Received: 1 April 2019 | Revised: 2 May 2019 | Accepted: 7 May 2019

DOI: 10.1002/ptr.6400

RESEARCH ARTICLE

WILEY

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

Enrico Sangiovanni[1] | Marco Fumagalli[1] | Barbara Pacchetti[2] | Stefano Piazza[1] | Andrea Magnavacca[1] | Saba Khalilpour[1] | Gloria Melzi[1] | Giulia Martinelli[1] | Mario Dell'Agli[1]

[1] Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Milan, Italy

[2] Linnea SA, Riazzino, Switzerland

**Correspondence**
Mario Dell'Agli, Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Via Balzaretti 9, Milan 20133, Italy.
Email: mario.dellagli@unimi.it

**Funding information**
Linnea SA; Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR), Grant/Award Number: Progetto Eccellenza-DiSFeB

Skin inflammatory diseases result from complex events that 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 antiinflammatory 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 the impairment of the nuclear factor-kappa B (NF-κB) pathway since the extract counteracted the tumor necrosis factor-alpha-induced NF-κB-driven transcription in both skin cell lines. *Cannabis* extract and cannabidiol showed different effects on the release of interleukin-8 and vascular endothelial growth factor, which are both mediators whose genes are dependent on NF-κB. The effect of cannabidiol on the NF-κB pathway and metalloproteinase-9 (MMP-9) release paralleled the effect of the extract thus making cannabidiol the major contributor to the effect observed. Down-regulation of genes involved in wound healing and skin inflammation was at least in part due to the presence of cannabidiol. Our findings provide new insights into the potential effect of *Cannabis* extracts against inflammation-based skin diseases.

**KEYWORDS**

cannabidiol, *Cannabis sativa* L., fibroblasts, keratinocytes, skin inflammation, wound injury

## 1 | INTRODUCTION

The skin responds to a variety of extrinsic and intrinsic physical stimuli that modify its 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 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 are deeply involved in wound healing and interact with keratinocytes and the other skin cells (Pastar et al., 2014). Keratinocyte function is regulated by a variety of growth factors, cytokines, and chemokines and, in turn, these cells release several proinflammatory mediators including interleukin-1 beta (IL-1β), IL-6, IL-8, tumor necrosis factor alpha (TNFα), and transforming growth factor alpha and beta, as well as

  

vascular endothelial growth factor (VEGF), a potent mitogen for endothelial cells, playing a pivotal 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 that 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, whereas MMP-9 contributes to the degradation of ECM. These proinflammatory mediators are regulated by different transcription factors, including nuclear factor-kappa B (NF-κB), which plays a 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 that include delta-9 tetrahydrocannabinol and cannabidiol (CBD), a cannabinoid without psychotropic activity. Chemistry and pharmacology of CBD, as well as its molecular targets, including CB receptors and other components of the endocannabinoid system, have been extensively reviewed (Izzo, Borrelli, Capasso, Di Marzo, & Mechoulam, 2009; Kogan & Mechoulam, 2007; Mechoulam & Hanus, 2002; Mechoulam, Parker, & Gallily, 2002; Mechoulam, Peters, Murillo-Rodriguez, & Hanus, 2007; Ujvary & Hanus, 2016). In addition, *Cannabis* extracts and pure compounds show various effects in a multitude of inflammatory-based diseases (Borrelli et al., 2013; Burstein, 2015; Izzo et al., 2012; Pagano et al., 2016).

Few studies demonstrated the antiinflammatory activity of CBD in animal models of skin inflammation (Lodzki et al., 2003; Tubaro et al., 2010). However, the molecular mechanisms underlying the antiinflammatory effect observed in vivo as well as 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 *C. sativa* L. ethanolic extract (CSE), standardized in CBD, as antiinflammatory agent in the skin, unraveling the molecular mechanisms of its action in human keratinocytes and fibroblasts. HaCaT cells, a spontaneously immortalized human keratinocyte line, have been used as a reliable in vitro model, with respect to normal keratinocytes, to test the antiinflammatory activity, according to Colombo et al. (2017).

## 2 | MATERIALS AND METHODS

### 2.1 | Plant material and CBD isolation

*C. sativa* L. oil extract, a dark green viscous liquid, containing 5% CBD and THC < 0.2% in medium chain triglycerides (MCT, Batch No. 74717009) and pure CBD (slightly yellow powder, Batch No. 74717009) were kindly provided by LINNEA SA (https://www.linnea.ch/). Certificates of analysis are provided as supporting information (Figure S1). CSE is a standardized commercial extract prepared from *C. 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 replaced with purified natural oil, such as MCT.

CBD (99.5% HPLC purity) was isolated and purified from *C. 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 extraction with final crystallization in an aliphatic hydrocarbon.

### 2.2 | Cell culture

HaCaT cells, spontaneously immortalized human keratinocyte line (Boukamp et al., 1988), were kindly provided by Cell Line Service GmbH (Eppelheim, Germany), whereas normal human dermal fibroblasts (HDFs) 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 4 days, at 80–90% of confluence, cells were detached from the 75-$cm^2$ flasks (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 $1.5 \times 10^6$ cells per flask, to allow the cell line growth. The remaining cells were seeded in 24-well plates (DB Falcon™) for the biological tests.

### 2.3 | Cell treatment

After 72 hr of growth, HaCaT and HDF cells (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). Based on the parameter to be evaluated, cells were subjected to treatment with the extract or individual compound and the proinflammatory stimulus for 6 hr (IL-8 release, nuclear factor-kappa B [NF-κB]-driven transcription, and mRNA levels) or 24 hr (VEGF and MMP-9 release). Ultraviolet radiation B (UVB) assays were performed following 1-hr 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.

### 2.4 | Cytotoxicity assay

The integrity of the morphology before and after the treatment was assessed by light microscopy. 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 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-hr treatment in both cell lines. At the end of the treatment, the culture medium was removed from each well, and 200 μl of 3,4,5-dimethylthiazol-2-yl-2-5-diphenyltetrazolium bromide solution was added for 30–40 min till the development of a violet color (formazan). Two hundred microliters 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 spectrophotometrically at 570 nm (Envision, PerkinElmer, USA).

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. CBD showed cytotoxic effects in HDF cells at concentrations higher than 2.5 μM. Absence of cytotoxicity was confirmed by lactate dehydrogenase (LDH) assay.

## 2.5 | 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™; 60,000 cells per well) for 72 hr. Then cells were treated with CSE or CBD along with the stimulus TNFα (10 ng/ml) for 6 or 24 hr. 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-hr treatment, whereas VEGF and MMP-9 release were analyzed after 24-hr treatment by an enzyme-linked immunosorbent assay (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) that were coated with the corresponding antibody contained in the kit, overnight at room temperature, whereas Human MMP-9 ELISA kit, containing a precoated 96-well plate, was provided by RayBiotech (Norcross, USA).

The amounts of IL-8, VEGF, and MMP-9 in the samples were detected by measuring the absorbance resulted from 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, USA) at 450 nm 0.1 s. IL-8, VEGF, and MMP-9 were quantified through a standard curve supplied in the ELISA kit (8–1,000 pg/ml for IL-8, 16–1,000 pg/ml for VEGF, and 10–6,000 pg/ml for MMP-9). The results are expressed as mean ± s.d. of at least three experiments. Epigallocatechin gallate (EGCG) (20 μM), resveratrol (50 μM), and quercetin (10 μM) were used as reference inhibitors of VEGF, MMP-9, and IL-8 release, respectively.

## 2.6 | NF-κB-driven transcription

HaCaT and HDF cells were seeded in 24-well plates (DB Falcon™) at a density of 60,000 cells per well for 72 hr. Then, the cells were transiently transfected, by lipofectamine method, with a reporter plasmid containing the luciferase gene under control of a promoter characterised by the presence of three responsive elements κB (NF-κB-LUC 250 ng per well). The plasmid NF-κB-LUC was a gift of Dr N. Marx (Department of Internal Medicine-Cardiology, University of Ulm, Ulm, Germany). After incubation overnight, the cells were treated with CSE or CBD and TNFα (10 ng/ml) for 6 hr. Luciferase produced into the cells was assessed using Britelite™ Plus reagent (PerkinElmer, Walthman, MA, USA) according to the manufacturer's instructions. The luminescence deriving from the reaction between luciferase and luciferin was measured through a spectrophotometer (Victor X3, PerkinElmer, USA). The results are expressed as mean ± s.d. of at least three experiments. EGCG (20 μM) was used as reference compound.

## 2.7 | UVB irradiation system

UVB-induced NF-κB nuclear translocation was measured following exposure of HaCaT cells to a UVB (40 mJ/cm$^2$) light source (Triwood 31/36, W36, V230, Helios Italquartz, Milano, Italy), over ice, and treated for 1 hr with increasing concentrations of CSE (1–25 μg/ml) or CBD (0.1–2.5 μM). Radiation time (about 50 s) was adjusted for each experimental day, measuring energy emission with an 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 pretreatment with CSE (25 μg/ml) or CBD (4 μM) for 1 hr; then cells were irradiated as above previously described and maintained in cell free-medium for 3 hr before the assay.

## 2.8 | Nrf-2 and NF-κB nuclear translocation assays

To assess the effect of the extract and the individual compounds on the Nrf-2 nuclear translocation, HaCaT cells were plated at a density of 1.5 × 10$^6$ cells/mL in 100-mm Petri dishes. After 48 hr, cells were treated for 1 hr with extract/compound under study and then exposed to UVB irradiation (40 mJ/cm$^2$). After 3 hr, nuclear extracts were obtained and stored at −80°C until assayed. The same amount of total nuclear proteins (80 μg per 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 spectrophotometric measurement at 450 nm, 0.1 s (Victor X3, 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 a density of 60,000 cells per well for 72 hr; then cells were exposed to UVB irradiation (40 mJ/cm$^2$) and treated for 1 hr 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 spectrophotometric measurement at 450 nm, 0.1 s (Victor X3, 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.

## 2.9 | Gene expression

### 2.9.1 | RNA extraction

HaCaT and HDF cells were grown in 24-well plates (DB Falcon™; 60,000 cells per well) for 72 hr; then, the cells were treated with the proinflammatory stimulus (TNFα, 10 ng/ml) and CSE (25 μg/mL) or CBD (4 μM). After 6-hr treatment, the medium was removed. The cells were lysed through the addition of the Qiazol lysis buffer (QIAGEN GmbH, Germany) according to the manufacturer's instructions, 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 spectrophotometrically (NanoDrop ND-1000, ThermoFisher Scientific). The purity of the samples was estimated by measuring the ratio between the absorbance of the samples at 260 nm and 280 or 230.

### 2.9.2 | 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, was used to obtain cDNA.

### 2.9.3 | Quantitative polymerase chain reaction

The analysis of gene expression was performed using two 384-well polymerase chain reaction (PCR) array, related to human genes involved in the inflammatory process and wound healing ($RT^2$ Profilet™ 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 (five 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™ 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™ (Bio-Rad), depending on the

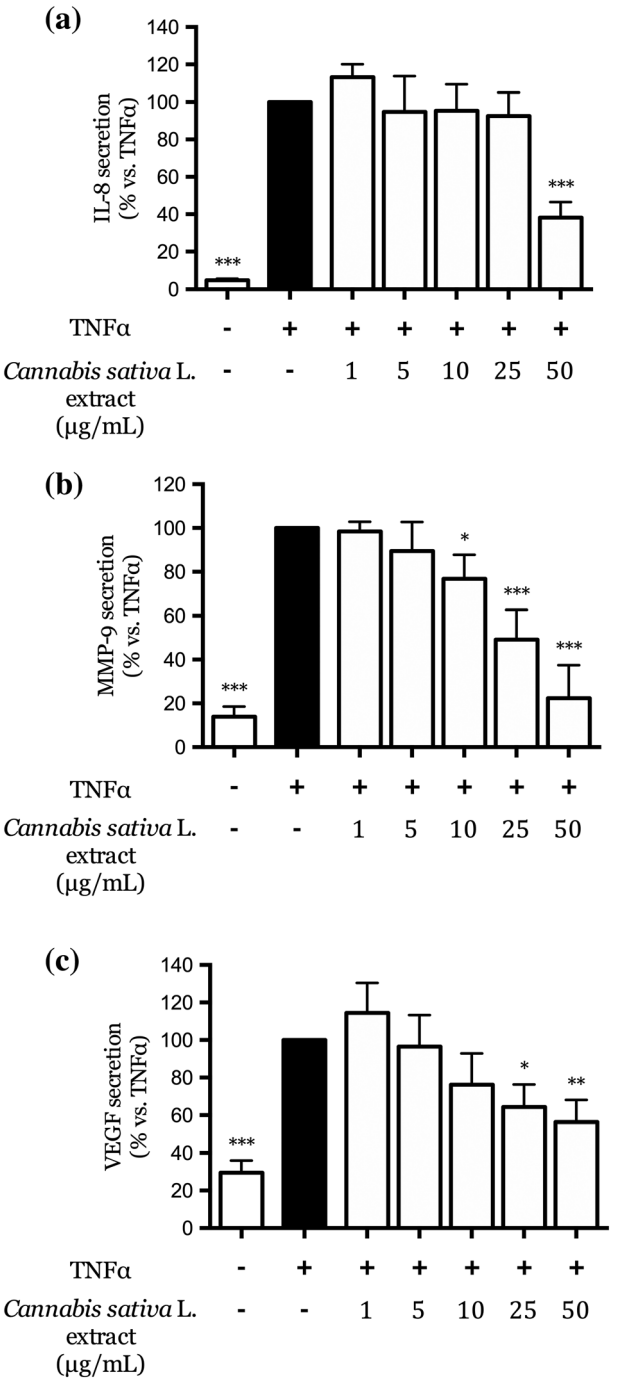


**FIGURE 1** Effect of CSE on TNFα-induced IL-8 (a), MMP-9 (b), and VEGF (c) release in HaCaT cells. The cells were treated with the stimulus (TNFα, 10 ng/ml) and the extract for 6 hr (IL-8 secretion) or 24 hr (MMP-9 and VEGF secretion). The release of these proinflammatory mediators was assessed through an ELISA assay. Data are expressed in percentage, relative to the stimulated control, to which is arbitrarily assigned the value of 100%. *$p < .05$, **$p < .01$, ***$p < .001$ versus TNFα

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 (QIAGEN Sciences, USA). The $C_t$ cut-off was set to 35. Data were normalized on the basis of housekeeping genes: hypoxanthine phosphoribosyltransferase 1, beta-2-microglobulin, ribosomal protein P0, beta-actin, and glyceraldehyde-3-phosphate dehydrogenase. In each experiment, the housekeeping genes with a variability higher than ±1 threshold cycle among the different experimental conditions were excluded from the analysis.

### 2.10 | 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 followed by Bonferroni 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. IC50s were calculated using GraphPad Prism 6.00 software.

## 3 | RESULTS AND DISCUSSION

### 3.1 | CSE inhibits TNFα-induced release of skin proinflammatory mediators in HaCaT and HDF cells

Skin inflammatory diseases are characterized by the overexpression of a multitude of proinflammatory mediators that impact on keratinocytes and fibroblasts. Among them, IL-8 is involved in neutrophil recruitment, and VEGF regulates the angiogenetic process, whereas MMP-9 contributes to the degradation of ECM. Thus, we investigated the ability of the extract to affect the release of these proinflammatory mediators in HaCaT and HDF cells.

In both cell models, TNFα considerably induced IL-8 release; in HaCaT cells, CSE was able to inhibit its secretion only at the highest concentration tested (50 μg/ml; Figure 1a), whereas in HDFs, CSE showed a more pronounced inhibitory effect, with low $IC_{50}$ (15.13 μg/ml; Figure 2a). 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 1b and 2b).

VEGF plays a crucial role during the pathogenesis of psoriasis, and increasing experimental evidences have shown the effectiveness of the targeting of VEGF for the treatment of psoriasis. Bevacizumab is effective against psoriasis, whereas the antibody G6-31, which is directed against human and murine VEGF, demonstrated a therapeutic effect in a mouse model of psoriasis-like skin inflammation (Schonthaler, Huggenberger, Wculek, Detmar, & Wagner, 2009). Thus, we investigated the effect of CSE on the impairment of TNFα-induced VEGF release in HaCaT and HDF cells. While in HDF TNFα did not induce VEGF release, on the other hand 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 1c).

### 3.2 | CBD shows different effect on TNFα-induced release of skin proinflammatory mediators in HaCaT and HDF cells

To test the contribution of CBD to the effect observed using the extract, both HaCaT and HDF cells were incubated with increasing concentrations of CBD, and the 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 non-toxic concentration tested (2.5 μM, data not shown). In HaCaT cells, CBD did not show any effect on IL-8 release (Figure 3a), whereas only an inhibitory trend could be observed on VEGF release (Figure 3c); CBD showed a concentration-dependent inhibition of MMP-9 with 50% inhibition at 5 μM thus reflecting inhibitory effect on MMP-9 release elicited by the extract (Figure 3b vs. Figure 1b).

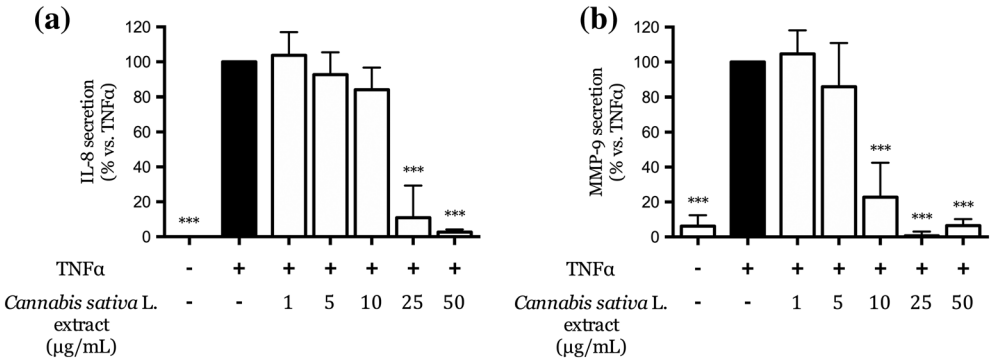


**FIGURE 2** Effect of CSE on TNFα-induced IL-8 (a) and MMP-9 (b) release in HDF cells. The cells were treated with the stimulus (TNFα, 10 ng/ml) and the extract for 6 hr (IL-8 secretion) or 24 hr (MMP-9 secretion). TNFα was not able to induce VEGF release in HDF cells. The release of these proinflammatory mediators was assessed through an ELISA assay. Data are expressed in percentage, relative to the stimulated control, to which is arbitrarily assigned the value of 100%. ***$p < .001$ versus TNFα

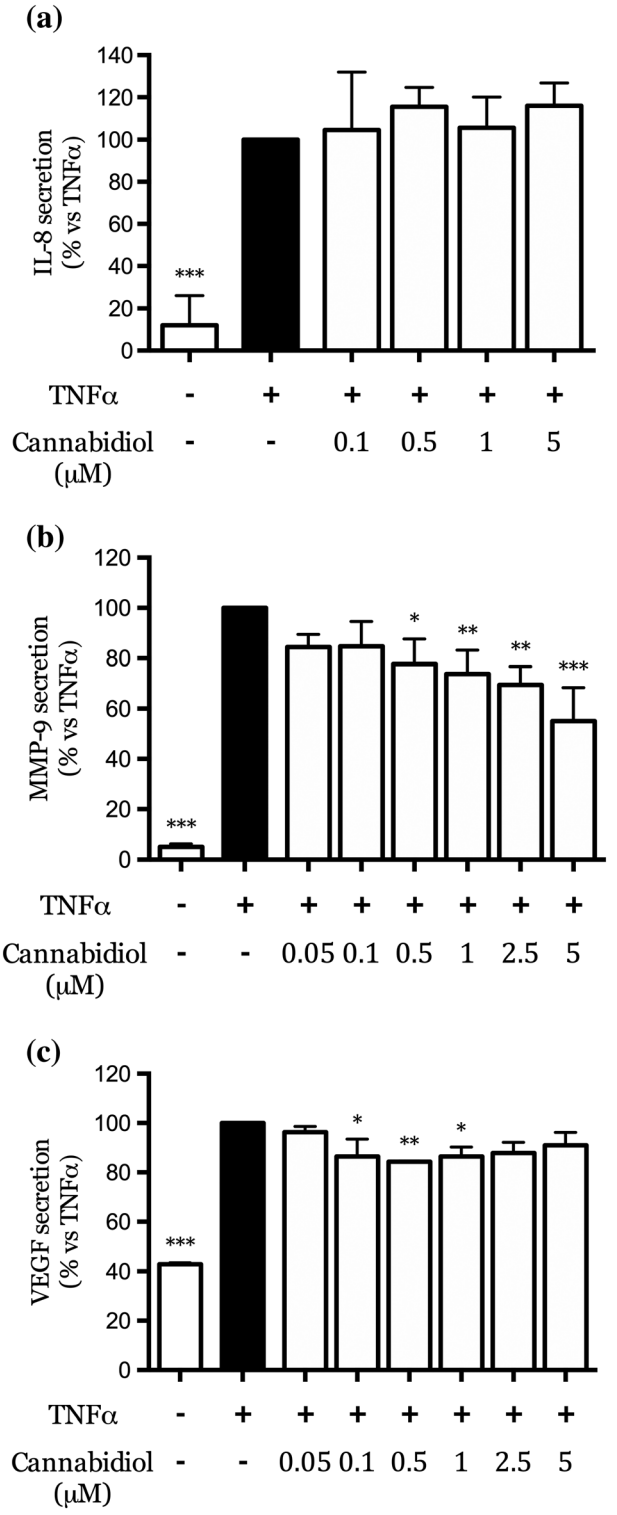


**FIGURE 3** Effect of CBD on TNFα-induced IL-8 (a), MMP-9 (b), and VEGF (c) release in HaCaT cells. The cells were treated with the stimulus (TNFα, 10 ng/ml) and the pure compound for 24 hr. MMP-9 release was assessed through an ELISA assay. Data are expressed in percentage, relative to the stimulated control, which is arbitrarily assigned to the value of 100%. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ versus TNFα

### 3.3 | 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 the activation of the NF-κB pathway. Therefore, we investigated if CSE and CBD might be able to counteract the NF-κB activation induced by TNFα. In HaCaT cells, CSE inhibited the NF-κB-driven transcription in a concentration dependent manner (Figure 4a), with $IC_{50}$ in the micromolar order (21.4 μg/ml). Since the extract contains significant amount of CBD, we assessed whether the antiinflammatory 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 4b). Considering the occurrence of CBD in the extract (5%) and its efficacy in impairing the NF-κB-driven transcription, it appears to give a significant contribution 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 4c,d). Previous papers have reported the ability of CBD to impair the NF-κB pathway both in vitro and in vivo (Esposito et al., 2006; Khaksar & Bigdeli, 2017), and the inhibition of the NF-κB by CBD infusion was found to ameliorate cerebral ischemia in rats (Khaksar & Bigdeli, 2017). It has been demonstrated that CBD inhibited the NF-κB pathway, upregulated the activation of the STAT3 transcription factor and decreased the activation of the STAT1 induced by LPS in BV-2 microglial cells (Kozela et al., 2010). Interestingly, the same authors demonstrated that LPS upregulated the expression of proinflammatory miRNAs associated to toll-like receptor and NF-κB signaling, including miR-21, miR-146a, and miR-155, whereas CBD inhibited LPS-stimulated expression of miR-146a and miR-155 (Juknat, Gao, Coppola, Vogel, & Kozela, 2019).

However, this is the first evidence assessing the ability to impair the NF-κB pathway in human skin cells.

### 3.4 | 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 hr 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 of 5–10 μg/ml or 0.5–1 μM, respectively (Figure S2A). Moreover, CSE (25 μg/ml) and CBD (4 μM) were not able to counteract inhibition of Nrf-2 nuclear translocation induced by UVB (Figure S2B). Collectively, our findings seem to suggest that both *Cannabis* extract, and CBD do not influence the NF-κB or Nrf-2 pathways in UVB-irradiated keratinocytes.

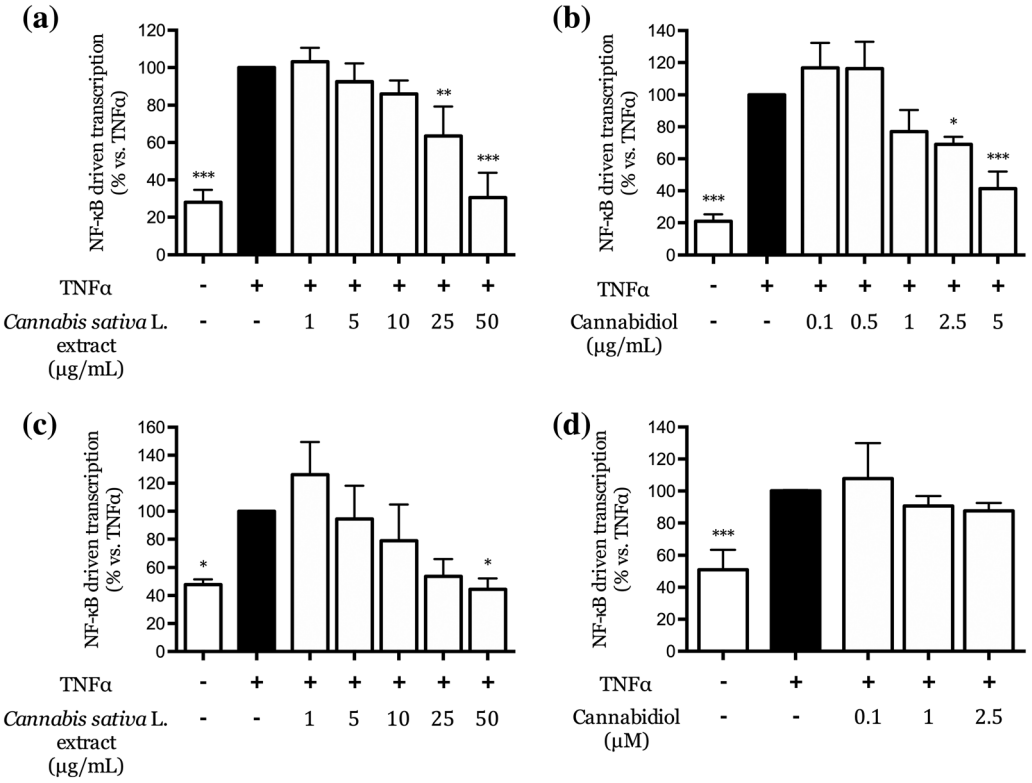


**FIGURE 4** Effect of CSE and CBD on TNFα-induced NF-κB-driven transcription in HaCaT (a–b) and HDF (c–d) cells. The cells were treated with the stimulus (TNFα, 10 ng/ml) and the extract or pure compound for 6 hr. NF-κB-driven transcription was assessed through luciferase method. Data are expressed in percentage, relative to the stimulated control, which is arbitrarily assigned to the value of 100%. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ versus TNFα

## 3.5 | 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 remodelling. Upon skin injury, inflammatory cells infiltrate the wound and release a multitude of cytokines and growth factors that promote the inflammatory process. In the granulation phase, fibroblasts are involved in the ECM laying-down, whereas keratinocytes proliferate and migrate close to the wound edges. During the final tissue remodeling phase, ECM remodeling to restore integrity of tissue occurs. Although both fibroblasts and keratinocytes are involved in skin inflammatory conditions and wound healing, the formers are mostly involved in the ECM remodeling, whereas the latters 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 Section 2. The corresponding concentration of CBD (4 μM) occurring in the extract at 25 μg/ml was also tested to verify the contribution of the pure compound to the activity of CSE. HaCaT and HDF cells were treated for 6 hr 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 (e.g., CXCL8 and CXCL10), interleukins (e.g., IL17C and IL1B), TNF family members (like TNF and LTB), and other genes such as VEGFA (Figure 5a). CSE decreased all the mRNA levels of the upregulated genes, whereas CBD was not able to fully explain the activity elicited by the extract as it was only able to downregulate 15 genes (Figure 5 b). Among the downregulated genes, 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 proinflammatory mediators.

In HDFs, the stimulus TNFα upregulated 16 genes involved in wound healing, in particular the most upregulated ones were ECM enzymes (e.g., MMP-1 and MMP-9), cytokines (e.g., CXCL11, CXCL2, and IL6), growth factors (such as TNF and CSF2), and signal transducers (like PTGS2; Figure 6a). As previously shown in HaCaT cells, CSE was able to counteract the transcription of 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

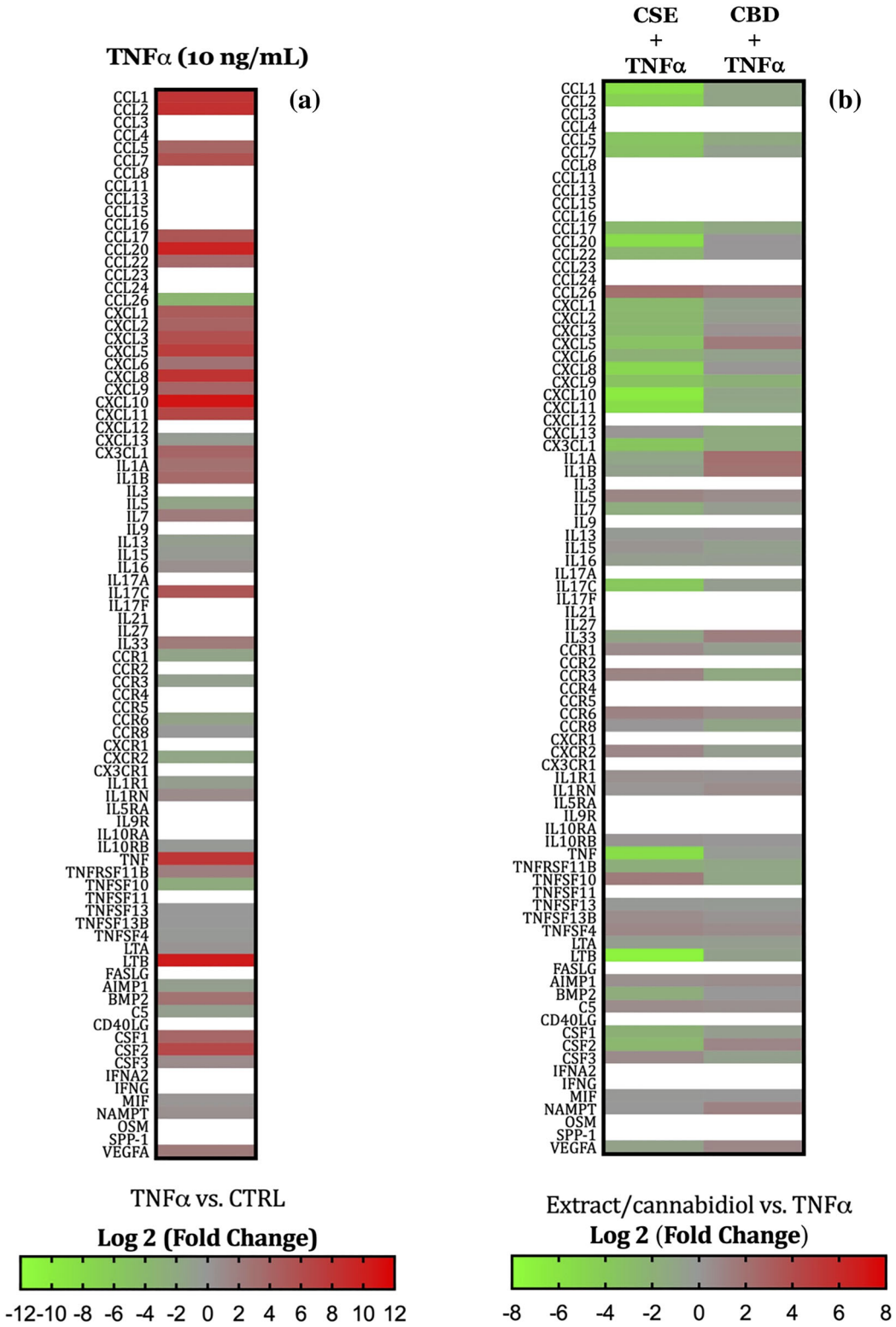


**FIGURE 5** Effect of CSE and CBD on TNFα-induced gene expression of 84 genes involved in the inflammatory process, in HaCaT cells. The cells were treated with the stimulus (TNFα, 10 ng/ml) and the extract (25 μg/ml) or pure compound (4 μM) for 6 hr. The mRNA levels were evaluated through quantitative PCR using an $RT^2$ Profiler PCR Array from Qiagen company [Colour figure can be viewed at wileyonlinelibrary.com]

than pure CBD, which failed to downregulate some genes playing pivotal roles in inflammation and matrix remodeling, including IL-6 and MMP-9 (Figure 6b).

CBD shows antiinflammatory activity in animal models including mouse challenged with Croton oil (Tubaro et al., 2010); moreover, transdermal application of CBD prevents inflammation and oedema

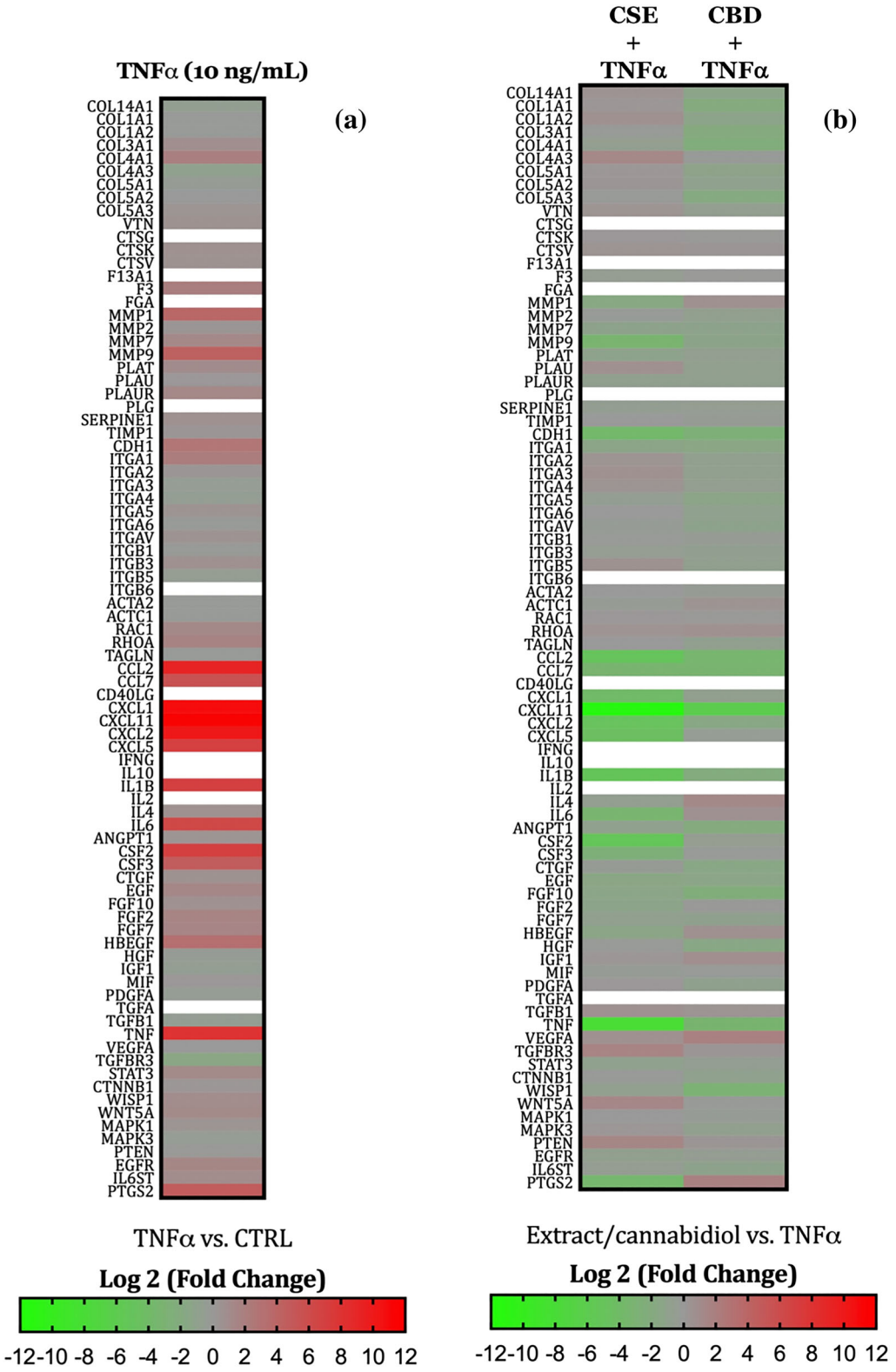


**FIGURE 6** Effect of CSE and CBD on TNFα-induced gene expression of 84 genes involved in the wound healing process, in HDF cells. The cells were treated with the stimulus (TNFα, 10 ng/ml) and the extract (25 μg/ml) or pure compound (4 μM) for 6 hr. The mRNA levels were evaluated through real-time PCR using an $RT^2$ Profiler PCR Array from Qiagen company [Colour figure can be viewed at wileyonlinelibrary.com]

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 agonists 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 because 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). Clues from previous studies suggest that CBD may exert its effect also acting on PPARγ or TRPV1 (Couch, Tasker, Theophilidou, Lund, & O'Sullivan, 2017; Hind, England, & O'Sullivan, 2016). However, no studies have demonstrated such effects in skin cells.

## 4 | 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 because 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, but of VEGF only in HaCaT cells, mediators whose genes are all 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 extract, including downregulation of genes involved in wound healing and skin inflammation, were not strictly associated with the presence of CBD. CSE is a complex mixture containing CBD and also other cannabinoids and flavonoids that may exert antiinflammatory activities thus contributing to the effect observed herein. 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.

## ACKNOWLEDGMENTS

The authors thank Professor Dr Petra Boukamp and Dr Norbert Fusenig from Deutsches Krebsforschungszentrum, Stiftung des öff entlichen Rechts (German Cancer Research Center), Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany, for providing HaCaT cell line. This research was supported by grants from MIUR Progetto Eccellenza. Dr B. Pacchetti is an employee of Linnea SA, Riazzino, CH, a pharmaceutical company that produces and commercializes *Cannabis* extracts. Linnea partially funded the research; however, this paper does not necessarily reflect the company's views of its future policy on this area.

## CONFLICT OF INTEREST

Dr B. Pacchetti is an employee of Linnea SA, Riazzino, CH, a pharmaceutical company that produces and commercializes Cannabis extracts. Linnea partially funded the research; however, this paper does not necessarily reflect the company's views of its future policy on this area. The other authors declare no conflict of interests.

## ORCID

*Enrico Sangiovanni* https://orcid.org/0000-0003-2811-8628
*Mario Dell'Agli* https://orcid.org/0000-0001-5378-402X

## REFERENCES

Baroni, A., Buommino, E., De Gregorio, V., Ruocco, E., Ruocco, V., & Wolf, R. (2012). Structure and function of the epidermis related to barrier properties. *Clinics in Dermatology*, *30*(3), 257–262. https://doi.org/10.1016/j.clindermatol.2011.08.007

Borrelli, F., Fasolino, I., Romano, B., Capasso, R., Maiello, F., Coppola, D., … Izzo, A. A. (2013). Beneficial effect of the non-psychotropic plant cannabinoid cannabigerol on experimental inflammatory bowel disease. *Biochemical Pharmacology*, *85*(9), 1306–1316. https://doi.org/10.1016/j.bcp.2013.01.017

Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A., & Fusenig, N. E. (1988). Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *The Journal of Cell Biology*, *106*(3), 761–771. https://doi.org/10.1083/jcb.106.3.761

Burstein, S. (2015). Cannabidiol (CBD) and its analogs: A review of their effects on inflammation. *Bioorganic & Medicinal Chemistry*, *23*(7), 1377–1385. https://doi.org/10.1016/j.bmc.2015.01.059

Colombo, I., Sangiovanni, E., Maggio, R., Mattozzi, C., Zava, S., Corbett, Y., … Dell'Agli, M. (2017). HaCaT cells as a reliable in vitro differentiation model to dissect the inflammatory/repair response of human keratinocytes. *Mediators of Inflammation*, *2017*, 7435621. https://doi.org/10.1155/2017/7435621

Couch, D. G., Tasker, C., Theophilidou, E., Lund, J. N., & O'Sullivan, S. E. (2017). Cannabidiol and palmitoylethanolamide are anti-inflammatory in the acutely inflamed human colon. *Clinical Science (London, England)*, *131*(21), 2611–2626. https://doi.org/10.1042/CS20171288

Denizot, F., & Lang, R. (1986). Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *Journal of Immunological Methods*, *89*(2), 271–277. https://doi.org/10.1016/0022-1759(86)90368-6

Esposito, G., De Filippis, D., Maiuri, M. C., De Stefano, D., Carnuccio, R., & Iuvone, T. (2006). Cannabidiol inhibits inducible nitric oxide synthase protein expression and nitric oxide production in beta-amyloid stimulated PC12 neurons through p38 MAP kinase and NF-kappaB involvement. *Neuroscience Letters*, *399*(1–2), 91–95. https://doi.org/10.1016/j.neulet.2006.01.047

Fumagalli, M., Sangiovanni, E., Vrhovsek, U., Piazza, S., Colombo, E., Gasperotti, M., … Dell'Agli, M. (2016). Strawberry tannins inhibit IL-8 secretion in a cell model of gastric inflammation. *Pharmacological Research*, *111*, 703–712. https://doi.org/10.1016/j.phrs.2016.07.028

Gjersvik, P. (2018). Biologics: Targeting systemic inflammation in psoriasis. *The British Journal of Dermatology*, *179*(2), 247–248. https://doi.org/10.1111/bjd.16775

Goldminz, A. M., Au, S. C., Kim, N., Gottlieb, A. B., & Lizzul, P. F. (2013). NF-kappaB: An essential transcription factor in psoriasis. *Journal of Dermatological Science*, *69*(2), 89–94. https://doi.org/10.1016/j.jdermsci.2012.11.002

Hanel, K. H., Cornelissen, C., Luscher, B., & Baron, J. M. (2013). Cytokines and the skin barrier. *International Journal of Molecular Sciences*, *14*(4), 6720–6745. https://doi.org/10.3390/ijms14046720

Hind, W. H., England, T. J., & O'Sullivan, S. E. (2016). Cannabidiol protects an in vitro model of the blood-brain barrier from oxygen-glucose deprivation via PPARgamma and 5-HT1A receptors. *British Journal of Pharmacology*, *173*(5), 815–825. https://doi.org/10.1111/bph.13368

Izzo, A. A., Borrelli, F., Capasso, R., Di Marzo, V., & Mechoulam, R. (2009). Non-psychotropic plant cannabinoids: New therapeutic opportunities

from an ancient herb. *Trends in Pharmacological Sciences*, *30*(10), 515–527. https://doi.org/10.1016/j.tips.2009.07.006

Izzo, A. A., Capasso, R., Aviello, G., Borrelli, F., Romano, B., Piscitelli, F., … Di Marzo, V. (2012). Inhibitory effect of cannabichromene, a major non-psychotropic cannabinoid extracted from Cannabis sativa, on inflammation-induced hypermotility in mice. *British Journal of Pharmacology*, *166*(4), 1444–1460. https://doi.org/10.1111/j.1476-5381.2012.01879.x

Johnston, A., Fritz, Y., Dawes, S. M., Diaconu, D., Al-Attar, P. M., Guzman, A. M., … Ward, N. L. (2013). Keratinocyte overexpression of IL-17C promotes psoriasiform skin inflammation. *Journal of Immunology*, *190*(5), 2252–2262. https://doi.org/10.4049/jimmunol.1201505

Juknat, A., Gao, F., Coppola, G., Vogel, Z., & Kozela, E. (2019). miRNA expression profiles and molecular networks in resting and LPS-activated BV-2 microglia-Effect of cannabinoids. *PLoS ONE*, *14*(2), e0212039. https://doi.org/10.1371/journal.pone.0212039

Khaksar, S., & Bigdeli, M. R. (2017). Intra-cerebral cannabidiol infusion-induced neuroprotection is partly associated with the TNF-alpha/TNFR1/NF-small ka, CyrillicB pathway in transient focal cerebral ischaemia. *Brain Injury*, *31*(13–14), 1932–1943. https://doi.org/10.1080/02699052.2017.1358397

Kogan, N. M., & Mechoulam, R. (2007). Cannabinoids in health and disease. *Dialogues in Clinical Neuroscience*, *9*(4), 413–430.

Kozela, E., Pietr, M., Juknat, A., Rimmerman, N., Levy, R., & Vogel, Z. (2010). Cannabinoids Delta(9)-tetrahydrocannabinol and cannabidiol differentially inhibit the lipopolysaccharide-activated NF-kappaB and interferon-beta/STAT proinflammatory pathways in BV-2 microglial cells. *The Journal of Biological Chemistry*, *285*(3), 1616–1626. https://doi.org/10.1074/jbc.M109.069294

Laprairie, R. B., Bagher, A. M., Kelly, M. E., Dupre, D. J., & Denovan-Wright, E. M. (2014). Type 1 cannabinoid receptor ligands display functional selectivity in a cell culture model of striatal medium spiny projection neurons. *The Journal of Biological Chemistry*, *289*(36), 24845–24862. https://doi.org/10.1074/jbc.M114.557025

Lodzki, M., Godin, B., Rakou, L., Mechoulam, R., Gallily, R., & Touitou, E. (2003). Cannabidiol-transdermal delivery and anti-inflammatory effect in a murine model. *Journal of Controlled Release*, *93*(3), 377–387. https://doi.org/10.1016/j.jconrel.2003.09.001

Marina, M. E., Roman, I. I., Constantin, A. M., Mihu, C. M., & Tataru, A. D. (2015). VEGF involvement in psoriasis. *Clujul Medical*, *88*(3), 247–252. https://doi.org/10.15386/cjmed-494

Mechoulam, R., & Hanus, L. (2002). Cannabidiol: An overview of some chemical and pharmacological aspects. Part I: Chemical aspects. *Chemistry and Physics of Lipids*, *121*(1–2), 35–43. https://doi.org/10.1016/S0009-3084(02)00144-5

Mechoulam, R., Parker, L. A., & Gallily, R. (2002). Cannabidiol: An overview of some pharmacological aspects. *Journal of Clinical Pharmacology*, *42*(S1), 11S–19S. https://doi.org/10.1002/j.1552-4604.2002.tb05998.x

Mechoulam, R., Peters, M., Murillo-Rodriguez, E., & Hanus, L. O. (2007). Cannabidiol—Recent advances. *Chemistry & Biodiversity*, *4*(8), 1678–1692. https://doi.org/10.1002/cbdv.200790147

Micali, G., Lacarrubba, F., Musumeci, M. L., Massimino, D., & Nasca, M. R. (2010). Cutaneous vascular patterns in psoriasis. *International Journal of Dermatology*, *49*(3), 249–256. https://doi.org/10.1111/j.1365-4632.2009.04287.x

Pagano, E., Capasso, R., Piscitelli, F., Romano, B., Parisi, O. A., Finizio, S., … Borrelli, F. (2016). An orally active Cannabis extract with high content in cannabidiol attenuates chemically-induced intestinal inflammation and hypermotility in the mouse. *Frontiers in Pharmacology*, *7*, 341. https://doi.org/10.3389/fphar.2016.00341

Pastar, I., Stojadinovic, O., Yin, N. C., Ramirez, H., Nusbaum, A. G., Sawaya, A., … Tomic-Canic, M. (2014). Epithelialization in wound healing: A comprehensive review. *Advanced Wound Care (New Rochelle)*, *3*(7), 445–464. https://doi.org/10.1089/wound.2013.0473

Schonthaler, H. B., Huggenberger, R., Wculek, S. K., Detmar, M., & Wagner, E. F. (2009). Systemic anti-VEGF treatment strongly reduces skin inflammation in a mouse model of psoriasis. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(50), 21264–21269. https://doi.org/10.1073/pnas.0907550106

Thomas, A., Baillie, G. L., Phillips, A. M., Razdan, R. K., Ross, R. A., & Pertwee, R. G. (2007). Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonists in vitro. *British Journal of Pharmacology*, *150*(5), 613–623. https://doi.org/10.1038/sj.bjp.0707133

Tubaro, A., Giangaspero, A., Sosa, S., Negri, R., Grassi, G., Casano, S., … Appendino, G. (2010). Comparative topical anti-inflammatory activity of cannabinoids and cannabivarins. *Fitoterapia*, *81*(7), 816–819. https://doi.org/10.1016/j.fitote.2010.04.009

Ujvary, I., & Hanus, L. (2016). Human metabolites of cannabidiol: A review on their formation, biological activity, and relevance in therapy. *Cannabis Cannabinoid Research*, *1*(1), 90–101. https://doi.org/10.1089/can.2015.0012

Wang, L. L., Zhao, R., Li, J. Y., Li, S. S., Liu, M., Wang, M., … Guan, D. W. (2016). Pharmacological activation of cannabinoid 2 receptor attenuates inflammation, fibrogenesis, and promotes re-epithelialization during skin wound healing. *European Journal of Pharmacology*, *786*, 128–136. https://doi.org/10.1016/j.ejphar.2016.06.006

## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**How to cite this article:** Sangiovanni E, Fumagalli M, Pacchetti B, et al. *Cannabis sativa* L. extract and cannabidiol inhibit in vitro mediators of skin inflammation and wound injury. *Phytotherapy Research*. 2019;33:2083–2093. https://doi.org/10.1002/ptr.6400