Abcam). Immunoreactivity was detected using a peroxidase-conjugated antibody and visualized with the ECL Plus Western Blotting Detection System (GE Healthcare). The blots were stripped before reprobing with antibody against actin (Santa Cruz Biotechnology). Quantification of Western blot bands was performed using ImageJ in duplicate.

Declarations

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Competing Interests: PathwayRX is a startup company engaged in medical cannabis and disease research.

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Tables

Table 1. Effects of novel *C. sativa* extracts on the expression of inflammation and fibrosis-related genes. Data are shown as log2 fold changes as compared to induced tissues. All changes shown here are statistically significant, p adj <0.05, ANOVA-like analysis and pair-wise comparison.

LINE	#4	#8	#14	#13	#6	#12	#15
Inflammation- and fibrosis-related genes							
TNF	-3.4	-3.6	-4.4	-2.9	-3.9		
IL1A	-1.6	-1.4	-1.8	-1.7	-1.6		
IL1B	-4.0	-3.6			-2.4		
IL6	-2.0	-1.8	-2.8		-2.4	1.3	
IL17C		-3.5		-7.4			
IL20		-4.8		-4.4	-3.2		
IL23A	-5.6	-5.2	-6.7	-6.0	-6.3	1.3	
IL24	-1.2	-1.1					
IL32	-2.0	-1.5	-2.7		3.2		
IL33		-1.0		-1.0			
IL37						-8.4	
CCL2	-2.5	-1.6	-3.1	-2.7			
CCL20	-4.2	-3.3	-5.7	-4.2			
CXCL1	-2.1	-1.4					
CXCL2	-2.9	-2.0	-3.5	-2.8			
CXCL3	-3.8	-3.3					
CXCL5	-3.9	-3.0		-4.0			
CXCL6	-1.8	-1.2		-1.3			
CXCL8	-4.1	-3.5					
CXCL10						2.3	
CXCL12				3.8	3.2		
CXCL14				5.0			
NFKB2	-1.0	-1.1	-1.5	-1.1			
TLR2	-1.8	-1.2	-2.0				
Fibrosis-related genes							
MMP1	-2.7	-1.8					
MMP3		-1.8					
MMP7		2.7		3.6	2.8		
MMP8		-1.5		-2.0			
MMP10	-1.7	-1.7	-1.5				
MMP11				3.2	2.9		
MMP19		-1.0		-1.1			
WNT2	-2.2	-1.5	-2.1	-1.5	-2.2		
WNT5A	-1.5	-1.2	-1.5	-1.4	-1.3		
FZD4	-1.2						
ICAM1	-1.5	-1.4	-2.2		-1.8		
ICAM5	-1.6	-2.0					

Table 2. Analysis of pathways affected by new *C. sativa* extracts. Downregulated pathways - green; upregulated pathways - red.

GID	Pvalue	Term	Genes
E			
UNREGULATED PATHWAYS			
323	1.79E-11	Rheumatoid arthritis	6374;1437;6364;51561;3553;2919;6347;4312;6372;3569;3576;2321;3383;7097;3552;3689;7124
60	4.22E-11	Cytokine-cytokine receptor interaction	6374;1437;6364;51561;2921;3553;2919;3976;57007;2920;6347;6372;3569;3575;7850;3576;2321;3552;7133;3082;7124;84957;1440;11009;23529
514	2.51E-06	Cell adhesion molecules (CAMs)	25945;4897;80380;3383;3696;214;3689;1364;257194;1366;29126;23562;3134
62	3.82E-05	Chemokine signaling pathway	6374;6364;2921;2919;2920;6347;6372;3055;2791;3576;4792;5908;57580;5604;114
630	0.000634346	Jak-STAT signaling pathway	3598;1437;51561;3976;3569;3575;6775;81848;1440;11009;23529
210	0.000993993	Apoptosis	3553;330;3656;3552;4792;11213;5533;637;7124
620	0.008055248	Toll-like receptor signaling pathway	3553;3569;3576;7097;4792;5604;7124
660	0.019535451	T cell receptor signaling pathway	1437;4792;5533;4773;5604;7124;4794
E			
UNREGULATED PATHWAYS			
621	5.24E-06	NOD-like receptor signaling pathway	6347;2920;3569;7128;330;8767;7124;4792
60	9.06E-06	Cytokine-cytokine receptor interaction	51561;6347;3976;2920;57007;3569;6364;7124;7133;23529;7850;84957;3552
323	4.01E-05	Rheumatoid arthritis	51561;6347;3383;3569;6364;7124;3552;7097
514	0.000993116	Cell adhesion molecules (CAMs)	3383;80380;25945;214;4897;1364;257194
620	0.010832243	Toll-like receptor signaling pathway	3569;7124;4792;5606;7097
62	0.04389252	Chemokine signaling pathway	6347;2920;3055;2791;6364;4792
210	0.003683526	Apoptosis	330;7124;4792;3656;5533;3552
E #8			
UNREGULATED PATHWAYS			
60	1.45E-13	Cytokine-cytokine receptor interaction	1440;6364;2921;3976;3553;51561;6374;2920;3589;57007;650;2919;6347;1437;3624;3576;3569;50604;3552;6372;3082;7124;51330;7133;84957;11009;23529
323	7.81E-12	Rheumatoid arthritis	6364;3553;51561;6374;3589;4312;2919;6347;1437;3576;3569;3552;6372;3383;7097;7124;3689
630	9.93E-05	Jak-STAT signaling pathway	1440;3976;51561;3589;3598;1437;3569;50604;81848;6775;11009;23529
514	0.000889035	Cell adhesion molecules (CAMs)	25945;23562;4897;80380;3383;257194;3696;3689;3134
62	0.000939189	Chemokine signaling pathway	6364;2921;6374;2920;2919;6347;2791;3576;6372;4792;9564;57580
620	0.005637004	Toll-like receptor	3553;3576;3569;4792;7097;148022;7124

		signaling pathway	
l210	0.010118422	Apoptosis	3553;3656;3552;4792;7124;330;5533
E #12			
EGULATED PATHWAYS			
l060	0.000239246	Cytokine-cytokine receptor interaction	3976;6376;6356;650;4982;7852;3627;6363;8995;3569;51561
l010	0.003164277	MAPK signaling pathway	1850;2353;1843;3164;2872;3725;3727;11221;1647;3303;22808
l323	0.008471825	Rheumatoid arthritis	2353;3725;5228;3569;51561
l512	0.045361608	ECM-receptor interaction	22801;1311;1301;1281
l510	0.047132189	Focal adhesion	80310;22801;3725;1311;1301;5228;1281
l620	0.047386067	Toll-like receptor signaling pathway	2353;3725;3627;3569

Figures

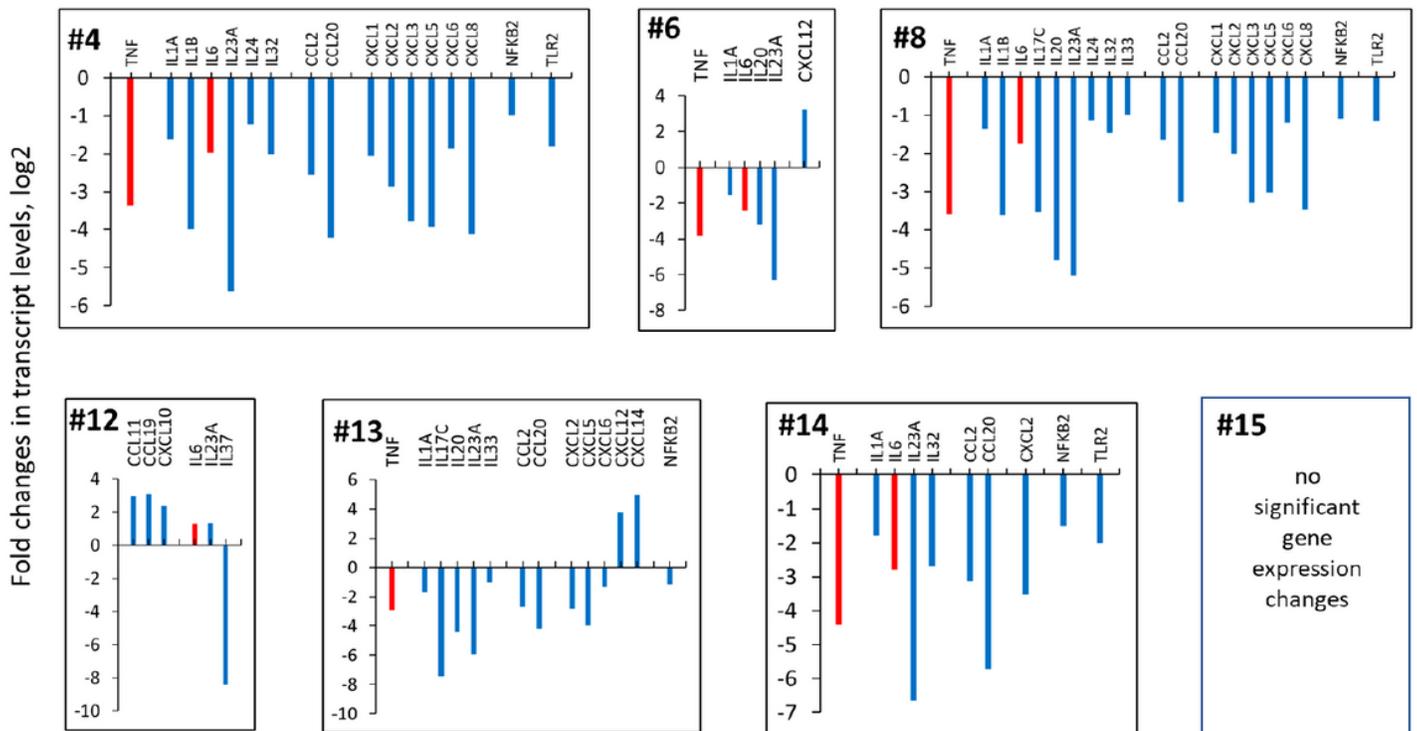


Figure 1

Effects of novel *C. sativa* extracts on the levels of pro-inflammatory cytokines. Extracts of cannabis lines decrease levels of TNF α , IL-6 and other cytokine and chemokine gene expression in human 3D EpiDermFT tissues. Data are shown as log₂ fold changes as compared to induced tissues. All changes shown here are statistically significant, p adj < 0.05, ANOVA-like analysis and pair-wise comparison.

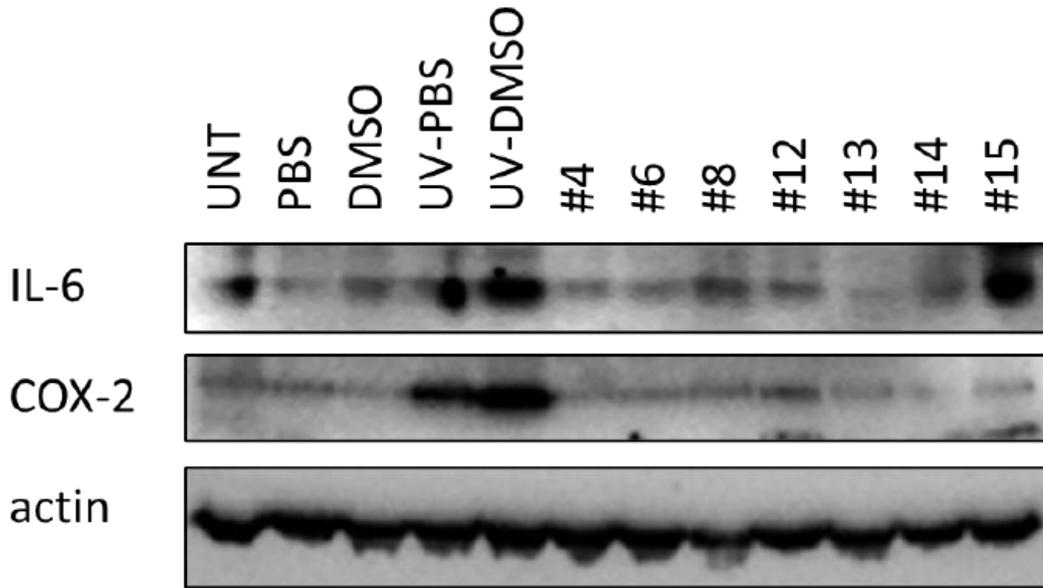


Figure 2

Effects of novel *C. sativa* extracts on the levels of IL-6 and COX2 in human EpiDerm FT tissues.

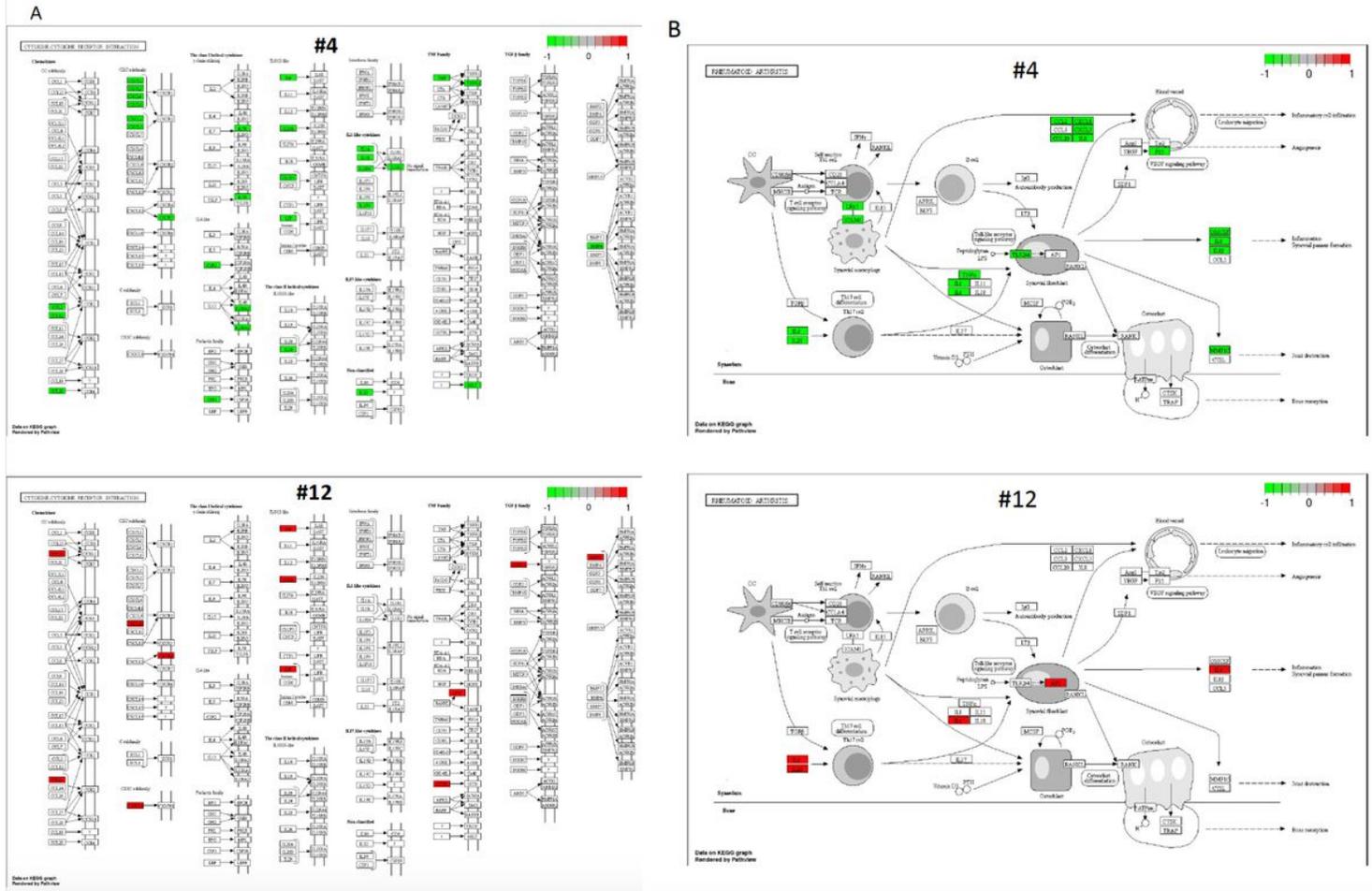


Figure 3

Effects of selected extracts on 3D tissues on cytokine-cytokine receptor interactions and rheumatoid arthritis pathways. (A) Changes in the cytokine-cytokine receptor interactions pathway caused by extracts #4 and #12. (B) Changes in the rheumatoid arthritis pathway caused by extracts #4 and #12.