Cannabis sativa L. extract and cannabidiol differently inhibit in vitro mediators of skin inflammation and wound injury

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Abstract

Skin inflammatory diseases result from complex events which include dysregulation and abnormal expression of inflammatory mediators or their receptors in skin cells. The present study investigates the potential effect of a *Cannabis sativa* L. ethanolic extract standardized in cannabidiol as anti-inflammatory agent in the skin, unraveling the molecular mechanisms in human keratinocytes and fibroblasts. The extract inhibited the

release of mediators of inflammation involved in wound healing and inflammatory processes occurring in the skin. The mode of action involved impairment of the NF-κB pathway since the extract counteracted the TNFα-induced NF-κB driven transcription in both skin cell lines. *Cannabis* extract and cannabidiol differently inhibited the release of IL-8, MMP-9 and VEGF, which are all mediators whose genes are dependent by NF-κB. The effect of cannabidiol on the NF-κB pathway and MMP-9 release paralleled the effect of the extract thus making this cannabinoid the major contributor to the effect observed; however, down-regulation of genes involved in wound healing and skin inflammation was not strictly associated to the presence of cannabidiol, suggesting that other unknown compounds occurring in the extract may exert anti-inflammatory effects. Our findings provide new insights into the potential effect of *Cannabis* extracts against inflammation-based skin diseases.

Introduction

The skin represents a continuously self-renewing organ managing the relationships between the human body and the environment and actively participating in the host defense. The skin responds to a variety of extrinsic and intrinsic physical stimuli that modify the chemical and biological properties of skin through activation of specific pathways. Skin epidermis and dermis show different cellular and extracellular compositions; the epidermis is mainly constituted of keratinocytes which represent the 95% of the epidermal cells. Keratinocytes resist the external insults and damages to the skin, and cooperate to maintain the structural and barrier function of the epidermis; moreover, their role in the initiation and perpetuation of skin inflammatory and

immunological responses is also well recognized (Baroni et al., 2012) (Hanel, Cornelissen, Luscher, & Baron, 2013).

The dermis contains the majority of the extracellular matrix (ECM), blood vessels, and other cells including fibroblasts which strictly interact with keratinocytes and the other skin cells and are deeply involved in wound healing (Pastar et al., 2014). Keratinocytes stimulate fibroblasts to acquire myofibroblast phenotype and to synthesize growth factors, which in turn stimulate keratinocyte proliferation, differentiation, and migration. Keratinocyte function is regulated by a variety of growth factors, cytokines, and chemokines and release several pro-inflammatory mediators including interleukin-1 beta (IL-1 beta), IL-6, IL-8, tumor necrosis factor alpha (TNF alpha), and TGF alpha and beta, as well as vascular endothelial growth factor (VEGF), a potent mitogen for endothelial cells, playing a central role in angiogenesis and psoriasis (Marina, Roman, Constantin, Mihu, & Tataru, 2015) (Micali, Lacarrubba, Musumeci, Massimino, & Nasca, 2010) (Hanel et al., 2013).

Skin inflammatory diseases, such as dermatitis and psoriasis, result from complex events which include dysregulation and abnormal expression of inflammatory mediators or their receptors in keratinocytes (Gjersvik, 2018). IL-8 is involved in neutrophil recruitment and VEGF regulates the angiogenesis process, while MMP9 contributes to the degradation of extracellular matrix. These pro-inflammatory mediators are regulated by different transcription factors, including NF-κB, which plays key role in inflammatory skin diseases including psoriasis (Goldminz, Au, Kim, Gottlieb, & Lizzul, 2013). The downregulation of keratinocytes inflammatory markers and the inhibition of

their interaction with immune cells may be an effective target in the treatment of skin inflammatory diseases.

Cannabis sativa L. (hemp) is an annual herbaceous plant belonging to the Cannabaceae Family. The flowered tops contain the highest concentration of cannabinoids which include delta-9 tetrahydrocannabinol (Δ9-THC) and cannabidiol (CBD), a cannabinoid without psychotropic activity. CBD is the second major cannabinoid occurring in *Cannabis sativa*; chemistry and pharmacology of CBD, as well as the molecular targets, including CB receptors and other components of the endocannabinoid system have extensively been reviewed (Mechoulam & Hanus, 2002) (Mechoulam, Parker, & Gallily, 2002) (Mechoulam, Peters, Murillo-Rodriguez, & Hanus, 2007) (Kogan & Mechoulam, 2007) (Ujvary & Hanus, 2016) (Izzo, Borrelli, Capasso, Di Marzo, & Mechoulam, 2009). Moreover, *Cannabis* extracts and pure compounds show anti-inflammatory effects in a variety of districts (Pagano et al., 2016) (Borrelli et al., 2013) (Izzo et al., 2012) (Burstein, 2015).

Few studies demonstrated the anti-inflammatory activity of CBD in animal models of skin inflammation (Lodzki et al., 2003) (Tubaro et al., 2010). However, the molecular mechanisms underlying the anti-inflammatory effect observed *in vivo*, in addition to the modulation of genes involved in skin inflammatory processes or wound healing have not been reported so far.

The aim of the present study was to investigate the potential effect of a *Cannabis sativa* L. ethanolic extract (CSE) standardized in CBD as anti-inflammatory agent in the skin, unraveling the molecular mechanisms in human keratinocytes and fibroblasts. HaCaT cells have been used as a reliable in vitro model, with respect to normal keratinocytes,

to test the anti-inflammatory activity, according to Colombo I. et al. (Colombo et al., 2017).

Materials and Methods

Plant material and CBD isolation

CSE is a standardized commercial extract prepared from *Cannabis sativa* L. flowers ("flos" or inflorescence) by solvent extraction with ethanol, followed by prolonged decarboxylation to transform cannabinoids acidic form in the neutral form. Then, ethanol is removed and substituted by purified natural oil, such as medium chain triglycerides (MCT).

CBD (99.5% HPLC purity) was isolated and purified by *Cannabis sativa* L. flowers using ethanol; material was subjected to prolonged decarboxylation to allow conversion of the acidic form (CBDA) to CBD. The procedure requires refining steps by liquid/liquid and liquid/solid purification with final crystallization in an aliphatic hydrocarbon.

Cell Culture

HaCaT cells, spontaneously immortalized human keratinocyte line (Boukamp et al., 1988), were kindly provided by Cell Line Service GmbH (Eppelheim, Germany), while normal human dermal fibroblasts (HDF) were provided by ECACC (Porton Down, UK). Cells were grown in DMEM (Gibco, Life Technologies, Monza, Italy) supplemented with 10% heat-inactivated fetal bovine serum (Euroclone S.p.A., Milan, Italy), L-glutamine (2 mM) (Gibco, Life Technologies, Monza, Italy), penicillin (100 U⁄mL) and streptomycin

(100 mg⁄mL) (Gibco, Life Technologies, Monza, Italy), at 37°C in humidified atmosphere containing 5% CO_2.

Every four days, at 80-90% of confluence, cells were detached from the 75 cm^2 flask (Euroclone S.p.A., Milan, Italy) using tripsin-EDTA 0.25% (Gibco, Life Technologies, Monza, Italy), counted and replaced in a new flask, at the density of 1.5 x 10^6 cells/flask, to allow the cell line growth. The remaining cells were seeded in 24-well plates (DB Falcon™) for the biological tests.

Cell treatment

After 72 hours of growth, HaCaT cells (CLS Cell Lines Service, GmbH, Eppelheim, Germany) and Human Dermal Fibroblasts (HDF, ATCC PCS-201-012™) were treated with CSE or CBD and TNFα (10 ng/mL) using DMEM medium (Gibco, Life Technologies, Monza, Italy) supplemented with L-glutamine (2 mM) (Gibco, Life Technologies, Monza, Italy), penicillin (100 U⁄mL) and streptomycin (100 mg⁄mL) (Gibco, Life Technologies, Monza, Italy). On the basis of the parameter evaluated, cells were subjected to treatment with the extract or individual compound and the pro-inflammatory stimulus for 6 (IL-8 release, NF-κB driven transcription and mRNA levels) or 24 hours (VEGF and MMP-9 release). UVB assays were performed following 1-hour treatment with the extract or CBD. At the end of the treatment, medium or cell lysates were collected and stored at -20°C till the biological assay.

Cytotoxicity assay

The integrity of the morphology before and after treatment was assessed by light microscope inspection. The cytotoxicity of CSE and CBD was evaluated, in HaCaT and HDF cells, by the 3,4,5-dimethylthiazol-2-yl-2-5-diphenyltetrazolium bromide (MTT) method (Sigma-Aldrich, Milan, Italy) (Denizot & Lang, 1986). This method evaluates cell viability by measuring the activity of the mitochondrial succinate dehydrogenase.

The influence of CSE and CBD on cell viability was tested after 6 and 24-hours treatment in both cell lines. At the end of the treatment, the culture medium was removed from each well and 200 μL of MTT solution was added for 30-40 minutes till the development of a violet color (formazan). 200 μL of a solution made by isopropanol:DMSO 90:10 was added in each well to extract formazan from the cells. The absorbance was read through spectroscopy at 570 nm (Envision, PerkinElmer, United States).

The extract and CBD did not show cytotoxic effects at the concentrations tested. CSE was assessed in the range 1 – 50 μg/mL in both HaCaT and HDF cells whereas CBD was evaluated in the range 0.05 – 5 μM in HaCaT cells and 0.1 – 2.5 μM in HDF cells.

Measurement of IL-8, VEGF and MMP-9 release

In order to measure IL-8, VEGF and MMP-9 release, HaCaT and HDF cells were seeded in 24-well plates (DB Falcon™) (60000 cells/well) for 72 hours. Then, cells were treated with CSE or CBD and the stimulus TNFα (10 ng/mL) for 6 or 24 hours. At the end of the treatment the culture medium was collected from each well and stored at -20°C till the biological test. IL-8 release was evaluated after 6 hours treatment whereas VEGF and MMP-9 release were analyzed after 24 hours treatment by an ELISA kit.

Human IL-8 and VEGF ELISA kits were provided by PeproTech (London, UK), corning 96-well EIA/RIA plates from Sigma-Aldrich (Milan, Italy) were coated with the corresponding antibody contained in the kit, overnight at room temperature; while Human MMP-9 ELISA kit, containing a pre-coated 96-well plate, was provided by RayBiotech (Norcross, United States).

The amount of IL-8, VEGF and MMP-9 in the samples was detected by measuring the absorbance produced by the colorimetric reaction between horseradish peroxidase enzyme and 3,3',5,5'-tetramethylbenzidine substrate (Sigma-Aldrich, St. Louis, MO, USA). Signal was read using a spectrophotometer (Victor X3, PerkinElmer, United States) at 450 nm 0.1 s. IL-8, VEGF and MMP-9 were quantified through standard curve supplied in the ELISA kit (8 – 1000 pg/mL for IL-8, 16 – 1000 pg/mL for VEGF, 10 – 6000 pg/mL for MMP-9). The results are expressed as mean ± s.d. of at least three experiments. EGCG (20 μM), Resveratrol (50 μM) and quercetin ($10\ \mu M$) were used as reference inhibitors of VEGF, MMP-9 and IL-8 release, respectively.

NF-κB driven transcription

HaCaT and HDF cells were seeded in 24-well plates (DB Falcon™) at the density of 60000 cells/well for 72 hours. Following, the cells were transiently transfected, by lipofectamine method, with a reporter plasmid containing luciferase gene under control of a promoter characterized by the presence of three responsive elements κB (NF-κB-LUC 250 ng/well). The plasmid NF-κB-LUC was a gift of Dr. N. Marx (Department of Internal Medicine-Cardiology, University of Ulm, Ulm, Germany). After overnight incubation, the cells were treated with CSE or CBD and TNFα 10 ng/mL for 6 hours.

Luciferase produced into the cells was assessed using Britelite™ Plus reagent (PerkinElmer, Walthman, MA, USA) according to the manufacturer's instructions. The luminescence derived from the reaction between luciferase and luciferin was read through a spectrophotometer (Victor X3, PerkinElmer, United States). The results are expressed as mean ± s.d. of at least three experiments. EGCG (20 μM) was used as reference compound.

UVB irradiation system

UVB-induced NF-κB nuclear translocation was measured following exposure of HaCaT cells to UVB (40 mJ/cm^2) light source (Triwood 31/36, W36, V230, Helios Italquartz, Milano, Italy) on ice and treated for 1 hour with increasing concentrations of CSE (1-25 μg/ml) or CBD (0.1-2.5 μM). Radiation time (about 50 seconds) was adjusted for each experimental day, measuring energy emission by LP 471 UVB probe (Delta OHM, Padova, Italy). After irradiation, fresh serum-free medium was immediately added. Nrf-2 nuclear translocation was measured following cell pre-treatment with CSE (25 μg/ml) or CBD (4 μM) for 1 hour; then cells were irradiated as above described and maintained in cell free-medium for three hours before assay.

Nrf-2 and NF-κB nuclear translocation assays

To assess the effect of the extract and individual compounds on the Nrf-2 nuclear translocation, HaCaT cells were plated at the density of 1.5 x 10^6 cells/mL in 100 mm Petri dishes. After 48 h, cells were treated for 1 hour with extract/compound under study, then exposed to UVB irradiation (40 mJ/cm^2). After 3 hours, nuclear extracts were

obtained and stored at −80 °C until assayed. The same amount of total nuclear proteins (80 μg/well), measured by the method of Bradford (Bio-Rad), was used to assess the nuclear translocation using the Nrf-2 transcription factor assay kit (cod. 600590, Cayman) followed by spectroscopy at 450 nm, 0.1 s (VictorX3, Perkin Elmer, Walthman MA, USA).

To assess the effect of the CSE and CBD on the NF-κB (p65) nuclear translocation, cells were plated in 24-well plates at the density of 60000 cells/well for 72 hours; then, cells were exposed to UVB irradiation (40 mJ/cm^2) and treated for 1 h with the extract or the pure compound. The same amount of total nuclear proteins (10 μg/well), measured by the method of Bradford (Bio-Rad), was used to assess the NF-κB nuclear translocation using the NF-κB (p65) transcription factor assay kit (Cayman) followed by spectroscopy at 450 nm, 0.1 s (VictorX3, Perkin Elmer, Walthman MA, USA) as previously described (Fumagalli et al., 2016). EGCG (20 μM) was used as reference inhibitor of the NF-κB nuclear translocation.

Gene expression

RNA extraction

HaCaT and HDF cells were grown in 24-well plates (DB Falcon™) (60000 cells/well) for 72 hours; then, the cells were treated with the pro-inflammatory stimulus (TNFα 10 ng/ml) and CSE (25 μg/mL) or CBD (4 μM). After 6 hours treatment, the medium was removed. The cells were lysed through the addition of the Qiazol lysis buffer (QIAGEN GmbH, Germany) according to the indications provided by the manufacturer, in order

to obtain cell lysis and inactivation of the endogenous RNases. The lysates were frozen at -80°C until the following RNA purification steps.

Total RNA was isolated from the cell lysates using the miRNeasy Mini Kit (QIAGEN GmbH, Germany), according to the manufacturer's protocol. A set of RNase-free DNase (QIAGEN GmbH, Germany) was used to ensure the complete elimination of genomic DNA. Total RNA was eluted in 35 µL of nuclease-free water and stored at -80°C.

The concentration of the isolated RNA was evaluated by spectrophotometry (NanoDrop ND- 1000, ThermoFisher Scientific). The purity of the samples was estimated by measuring the ratio between the optical density of the samples at 260 nm and 280 or 230.

cDNA synthesis

cDNA was synthetized, after elimination of any residual genomic DNA, using the RT^2 First Strand kit (QIAGEN, GmbH, Germany), according to the manufacturer's indications. 400 ng of total RNA, of each sample, were used to produce cDNA.

qPCR

The analysis of gene expression was performed using two 384-well PCR array, related to human genes involved in the inflammatory process and wound healing (RT^2 Profilet^{TM} PCR array: PAHS-011ZE Human Inflammatory Cytokines and Receptors, PAHS-121Z Human Wound Healing; QIAGEN Sciences, USA). In this array, each well contained the primers for a specific target gene (in total 84 different target genes), or housekeeping gene for data normalization (5 different housekeeping genes). Moreover, the array included some controls: one control for genomic DNA contamination, three controls for

the repeatability of the reverse transcription reaction and three controls for the repeatability of the PCR reaction.

A diluted aliquot of cDNA, equivalent to 400 ng total RNA, was mixed with the SYBR Green Master Mix RT^2 reagent (QIAGEN Sciences, USA) according to the manufacturer's instructions and loaded into the 384-well array. The real-time PCR was performed using the CFX384^{TM} Real-Time PCR Detection System (coupled to C1000TM Thermal Cycler) (Bio-Rad Laboratories Srl, Segrate, Italy). The threshold cycle value for each gene (Ct) was automatically provided by the management software CFX Manager^{TM} (Bio-Rad), depending on the amplification curves. The baseline and the threshold values were set manually as recommended by the PCR array manual. The analysis of the data was performed using the web portal SABiosciences company (QIAGEN Sciences, USA). The C_t cut-off was set to 35. Data were normalized on the basis of housekeeping genes: hypoxanthine phosphoribosyltransferase 1 (HPRT1), beta-2-microglobulin (B2M), ribosomal protein P0 (RPLP0), beta-actin (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In each experiment, the housekeeping genes with a variability higher than ±1 threshold cycle among the different experimental conditions, were excluded to the analysis.

Statistical analysis

All data are expressed as mean ± s.d. of at least four experiments. Data were analyzed by unpaired one-way analysis of variance (ANOVA) followed by Bonferroni as post-hoc test. Statistical analyses were done using GraphPad Prism 6.0 software (GraphPad

Software Inc., San Diego, CA, USA). $p < 0.05$ was considered statistically significant. IC_{50}s were calculated using GraphPad Prism 6.00 software.

Results and discussion

CSE inhibits TNFα-induced release of skin pro-inflammatory mediators in HaCaT and HDF cells

Skin inflammatory diseases are characterized by over-expression of a multitude of pro-inflammatory mediators which impact on keratinocytes and fibroblasts. Among them, IL-8 is involved in neutrophil recruitment and VEGF regulates the angiogenesis process, while MMP9 contributes to the degradation of extracellular matrix. Thus, we investigated the ability of the extract to affect the release of these pro-inflammatory mediators in HaCaT and HDF cells.

In both cell models, TNFα highly induced IL-8 release; in HaCaT cells, CSE was able to inhibit secretion only at the highest concentration tested (50 μg/ml, Figure 1, panel A) whereas in human dermal fibroblasts CSE showed a more pronounced inhibitory effect, with low IC_{50} (15.13 μg/ml, Figure 2, panel A). The effect of CSE on TNFα-induced MMP-9 release in HaCaT and HDF cells was significant, with inhibition starting from 10 μg/ml (IC_{50} 18.0 and 7.21, respectively), thus implying that the extract may counteract matrix degradation induced by metalloproteases in the skin dermis and epidermis (Figures 1 and 2, panels B).

VEGF plays a crucial role during the pathogenesis of psoriasis and increasing experimental data have shown the effectiveness of anti-VEGF strategy for the treatment of psoriasis. Bevacizumab is effective for psoriasis whereas the antibody G6-31, which is

potently against human and murine VEGF, demonstrated a therapeutic effect in a mouse model which had psoriasis-like skin inflammation (Schonthaler, Huggenberger, Wculek, Detmar, & Wagner, 2009). Thus, we investigated the effect of CSE in impairing TNFα-induced VEGF in HaCaT and HDF cells. In HDF, TNFα did not induce VEGF whereas in HaCaT cells TNFα induced high release of VEGF, and the extract showed a concentration dependent inhibition, with an IC_{50} of 26.8 μg/ml (Figure 1, panel C).

CBD shows different effect on TNFα-induced release of skin pro-inflammatory mediators in HaCaT and HDF cells

To test contribution of CBD to the effect observed with the extract, both HaCaT and HDF cells were incubated with increasing concentrations of CBD, and release of inflammatory mediators was assayed. CBD did not show any inhibitory effect on IL-8 and MMP-9 release in HDF cells at the highest no toxic concentration tested (2.5 μM, data not shown). In HaCaT cells, CBD did not show effects on IL-8 release (data not shown) whereas only an inhibitory trend was observed on VEGF release; CBD showed a concentration dependent inhibition of MMP-9 with 50% inhibition at 5 μM thus reflecting inhibitory effect of MMP-9 release elicited by the extract (Figure 3 vs. Figure 1, panel B).

Effect of CSE and CBD on TNFα-induced NF-κB driven transcription in HaCaT and HDF cells

NF-κB represents a key factor in a variety of skin inflammatory conditions including psoriasis (Goldminz et al., 2013), and TNFα strongly induces activation of the NF-κB

pathway. Thus, we investigated if CSE and CBD might be able to counteract the NF-κB induced by TNFα. In HaCaT cells, CSE inhibited the NF-κB driven transcription in a concentration dependent way (Figure 4, panel A), with IC_{50} in the micromolar order (21.4 μg/ml). Since the extract contains significant amount of CBD, we assessed if the anti-inflammatory effect of CSE could be due to the presence of this compound. Pure CBD was able to inhibit TNFα-induced NF-κB driven transcription in HaCaT cells in a concentration dependent fashion, with low IC_{50} (2.85 μM) (Figure 4, panel B). Considering the occurrence of CBD in the extract (5%) and its efficacy in impairing the NF-κB driven transcription, it appears to highly contribute to the effect. CSE also inhibited the transcription in HDF cells with lower IC_{50} (12.3 μg/ml); however, CBD failed to elicit such effect in HDF cells at 2.5 μM, a concentration which is very close to the IC_{50} obtained in HaCaT cells (Figure 4, panel C and D). Previous papers have reported the ability of CBD to impair the NF-κB pathway both *in vitro* and *in vivo* (Khaksar & Bigdeli, 2017) (Esposito et al., 2006), and the inhibition of the NF-κB by CBD infusion was found to ameliorate cerebral ischemia in rats (Khaksar & Bigdeli, 2017). However, this is the first evidence assessing the ability to impair the NF-κB pathway in human skin cells.

CSE and CBD show negligible protective effects in HaCaT cells exposed to UVB irradiation

To provide insights into the possible protective role of CSE and CBD in keratinocytes, cells were exposed to UVB and treated for 1 hour with the extract or pure CBD; then, NF-κB nuclear translocation was measured by ELISA assay. Both CSE and CBD showed negligible protective effects on oxidative stress induced by UVB irradiation at

concentrations 5-10 μg/ml or 0.5-1 μM, respectively (Figure S1, panel A). Moreover, CSE (25 μg/ml) and CBD (4 μM) were not able to counteract inhibition of Nrf-2 nuclear translocation induced by UVB (Figure S1, panel B). Collectively, our findings suggest that both *Cannabis* extract and CBD do not protect human keratinocytes by oxidative stress through modulation of the NF-κB or Nrf-2 pathways.

Effect of CSE and CBD on inflammatory or wound healing genes overexpressed by TNFα

Wound healing is a complex process occurring via three overlapping phases: inflammation, granulation and tissue remodeling. During skin injury inflammatory cells infiltrate the wound and release a multitude of cytokines and growth factors which promote the inflammatory process. In the granulation phase, fibroblasts are involved in the extracellular matrix (ECM) deposit whereas keratinocytes proliferate and migrate close to the wound. During the final tissue remodeling phase, ECM remodeling to resemble integrity of tissue occurs. Although both fibroblasts and keratinocytes are involved in skin inflammatory conditions and wound healing, the former is mostly involved in the ECM remodeling whereas the latter are the major contributors to the inflammatory processes.

Then, the ability of CSE (25 μg/mL) to reduce the mRNA levels of 84 genes involved in the inflammatory response (in HaCaT cells) or in wound healing (in HDF cells) was assessed. These experiments were performed using two different RT^2 Profiler PCR Array from Qiagen company as reported in Materials and Methods section. The corresponding concentration of CBD occurring in the extract (4 μM), was also tested to verify the

contribution of the pure compound to the activity of the extract. HaCaT and HDF cells were treated for 6 hours with TNFα (10 ng/mL) and CSE (25 μg/mL) or CBD (4 μM).

In HaCaT cells, following TNFα treatment, expression of 26 genes was more than five-fold higher; these genes included chemokines (ex. CXCL8 and CXCL10), interleukins (ex. IL17C and IL1B), TNF family members (like TNF and LTB), and other genes such as VEGFA (Figure 5, panel A). CSE decreased all the mRNA levels of the up-regulated genes whereas CBD was not able to fully explain the activity elicited by the extract since it was able to down-regulate 15 genes (Figure 5, panel B). Among the genes down-regulated we found IL-17C which is considered an interesting target for psoriasis (Johnston et al., 2013), and IL17C inhibitors may be useful to treat the disease.

According to the results obtained on IL-8 and VEGF release, CSE was also able to downregulate the corresponding genes in HaCaT cells whereas pure compound CBD was inactive; these results seem to suggest that compounds other than CBD may contribute to the inhibition of these pro-inflammatory mediators.

In human dermal fibroblasts, the stimulus TNFα up-regulated 16 genes involved in the wound healing, in particular the most up-regulated genes were extracellular matrix enzymes (ex. MMP1 and MMP9), cytokines (ex. CXCL11, CXCL2 and IL6), growth factors (such as TNF and CSF2) and signals transduction (like PTGS2) (Figure 6, panel A). As previously shown in HaCaT cells, CSE was able to counteract all the genes induced by TNFα. In this cell model, the contribution of CBD to the activity of the extract was evident on 11 genes, showing a more pronounced activity than in HaCaT cells; however, the effect of CSE was still higher than pure CBD, which failed to downregulate some

genes playing pivotal roles in inflammation and matrix remodeling, including IL-6 and MMP-9 (Figure 6, panel B).

CBD shows anti-inflammatory activity in animal models including mouse challenged with Croton oil (Tubaro et al., 2010); moreover, transdermal application of CBD prevents inflammation and edema induced by carrageenan injection in a murine model of skin inflammation (Lodzki et al., 2003); however, the mechanism of action is still controversial. CB2 receptors are expressed by a variety of cells during the wound healing process, and CB2 agonist were found to decrease inflammatory response in a mouse model of wound healing promoting re-epithelization (Wang et al., 2016). This mechanism seems not to be mediated by CBD since previous studies demonstrated that CBD antagonizes CB1/CB2 receptors agonists, with effects on intracellular signaling highly independent of CB1 receptors (Laprairie, Bagher, Kelly, Dupre, & Denovan-Wright, 2014) (Thomas et al., 2007).

Conclusions

This study demonstrates that CSE is able to inhibit the release of mediators of inflammation involved in wound healing and inflammatory processes occurring in the skin. The mode of action seems to involve impairment of the NF-κB pathway since the extract was able to inhibit the TNFα-induced NF-κB driven transcription both in HDF and HaCaT cells. This is also corroborated by the evidence that CSE inhibits the release of IL-8 and MMP-9 in both cell lines, and VEGF just in HaCaT cells, which are all mediators whose genes are dependent by NF-κB. The effect of CBD on the NF-κB pathway and MMP-9 release paralleled the effect of *Cannabis* extract thus making this cannabinoid

the major contributor to the effect observed; however, the other effects elicited by the extracts, including down-regulation of genes involved in wound healing and skin inflammation, were not strictly associated to the presence of CBD, suggesting that other unknown compounds occurring in the extract may exert anti-inflammatory effects. Information collected through the arrays will help better address future investigations. Our findings provide new insights into the potential effect of *Cannabis* extracts against inflammation-based skin diseases.

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Figure legends

Figure 1. Effect of CSE on TNFα-induced IL-8 (A), MMP-9 (B) and VEGF (C) release in HaCaT cells. The cells were treated with the stimulus (TNFα 10 ng/mL) and the extract for 6 hours (IL-8 secretion) or 24 hours (MMP-9 and VEGF secretion). The release of these pro-inflammatory mediators was assessed through an ELISA assay. Data are

expressed in percentage, relative to the stimulated control, which is arbitrarily assigned to the value of 100%. *p < 0.05 **p < 0.01, ***p < 0.001 versus TNFα.

Figure 2. Effect of CSE on TNFα-induced IL-8 (A), MMP-9 (B) and VEGF (C) release in HDF cells. The cells were treated with the stimulus (TNFα 10 ng/mL) and the extract for 6 hours (IL-8 secretion) or 24 hours (MMP-9 secretion). The release of these pro-inflammatory mediators was assessed through an ELISA assay. Data are expressed in percentage, relative to the stimulated control, which is arbitrarily assigned to the value of 100%. ***p < 0.001 versus TNFα.

Figure 3. Effect of CBD on TNFα-induced MMP-9 release in HaCaT cells. The cells were treated with the stimulus (TNFα 10 ng/mL) and the pure compound for 24 hours. MMP-9 release was assessed through an ELISA assay. Data are expressed in percentage, relative to the stimulated control, which is arbitrarily assigned to the value of 100%. *p < 0.05 **p < 0.01, ***p < 0.001 versus TNFα.

Figure 4. Effect of CSE and CBD on TNFα-induced NF-κB driven transcription in HaCaT (A-B) and HDF (C-D) cells. The cells were treated with the stimulus (TNFα 10 ng/mL) and the extract or pure compound for 6 hours. NF-κB driven transcription was assessed through luciferase method. Data are expressed in percentage, relative to the stimulated control, which is arbitrarily assigned to the value of 100%. *p < 0.05 **p < 0.01, ***p < 0.001 versus TNFα.

Figure 5. Effect of CSE and CBD on TNFα-induced gene expression of 84 genes involved in the inflammatory process, in HaCaT cells. The cells were treated with the stimulus (TNFα 10 ng/mL) and the extract (25 μg/mL) or pure compound (4 μM) for 6 hours. The mRNA levels were evaluated through real time PCR using an RT^2 Profiler PCR Array from Qiagen company.

Figure 6. Effect of CSE and CBD on TNFα-induced gene expression of 84 genes involved in the wound healing process, in HDF cells. The cells were treated with the stimulus (TNFα 10 ng/mL) and the extract (25 μg/mL) or pure compound (4 μM) for 6 hours. The mRNA levels were evaluated through real time PCR using an RT^2 Profiler PCR Array from Qiagen company.

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