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Article

In Search of Preventative Strategies: Novel Anti-Inflammatory High-CBD *Cannabis Sativa* Extracts Modulate ACE2 Expression in COVID-19 Gateway Tissues

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Abstract: With the rapidly growing pandemic of COVID-19 caused by the new and challenging to treat zoonotic SARS-CoV2 coronavirus, there is an urgent need for new therapies and prevention strategies that can help curtail disease spread and reduce mortality. Inhibition of viral entry and thereby spread constitute plausible therapeutic avenues. Similar to other respiratory pathogens, SARS-CoV2 is transmitted through respiratory droplets, with potential for aerosol and contact spread. It uses receptor-mediated entry into the human host via angiotensin-converting enzyme II (ACE2) that is expressed in lung tissue, as well as oral and nasal mucosa, kidney, testes, and the gastrointestinal tract. Modulation of ACE2 levels in these gateway tissues may prove a plausible strategy for decreasing disease susceptibility. *Cannabis sativa*, especially one high in the anti-inflammatory cannabinoid cannabidiol (CBD), has been proposed to modulate gene expression and inflammation and harbour anti-cancer and anti-inflammatory properties. Working under the Health Canada research license, we have developed over 800 new *Cannabis sativa* lines and extracts and hypothesized that high-CBD *C. sativa* extracts may be used to modulate ACE2 expression in COVID-19 target tissues. Screening *C. sativa* extracts using artificial human 3D models of oral, airway, and intestinal tissues, we identified 13 high CBD *C. sativa* extracts that modulate ACE2 gene expression and ACE2 protein levels. Our initial data suggest that some *C. sativa* extract down-regulate serine protease TMPRSS2, another critical protein required for SARS-CoV2 entry into host cells. While our most effective extracts require further large-scale validation, our study is crucial for the future analysis of the effects of medical cannabis on COVID-19. The extracts of our most successful and novel high CBD *C. sativa* lines, pending further investigation, may become a useful and safe addition to the treatment of COVID-19 as an adjunct therapy. They can be used to develop easy-to-use preventative treatments in the form of mouthwash and throat gargle products for both clinical and at-home use. Such products ought to be tested for their potential to decrease viral entry via the oral mucosa. Given the current dire and rapidly evolving epidemiological situation, every possible therapeutic opportunity and avenue must be considered.

1. Introduction

Currently, there is a global pandemic of COVID-19 disease caused by the SARS-CoV2 zoonotic coronavirus. The disease started with a fast-growing outbreak in Wuhan, China, in December 2019,

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and by April of 2020 spread throughout the entire world, affecting over 1.42 million people and killing over 82,000. Worldwide, death rates vary between 2 and 10 percent.

SARS-CoV2 possesses fast human-human transmission, with a doubling time of around 6-7 days and a R_o of around 2.2 [1,2]. Similar to other respiratory pathogens, SARS-CoV2 is transmitted through respiratory droplets from coughing and sneezing. However, aerosol transmission and close contact transmission cannot be ruled out as a means of disease spread [2].

While COVID-19 symptoms are many, they can be broken up into several categories: typical influenza-like symptoms, such as fever, fatigue, myalgia, and headache; respiratory symptoms, such as dry cough and dyspnea; and gastrointestinal symptoms, such as diarrhea and nausea [3]. Anosmia and ageusia, the loss of the senses of smell and taste, respectively, are also common. Overall, COVID-19 has a broad clinical spectrum, ranging from asymptomatic and mild disease to pneumonia that often progresses to respiratory failure, major organ failure, and death [2,4]. Up to 20% of cases are severe and require hospital-admission, and, currently, there exists no vaccine for this virus, nor any known approved drug therapy.

SARS-CoV2 was first isolated from human airway epithelial cells [5] and found to be similar to the severe acute respiratory syndrome coronavirus (SARS-CoV) [6], and hence was named SARS-CoV2. It is now well accepted that angiotensin-converting enzyme II (ACE2) is the cell receptor of SARS-CoV2 and the main route for receptor-mediated entry of the virus into the human host [7]. Since ACE2 plays a pivotal role in cellular entry, ACE2 expressing cells serve as critical viral gateways [8].

To date, ACE2 expression was found in lung tissue, nasal mucosa, kidney, testes, and the gastrointestinal tract. High levels of ACE2 were seen in lung and intestinal epithelia [9]. An in-depth analysis of The Cancer Genome Atlas (TCGA) and Functional Annotation of The Mammalian Genome Cap Analysis of Gene Expression (FANTOM5 CAGE) datasets revealed that ACE2 is expressed in oral mucosa and is enriched in the epithelial cells of the tongue. ACE2 expression in oral, lung, and intestinal epithelia may thus constitute important routes of SARS-CoV2 entry into hosts [10].

A recent study by Xu and colleagues reported high levels of ACE2 expression in oral epithelial tissues and suggested that the oral cavity could be regarded as a high-risk target for SARS-CoV2 infectious susceptibility, and thereby an important target for prevention strategies [10]. Similarly, numerous studies have reported high levels of ACE2 in the lower respiratory tract, and higher levels of ACE2 expression, such as those seen in smokers and patients with chronic obstructive pulmonary disease (COPD), were associated with higher COVID-19 predisposition and more severe disease [11].

Modulation of ACE2 levels in gateway tissues may thus prove a plausible strategy to decrease disease susceptibility. These strategies ought to be accessible, easy to use, and, ideally, should fall into the generally regarded as safe (GRAS) category.

Cannabis sativa, especially one high in the anti-inflammatory cannabinoid cannabidiol (CBD), has been proposed to modulate gene expression and inflammation and is under investigation for several potential therapeutic applications against cancer and various inflammatory diseases [12,13].

Working under the Health Canada research license, we have developed over 800 new *Cannabis sativa* lines and extracts, as well as a method of using them as a means to regulate gene expression and molecular cascades that drive inflammation and other vital cellular processes (PCT/IL2019/051340; US16/711,647; PCT/IL2019/051342; US16/713,029; PCT/IL2019/051341; US16/711,655; PCT/IL2019/051343; US16/713,030). Serendipitously, we noted that cannabis may also affect ACE2. Here, we hypothesized that high-CBD *C. sativa* extracts may be used to modulate ACE2 expression in COVID-19 target tissues.

2. Materials and methods

2.1. 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 #1, #5, #7, #9, #10, #31, #45, #49, #81, #90, #114, #115, #129, #130, #131, #155, #157, #166, #167, #169, #207, #274 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

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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 21C 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.

2.2. Tissue models and treatments

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

EpiAirway Tissues(AIR-100): Mattek's EpiAirway tissue model is a human 3D mucociliary tissue model that consists of normal, human-derived tracheal/bronchial epithelial cells, is cultured at the air-liquid interface and fully recapitulates the barrier, mucociliary responses, infection, toxicity responses of human airway tissues *in vivo* (Mattek Life Sciences, MA).

EpiOral Tissues (ORL-200): MatTek's EpiOral tissues consist of normal, human-derived oral epithelial cells. The cells have been cultured to form multilayered, highly differentiated models of the human buccal (EpiOral) phenotypes. The tissues are cultured on specially prepared cell culture inserts using serum free medium and attain levels of differentiation on the cutting edge of in vitro cell culture technology. The EpiOral tissue models exhibit in vivo-like morphological and growth characteristics which are uniform and highly reproducible (Mattek Life Sciences, MA).

EpiIntestinal Tissues (SMI-100): EpiIntestinal tissues are 3D highly differentiated tissue models produced from normal, human cell-derived small intestine epithelial and endothelial cells and fibroblasts. Grown at the air-liquid interface, EpiIntestinal tissue models are similar to in vivo human epithelial tissues and exhibit columnar shaped basal cells and Kerckring folds, as well as brush borders, functional tight junctions and mucous secreting granules (Mattek Life Sciences, MA).

EpiAirway tissue treatment: The extracts or vehicle (DMSO) were dissolved in media and applied to the media surrounding the tissues. Tissues were incubated with extracts for 24 hours and flash frozen for RNA and protein analysis.

EpiIntestinal and EpiOral tissue treatments: Inflammation was induced by treatment with proinflammatory cytokines (TNF-α and IFN-γ), as recommended by the manufacturer, then the extracts or vehicle (DMSO) were dissolved in media and applied to the media surrounding the tissues. Tissues were incubated with extracts for 24 hours and flash frozen for RNA and protein analysis.

2.3. Gene expression analysis

RNA extraction: Two 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 RNAesy 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

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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 2.0.5 [14]. Counts of reads mapping to the gene as a meta-feature were obtained using featureCounts v.1.6.1 [15] 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 manual [16]. 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 procedure [17]. Specific comparisons between groups were extracted using *results()* function with *contrast* argument specified. Genes with adjusted p-values below 0.05 were considered significant. The results of statistical tests for the ACE2 receptor (Ensembl gene identifier: ENSG00000130234) were selected from the list of significant genes.

2.4. 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 ACE2 (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 GAPDH (Santa Cruz Biotechnology). Quantification of Western blot bands was performed using ImageJ in duplicate. Statistics: The student's t test was used to determine the statistical significance of differences between groups in ACE2 expression. A value of $p < 0.05$ was considered statistically significant.

3. Results

To test our hypothesis, we used artificial human 3D tissue models of oral, airway, and intestinal tissues. EpiOral tissues consist of healthy, human-derived oral epithelial cells that have been cultured to form multilayered, highly differentiated models of human buccal phenotypes. EpiAirway tissue models are human 3D mucociliary tissues that consist of healthy, human-derived tracheal/bronchial epithelial cells. EpiAirway tissues exhibit the barrier, mucociliary, infection, and toxicity responses of human airway tissues in vivo. EpiIntestinal 3D tissues are highly differentiated tissue models produced from healthy, human cell-derived small intestine epithelial and endothelial cells and fibroblasts. They are similar to in vivo human epithelial tissues, possessing columnar shaped basal cells and Kerckring folds, as well as brush borders, functional tight junctions, and mucous secreting granules. These models are well-established and well-accepted for pathophysiology, toxicology, inflammation, virus infection, and drug development studies.

To analyze the effects of *C. sativa* on ACE2 expression, the extracts of twenty two novel *C. sativa* lines (#1, #5, #7, #9, #10, #31, #45, #49, #81, #90, #114, #115, #129, #130, #131, #155, #157, #166, #167, #169, #207, #274) (Table 1) were used to treat 3D EpiOral, EpiIntestinal, and EpiAirway tissues that were previously treated with the proinflammatory cytokines TNF-α and IFN-γ (TNF-IFN) to induce inflammation [18] (Fig. 1).

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Table 1. Content of major cannabinoids – THC and CBD, and THC: CBD ratios in tested C. sativa lines.

Extract	THC	CBD	THC:CBD ratio
#1	0.25%	6.79%	1:27
#5	0.25%	8.5%	1:35
#7	0.21%	7.2%	1:34
#9	0.22%	6.91%	1:31
#10	0.45%	9.5%	1:21
#31	4.5%	6.7%	1:1.5
#45	0.03%	1.61%	1:54
#49	0.15%	3.1%	1:9
#81	0.55%	11.5%	1:21
#90	0.99%	4.58%	1:5
#114	0.22%	6.8%	1:31
#115	0.4%	9.54%	1:24
#129	0.34%	6.75%	1:20
#130	0.86%	2.63%	1:3
#131	0.44%	6.1%	1:14
#155	0.22%	4.59%	1:21
#157	0.2%	3.78%	1:19
#166	0.1%	2.5%	1:25
#167	0.08%	2.25%	1:28
#169	0.21%	1.88%	1:9
#207	4.34%	4.68%	1:1
#274	0.44%	9.02%	1:21

Lines indicated in bold have less then 0.3% of total THC and therefore can be classified as CBD Hemp in Canada and USA. Lines that were shown to affect ACE2 expression (Fig. 2-4) are indicated in red.

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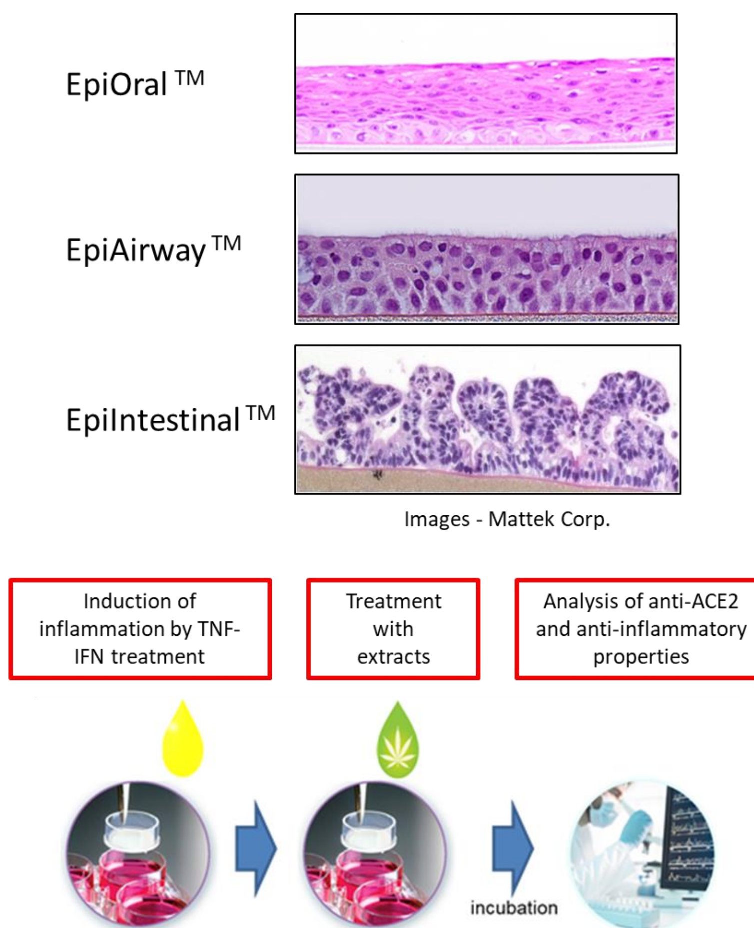

Figure 1. Experimental models and experimental set up.

In an initial experiment, we used RNA-seq to analyze the effects of four (#81, #90, #130, #131) extracts of novel cannabis lines on the levels of ACE2 gene expression in EpiOral tissues after inflammation induction by TNF-IFN. We noted that extracts #81 and #130 significantly down-regulated the levels of ACE2 transcript levels in EpiOral tissues (ANOVA-like analysis, padj=2.14e-06 for both extracts, and pair-wise comparison between DMSO and extract #130 and DMSO and extract #81, padj <0.05) (Fig. 2A).

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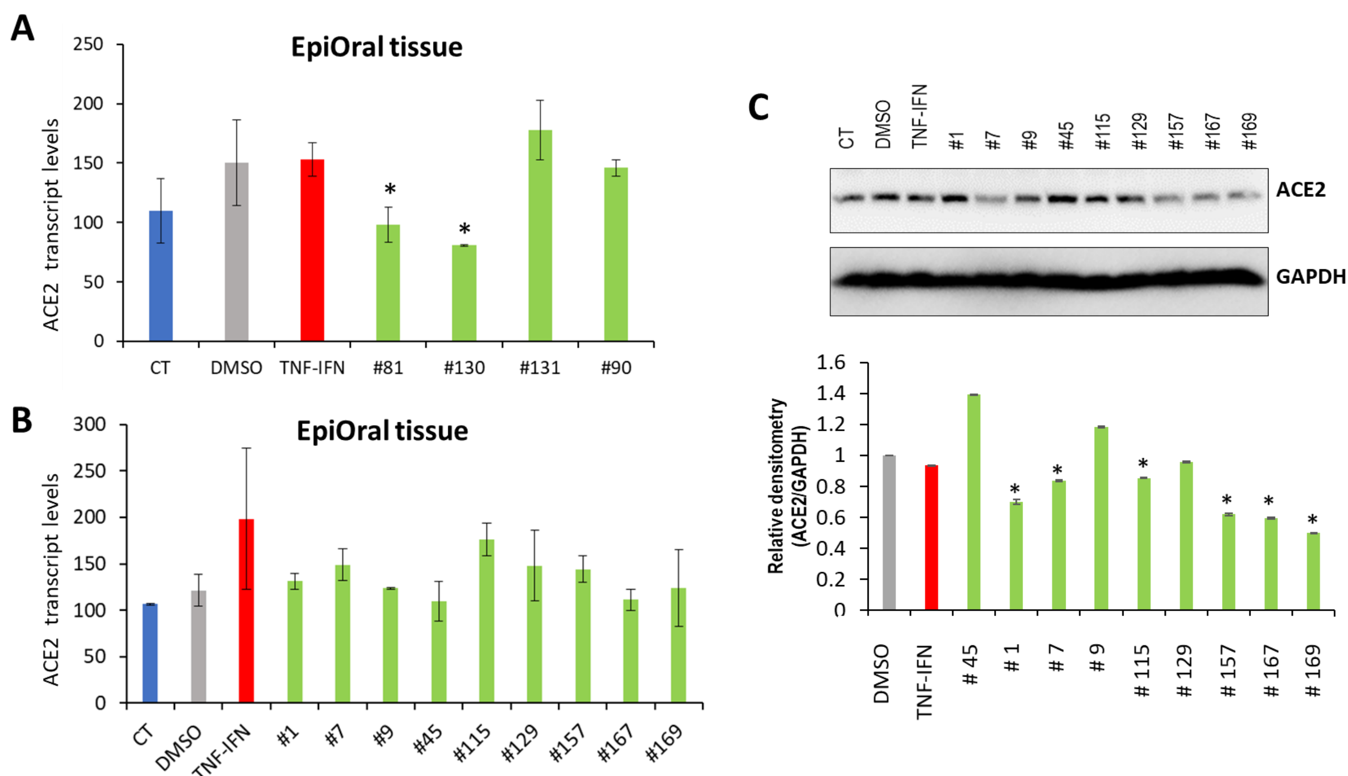

Figure 2. Effects of novel *C. sativa* extracts on the levels of ACE2 in human EpiOral tissues. A Extracts of cannabis lines #81 and #130 decrease levels of ACE2 gene expression in human 3D EpiOral tissues. Data are shown as an average (with SD) from two samples. * - statistically significant, ANOVA-like analysis and pair-wise comparison. B Effects of cannabis extracts #1, #7, #9, #45, #115, #129, #157, #167, #169 on the levels of ACE2 gene expression in human 3D EpiOral tissues. Data are shown as an average (with SD) from two samples. C. Cannabis extracts #1, #7, #115, #157, #167, #169 down-regulate the levels of ACE2 protein in human 3D EpiOral tissues. * - $p<0.05$, student's t-test. Data are shown as an average (with SD) from two samples.

In a larger-scale follow-up experiment, we investigated the effect of nine extracts of novel cannabis lines (#1, #7, #9, #45, #115, #129, #157, #167, #169), and noted that extracts #9, #45, and #167 down-regulated the levels of ACE2 RNA in EpiOral tissues (Fig. 2B). Next, we investigated the levels of ACE2 protein in treated tissues and found that the levels of ACE2 protein in EpiOral tissues were significantly ($p<0.05$) down-regulated by extracts #7, #9, #157, #167 and #169 (Fig. 2C).

We proceeded to analyze the effects of *C. sativa* extracts (#5, #10, #31, #49, #81, #114, #155, #166, #169 and #207) on the levels of ACE2 protein in the EpiAirway tissues. We found that application of extracts # 5, #10, #31, and #114 significantly ($p<0.05$) down-regulated ACE2 levels in EpiAirway tissues (Fig. 3).

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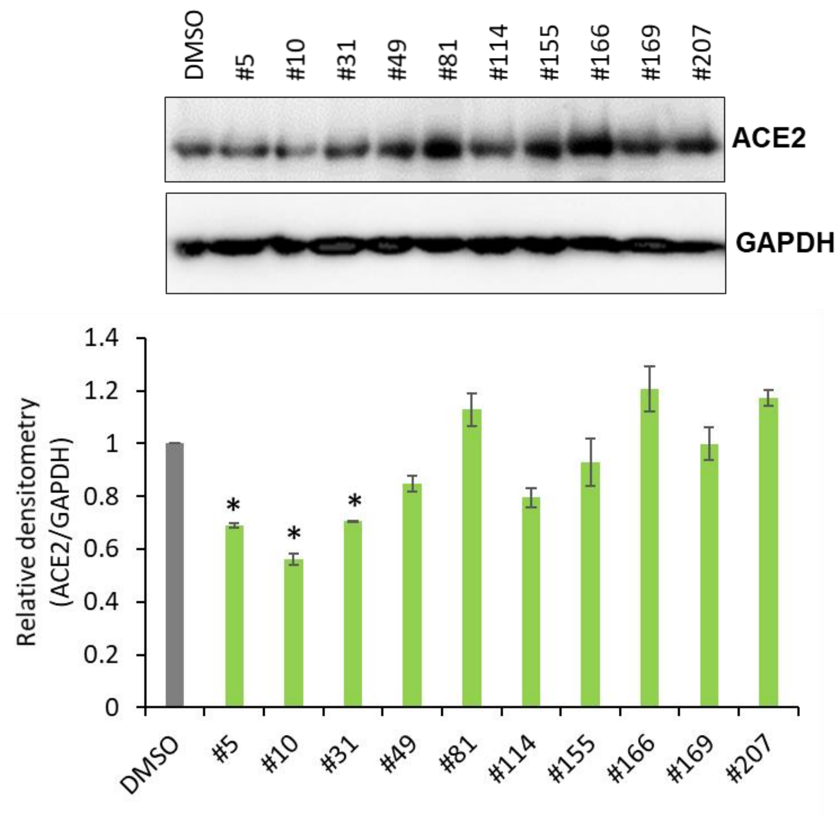

Figure 3. Effects of novel *C. sativa* extracts on the levels of ACE2 in human EpiAirway tissues. Cannabis extracts #5, #10, #31, as well as #114 and #49 down-regulate the levels of ACE2 protein in human 3D EpiAirway tissues. Data are shown as an average (with SD) from two samples. * - $p<0.05$, student's t-test.

An analysis of the effects of *C. sativa* extracts in the EpiIntestinal tissues revealed that the extract of line #45 significantly down-regulated the levels of ACE2 mRNA (ANOVA-like analysis, padj=0.00623, and pair-wise comparison between CT and extract #45, padj=0.000626 (Fig. 4A). This result was further confirmed on the protein level, whereby ACE2 down-regulation was also achieved by extracts #1, #7, #129, and #169 (Fig. 4B). Along with the down-regulation of ACE2, *C. sativa* extracts exerted anti-inflammatory effects on EpiOral, EpiAirway, and EpiIntestinal tissues (data not shown).

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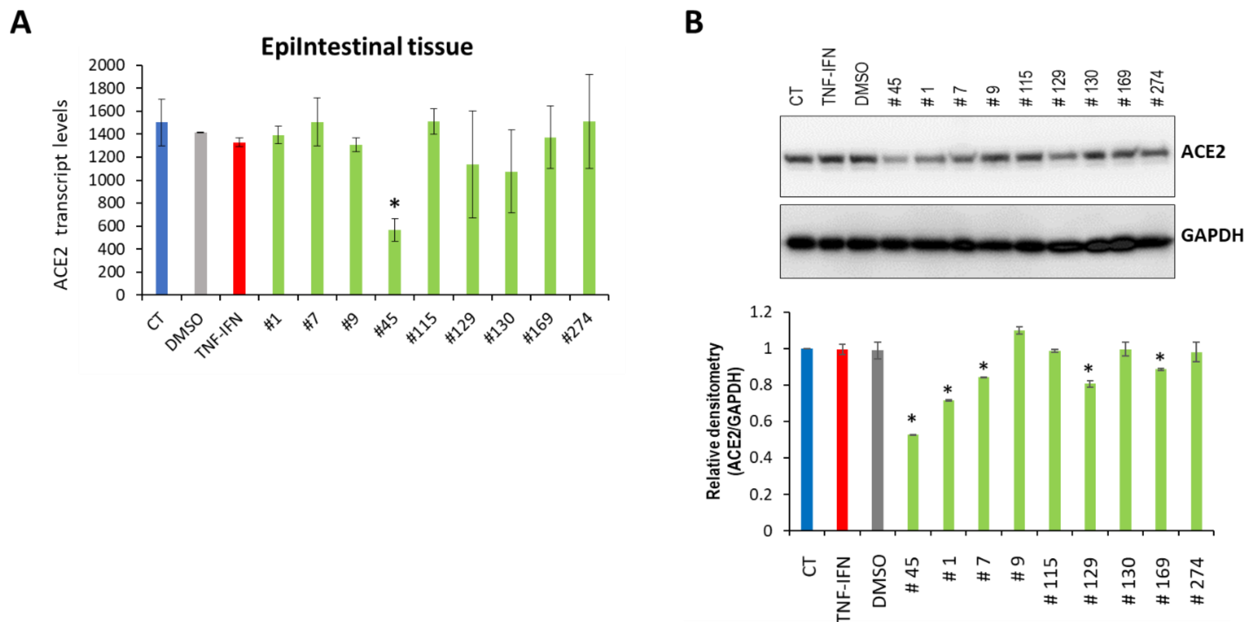

Figure 4. Effects of novel *C. sativa* extracts on the levels of ACE2 in human EpiIntestinal tissues. **A** Extract of cannabis line #45 decreases the levels of ACE2 gene expression in human 3D EpiIntestinal tissues. Data are shown as an average (with SD) from two samples. * - statistically significant, ANOVA-like analysis and pair-wise comparison. B Cannabis extracts #45, #1, #7, #129, #169 down-regulate the levels of ACE2 protein in human 3D EpiIntestinal tissues. Data are shown as an average (with SD) from two samples. * - $p<0.05$, student's t-test.

Along with ACE2, serine protease TMPRSS2 plays an important role in the SARS-CoV2 infection process. While ACE2 is the receptor for viral entry, TMPRSS2 primes viral spike proteins, and is therefore crucial for SARS-CoV2 entry into host cells. Recent studies revealed that TMPRSS2 inhibitors blocked virus entry [19]. We noted that several *C. sativa* extracts down-regulated TMPRSS2 gene expression in EpiOral and EpiIntestinal tissues (Fig.5). In light of this finding, the effects of *C. sativa* extracts on TMPRSS2 protein levels in all tissue models have to be further elucidated.

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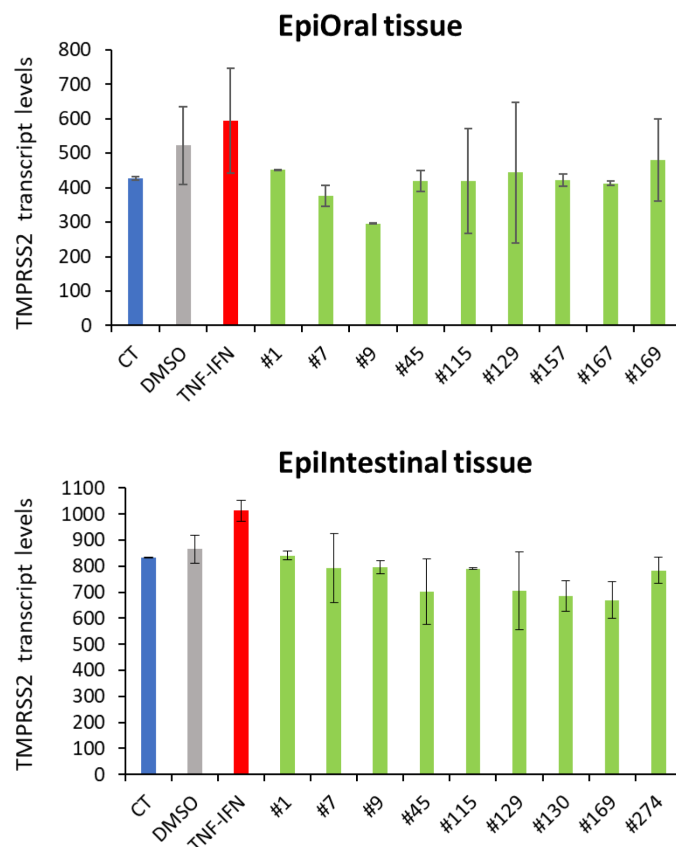

Figure 5. Effects of nova *C. sativa* extracts on the levels of TMPRSS2 gene expression in EpiOral and EpiIntestinal tissues. Data are shown as an average (with SD) of two samples. Y axis shows the arbitrary units of TMPRSS2 gene expression, while X axis show the samples.

4. Discussion

The observed down-regulation of ACE2 gene expression by several tested extracts of *C. sativa* is a novel and crucial finding. Our results lay a foundation for further in-depth analysis of the effects of *C. sativa* on the molecular etiology and pathogenesis of COVID-19, as well as other viral diseases in which viruses use the ACE2 receptor as a molecular gateway. If further confirmed, select high-CBD cannabis extracts can be used to develop prevention strategies directed at lowering or modulating ACE2 levels in high-risk tissues. ACE2 level modulation is of particular importance since it appears to change throughout disease progression, and some studies show that ACE2 is essential for lung function in animal models of SARS[20,21]. It would also be important to test the effects of *C. sativa* lines on other receptors involved in SARS-CoV2 entry, as well as for their anti-inflammatory potential.

Furthermore, cannabis has over 100 phytocannabinoids [22], of which the main ones are delta-9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD) [23]. Beyond these main cannabinoids, cannabis possesses many minor cannabinoids, as well as numerous terpenes. The latter are responsible for variations in scent and may act synergistically with cannabinoids, with the potential to strongly enhance cannabinoid effects. Terpenes and minor cannabinoids are responsible for the 'entourage' effect [23], whereby whole plant extracts have more pronounced biological effects than individual cannabinoids. In the future, it would be important to identify the cannabinoids and terpenes responsible for the observed effects, albeit, based on the entourage effects, one could predict that whole flower extracts may be more potent than single compounds[24,25]. An exciting aspect of our study is that, while the ratios of the major cannabinoids (THC and CBD) are similar between the analyzed lines (Table 1), not all extracts were equally effective, and some produced undesired molecular effects. Such a finding emphasizes that medical cannabis is not generic, and each cannabis line has to be analyzed in detail to establish the ones that

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are the most potent. Most importantly, seven active lines have less than 0.3% of total THC and therefore can be classified as CBD Hemp in Canada and USA, allowing for easier implementation.

Within this study, extracts were applied via media. Application was intended to model medical delivery, such as local mouth wash applications, encapsulated extracts and dosed oils, and inhalers or nebulizers, and therefore these results cannot be extrapolated to the effects of cannabis smoking. Moreover, in light of recent findings that tobacco smoking increases ACE2 levels and exacerbates clinical outcomes of COVID-19[26], the effects of cannabis smoking on the levels of ACE2 expression should be carefully investigated.

However, our study is not without limitations. Our original experiments were designed for screening of the biological activities of novel cannabis extracts in human 3D tissue models. They allowed us to pinpoint the important effects of cannabis on the levels of ACE2 and TMPRSS2 expression, and inflammation markers (data not shown). While our most efficacious extracts require further validation in a large-scale analysis and an animal model, our study is crucial for the future analysis of the effects of medical cannabis on COVID-19. Given the current dire and rapidly developing epidemiological situation, every possible therapeutic opportunity and avenue needs to be considered.

5. Conclusions

Based on our preliminary data, extracts of novel efficacious *C. sativa* lines, pending further investigations, may become a useful addition to the treatment of COVID-19, and an excellent GRAS adjunct therapy. They may also be used to develop additional easy-to-use preventative strategies such as mouth wash and throat gargle products that may be tested for their potential to decrease viral entry via the oral cavity and may be used both in clinical practice and at-home treatment.

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Author Contributions: A.K., O.K. developed the idea; A.K. O.K. I.K, and B.W. planned the experiments; B.W., D.L. conducted experiments; A.K, S.Y., B.W, O.K. conducted initial data analysis; A.K, B.W, D.L., S.Y, I.K and O.K. conducted further data evaluation; A.K. and O.K. drafted the manuscript; all authors contributed to manuscript preparation and revision.

COMPETING INTERESTS: Pathway Research and Swysh are startup companies engaged in medical cannabis and disease research.

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