Original Research

Cannabidiol (CBD) with 4’,7-Isoflavandiol (Equol) Efficacy is Greater than CBD or Equol Treatment Alone via Human Skin Gene Expression Analysis

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Abstract

Background: While cannabidiol (CBD) and 4’,7-isoflavandiol (Equol) have been examined individually in various skin studies, the present investigation tested whether topically applied CBD with Equol may yield enhanced effects on human skin biomarkers.

Methods: After 24 hours exposure human skin gene expression was measured by quantitative polymerase chain reaction-messenger ribonucleic acid (qPCR-mRNA) analysis across 9 functional skin categories covering 97 biomarkers.

Results: In general, among the biomarkers analyzed the CBD with Equol treatment displayed greater efficacy compared to CBD only or the Equol treatment alone (e.g., 4 out 5 for anti-acne, 15 out of 17 for anti-aging [e.g., collagen, elastin, calcium binding protein A7, tissue inhibitor of matrix metalloproteinase 1 (TIMP 1), etc.], 19 out of 21 for anti-inflammatory/pain, 10 out of 11 for antioxidants to protect against oxidative stress, 6 out of 6 for circadian rhythm regulation for cell repair/restoration, 10 out of 15 for anti-pigmentation properties, 4 out of 5 for skin hydration, 6 out of 6 for tissue integrity, and 11 out of 12 for wound healing properties). Conclusions: CBD with Equol displayed synergistic effects that may be an effective topical treatment for dermatology and cosmetic applications to improve human skin health and reduce photo-aging.

Keywords: cannabidiol (CBD); isoflavandiol; Equol; human; skin; gene expression; microarray; photo-aging; antioxidant

1. Introduction

Cannabis has been used for medicinal purposes for centuries, and to date more than 500 biologically active compounds have been identified [1]. In brief, Cannabis sativa is composed of two different strains: (a) marijuana that has high tetrahydrocannabinol (THC), the psychoactive phytocannabinoid responsible for the intoxicating euphoric “high”, whereas, (b) hemp contains high amounts of cannabidiol (CBD), the biologically active anti-inflammatory/antioxidant phytocannabinoid that has less than 0.3% of THC and therefore not intoxicating with low abuse potential [1].

CBD was first isolated in 1940 by Roger Adams at the University of Illinois, and the level of research interest was a non-entity until the first journal report was published in 1963 on CBD [1]. Thereafter, published journal reports on CBD remained very low until 2010, but after 2018 (coinciding with the 2018 United States Farm Bill that made hemp an ordinary agricultural commodity), there has been a dramatic increase in CBD research. In 2021 and 2022 more than 1000 journal reports were published on this topic (Fig. 1, Ref. [2]).

Together with the discovery of the endocannabinoid system that has been proven to be an important, multifaceted homeostatic regulator, which influences a wide-variety of physiological functions throughout the body via the main endocannabinoid receptors (CB1 found mostly in the central nervous system and CB2 found in the peripheral nervous system, particularly in immune cells and is also located in many organ and body areas) [3,4]. In general, CBD is known for its anti-inflammatory and anti-nociceptive actions with broad therapeutic potential [3,4]. Furthermore, CBD acting via the CB1 and CB2 receptors located in many different types of skin cells and components have been shown to be involved in skin disorders/diseases (i.e., atopic dermatitis, psoriasis, scleroderma, barrier formation, acne, hair growth, abnormal pigmentation, keratin conditions, itch, wound healing, etc.) [4–7].

As displayed in Fig. 2 (Ref. [4–8]), a general overview of CB1 and CB2 receptors along with immune cells located in human skin layers is presented. One of the first reports of the effects of CBD on human skin gene expression was reported by Langerveld et al. [9] in 2019, where it was shown to regulate over 100 genes that influence, wound healing, pigmentation, aging, acne, and hydration etc. Subsequently, several recent reviews have been published about CBD and skin/cosmetic disorders that highlight its use as an ingredient in dermatology and skin care products [4–8,10].

Conversely, plant-derived phytochemicals have been incorporated into skin care products for several years that possess antioxidant, anti-inflammatory, anti-aging properties (intrinsic/chronological and extrinsic/photo-aging), which have been extensively researched (covering skin pen-
and EquolareshowninFig.

Finally, the chemical structures and characteristicsof CBDray analysis compared to CBD or Equol treatments alone.

biomarkers covering a comprehensive profile via microar-
to test the hypothesis that topically applied CBD
long-lasting effectsafterasingletopicaldose.

While CBD and Equol have been studied individually
in various skin studies, the present investigation was un-
derstood to test the hypothesis that topically applied CBD
with Equol may yield enhanced effects on human skin gene
biomarkers covering a comprehensive profile via microar-
ray analysis compared to CBD or Equol treatments alone.

Finally, the chemical structures and characteristics of CBD
and Equol are shown in Fig. 4.

2. Materials and Methods

2.1 Test Materials, Control(s)—Untreated Tissues,
Transcutol Vehicle Testing; Human Skin Tissue Cultures,
Viability, Validation and Assessment

Transcutol CG was purchased from Gattefosse SAS
(Cedex, France). CBD (99% purity, #191012709) was pur-
chased from Extract Labs (Lafayette, CO, USA). Equol
(CAS 94105-90-5, ≥98% purity) was purchased from Santa
Cruz Biotechnology (Dallas, TX, USA). All other chem-
icals: tissue culture medium, and regents, etc., were also
purchased from Sigma-Aldrich Chemical Company.
The epidermal full-thickness human skin cultures (EFT-400)
from MatTek (Ashland, MA, USA) were maintained at 37
°C with 5% CO₂ and 95% humidity according to Mat-
Tek instructions. The EFT cultures represent human skin
barrier equivalents, the concentration of CBD or Equol
or CBD with Equol combined was applied topically via tran-
scutol (vehicle control) or no material application in un-
treated controls. The total volume of the transcutol con-
tral or treatments dissolved in transcutol that were applied
to the EFT human skin cultures was 20 μL (single appli-
cation), and the exposure time was 24 hours as described
previously [12,18]. Notably, transcutol is considered as a
safe skin penetrating agent and is commonly used in the
formulation or manufacturing process of pharmaceuticals,
cosmetics, and food additives [19]. In order to select con-
centrations of the treatments that did not cause cytoxic-
ity, an MTT viability assay was performed as described
elsewhere [12,18]. In brief, MTT (3-(4,5-dimethylthiazolyl-
2)-2, 5-diphenyltetrazolium bromide) is a yellow colored
tetrazolium salt that is converted to purple formazan by liv-
ing cells. The assay measures the amount of formazan pro-
duced in the cells [12,18]. In this study, the controls (un-
treated, n = 3 served as the positive control; transcutol, n = 3
as the vehicle control; Triton X-100, n = 2 as the negative
control) and test materials (n = 3 for all treatment groups).
For the MTT results (epidermis, dermis and full-thickness)
following 24 hour exposure were quantified. To separate
the epidermis from the dermis, an enzymatic digestion us-
ing Dispase (Corning Life Sciences, Durham, NC, USA)
was used by placing the tissue cultures over night (for 14–
16 hours at 4 °C), then placing the cultures in an incubator
at 37 °C where the epidermis was peeled from the dermis
then assayed.

For all the EFT skin cultures, after the application
of the treatment(s), sections of the skin sample were pre-
pared and stained with hematoxylin/eosin that revealed cel-
lar components [epidermal layers (stratum corneum and
keratinocytes), dermal (fibroblasts), and epidermal/dermal
borders. This method has been reported elsewhere [12,17]
where the histological sections were recorded using a Nikon
microscope camera (DS-Fi3; Melville, NY, USA). Notably,
it is well established that transcutol readily penetrates the
stratum corneum as a skin penetrating agent [19,20].

2.2 Gene Array/mRNA Quantification of Human Skin
Biomarkers

This was accomplished through experiments using
gene (array/mRNA levels) expression, where several skin-
related genes could be examined at the same time using hu-
man skin (EFT) cultures, as preformed previously [12,18].
A total of 163 genes were tested. However, 4 genes did not
amplify well (CNR1, CRN2, NDRG2, ELOVL4), 34 genes
did not display significant alterations with any of the treat-
ments and 9 genes have not been well studied in human
skin (dependent upon stimulation or inhibition of the gene
expression recorded). Therefore, 97 gene biomarkers out of
116 genes or 84 percent were included in the current study.
This gene expression experiment was performed to com-
pare CBD (0.3%), Equol (0.5%) or CBD (0.3%) with Equol
(0.5%) versus transcutol control values for each parameter
(at 24 hour topical exposure), plus untreated controls, n =
4 across all treatment groups. Validation of these methods
used have been reported elsewhere [12–18]. However, in

Fig. 1. Number of Cannabidiol Journal Articles Published from 1960 through 2022 (via PubMed search using cannabidiol as the key term) [2].
Fig. 2. Cartoon displays skin layers/components/immune cells and the location of endocannabinoid receptors (CB1 and CB2). This is a general overview based upon the limitations of space that does not allow labeling of all CB1 and CB2 receptors, immune cells as well as other receptors and enzymes in human skin. 2-AG = 2-arachidonoylglycerol and AEA = arachidonoylethanolamide, which are endocannabinoid-like compounds that bind to CB1 receptors [4–8].

Fig. 3. Cartoon displays skin layers and dermal actions of 4’,7-Isoflavandiol (Equol) via *in vitro* and *ex vivo* investigations.

Inhibits:
1. MMPs and elastase that breakdown collagen & elastin and the 5α-reductase enzyme to decrease androgen hormone actions
2. The inflammatory response & NkappaB to ↓ROS
3. AP-1 & neoplastic cell growth via ERγamma to protect against OS

Stimulates:
1. Collagen, Elastin & TIMP1 for skin integrity
2. Nrf2 to ↑Antioxidants to protect against OS & ROS

Binds to:
1. 5α-DHT to decrease androgen hormone actions in skin
2. Estrogen Receptor Beta to enhance skin health
3. Protects DNA & enhances nerve/tissue repair to guard against OS

The reservoir-forming properties of Equol in the epidermis allow for novel delivery over time of its positive actions to improve skin health and regeneration.

brief, after the topical application of the 20 µL test samples onto the EFT cultures and at the end of the 24 h incubations, total RNA was isolated using Maxwell 16 Simply RNA Tissue kit (Promega, Madison, WI, USA). RNA concentration and purity were determined using a Nanodrop 200 spectrophotometer (A260/A280), all samples had values from 1.8 to 2.1. Also, for quantitative polymerase chain reaction analysis, 1 µg of total RNA from each tissue EFT sample was converted to cDNA using High Capacity DNA Synthesis Kits (Applied Biosystems, Foster City, CA, USA) for open array processing and qPCR reactions were run using validated Taqman gene expression assays, which were analyzed in a Life Technologies QuantStudio 12 K Flex instrument (Thermo Fisher Scientific, Carlsbad, CA, USA). GUSB was the most stable (control) endogenous gene (among 5 control gene tested) and served as the control gene for all samples. Cycling conditions were 50 °C for 2 minutes, 94.5 °C for 10 minutes, followed by 40 cycles of 97 °C for 30 seconds, then 59.7 °C for 1 minute. mRNA levels were quantified using Applied Biosystem’s
Fig. 4. Chemical Structures and Characteristics of Cannabidiol (CBD) and 4',7-Isoflavandiol (Equol). $\text{CLogP} = \log P$ value of a compound representing its partition coefficient and lipophilicity. Polar Surface Area = sum over all polar atoms or molecules, primarily oxygen and nitrogen (and associated hydrogen atoms). Molecules with a polar surface area of greater than 140 angstroms squared ($\text{A}^2$) tend to be poor at permeating cell membranes.

Sequence Detection Software (SDS, v5.0) and RQ Manager (v2.2) programs (Foster City, CA, USA). Inhibition of gene expression was detected by significant lower copy numbers and significant stimulation of gene expression was detected by higher copy numbers compared to vehicle control values for each biomarker (see Data and Statistical Analysis section), plus dCT values normalized to the GUSB values for each biomarker was performed.

2.3 Data and Statistical Analysis

Real-Time RT-PCR data were analyzed using Real-Time StatMiner software v4.2 (Thermo Fisher Scientific, Waltham, MA, USA) for statistical analysis using the relative quantitation (RQ) method. The cycle threshold (CT) value of the target was normalized to the CT value of a selected endogenous control. RQ value was calculated and converted to linear fold changes.

Unpaired $t$-tests were performed, and a $p$-value of less than or equal to 0.05 was reported as statistically significant results ($p \leq 0.05$), as reported previously [12,18]. Treatment groups were compared to the transcutol control results, and a $p$-value of less than 0.05 was reported as statistically significant results ($p < 0.05$). Due to the number of statistical comparisons in the gene array investigation the individual $p$-values were not stated herein. Usually the results are typically expressed as mean ± standard error of the mean (SEM). However, due to the large number of genes tested the SEM is stated herein (the SEM values were less then 6%, which ranged from 0.11 to 3.14 for means ranging from 2 to 57; i.e., depending upon the value of the mean).

3. Results

A total of 163 genes were tested. However, 4 genes did not amplify well, 43 genes were omitted (see methods), therefore, 97 gene biomarkers out of 116 genes or 84 percent were included in the current study regardless of whether the 2-fold or greater change in gene expression had a positive or negative impact on skin health. The 97 gene biomarkers covered nine different skin functions (i.e., acne regulation, anti-aging, anti-inflammatory (pain), antioxidant, circadian rhythm regulation, pigmentation, skin hydration, tissue integrity and wound healing); stimulation or inhibition of certain genes (in most cases), enhanced dermal health.

The MTT results that indicated cell viability (epidermis, dermis and full-thickness) following 24 hour exposure to the treatments are displayed in Fig. 5A–C.

When the skin sections were stained with hematoxylin/eosin, all of the treatments slides displayed the dermal layers and cellular components such as the stratum corneum, keratinocytes, fibroblasts and the epidermal/dermal borders (see Fig. 6).

As shown in Table 1, the skin genes (biomarker names) associated with acne regulation, anti-aging and anti-inflammatory (pain) are displayed along with each gene’s skin function and where the gene is located by skin cell type (either fibroblasts, keratinocytes or both). Among the skin gene analysis involved in acne regulation, CBD with Equol displayed the greatest significant values for four out of the five biomarkers tested. The anti-acne and anti-inflammatory skin effects ranging from −3.77 to −5.31-fold change over CBD or Equol only treatments for ($\text{KLK5}$, $\text{KLK7}$, $\text{PSAPL1}$). Additionally, CBD with Equol significantly stimulated gene expression for $\text{TRIB3}$ above CBD or Equol treatments only. Only the Equol treatment alone showed a significant −2.19-fold change (or inhibition of 219%) for the gene expression of $\text{MAPK3}$ over CBD alone or CBD with Equol values, which also had an anti-acne and anti-inflammatory influence. The CBD treatment group
Fig. 5. (A–C) Testing Cell Viability (expressed as relative percent) via the MTT assay. Topical applications of the treatments (single dose of 20 µL) for 24 hours. The data are expressed as the mean ± SEM for all treatments. The controls (untreated, n = 3 as the positive control; transcutol, n = 3 as the vehicle control; Triton X-100, n = 2 as the negative control; which was untreated controls plus Triton-X 100) and test materials (n = 3 for all treatment groups).
only displayed either no significant alterations in skin gene expression or displayed the lowest fold-change values compared to the Equol only or CBD with Equol levels for this skin function category.

When the anti-aging genes were analyzed (see Table 1), 15 out of the 17 biomarkers showed significant stimulation ranging from 2.07 to 12.63-fold changes (or 207% to 1263%) in the CBD with Equol treatment group compared to the CBD or Equol treatments alone for ADH1B, CASP3, CST6, DNASE1L2, DSC1, DSG1, ELN, IGFL3, PPARG1A, S100A7, SERPINH1, SDR16C5, TGM5 and TIMP1, whereas the CBD with Equol displayed a significant inhibition of GSDMC compared to the CBD and Equol only treatments. Only the Equol treatment alone displayed the greatest significant fold-inhibition for the matrix metalloproteinase 10 (MMP10) biomarker (at 7.48 or 748%) compared to CBD alone (–3.41) or CBD with Equol (at –3.57). Finally, CBD alone was not significant for the collagen biomarker (COL1A1) while Equol alone and CBD with Equol display similar significant levels at 2.10 or 210% vs. 2.07 or 207%, respectively, for the stimulation of this skin gene profile. Moreover, CBD treatment alone in 13 out of the 17 biomarkers displayed no significant changes compared to control values for anti-aging gene expression (Table 1).

Finally, when the anti-inflammatory (pain) genes were tabulated (see Table 1), 18 out of the 21 biomarkers showed significant changes (either stimulation or inhibition) ranging from 2.10 to 19.16-fold (or 210% to 1916%) in the CBD with Equol treatment group compared to the CBD or Equol treatments alone. Notably, one biomarker (IL23A) an interleukin with pro-inflammatory properties was significantly stimulated in the CBD with Equol group compared to the other two treatment groups. Only 2 biomarkers (APOB and IL37) displayed the greatest significant change in gene expression in the Equol only treatment group compared to the CBD only or CBD with Equol values. In general, while the CBD only treatment displayed anti-inflammatory properties among the skin biomarkers tested, this treatment group showed the most non-significant or lowest gene expression levels among the 20 anti-inflammatory genes analyzed (Table 1).

The genes involved as antioxidants, in circadian rhythm regulation and pigmentation are shown in Table 2. In the CBD only treatment group for the antioxidant biomarkers 5 out of 11 genes displayed protection against oxidative stress (OS), however, in this treatment group only the MT1G biomarker showed significantly greater stimulation compared to the Equol only group. Furthermore, the Equol only treatment displayed the greatest significant stimulation of MT1A compared to the CBD only or the CBD with Equol values. Notably, in the CBD with Equol group 10 out of the 11 biomarkers displayed higher numerical values with 9 skin genes that were significantly (stimulated or inhibited) compared to the CBD only or Equol only values that included activation of Nrf2, anti-inflammatory, pro-survival, wound healing and protection against radical oxidative stress (OS) (see Table 2).

Skin cells have specialized “clock” genes that when synchronized can maximize DNA repair by providing nutrients and compounds to assist in skin cell restoration. When 6 clock genes were tested, the CBD only treatment group did not significantly alter any of these genes compared to vehicle control values (see Table 2). In the Equol only trea-
<table>
<thead>
<tr>
<th>Gene name</th>
<th>CBD</th>
<th>Equol</th>
<th>CBD + Equol</th>
<th>Skin function</th>
<th>Skin cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ACNE REGULATION</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. KLK5: Kallikrein Related Peptidase 5</td>
<td>NS</td>
<td>NS</td>
<td>–4.67^</td>
<td>anti-acne, anti-inflammatory</td>
<td>K</td>
</tr>
<tr>
<td>2. KLK7: Kallikrein Related Peptidase 7</td>
<td>–2.34*</td>
<td>NS</td>
<td>–5.31^</td>
<td>anti-acne, anti-inflammatory</td>
<td>K</td>
</tr>
<tr>
<td>3. MAPK3: Mitogen-Activated Protein Kinase 3</td>
<td>NS</td>
<td>–2.19#</td>
<td>NS</td>
<td>anti-acne, anti-inflammatory</td>
<td>F, K</td>
</tr>
<tr>
<td>4. PSAPL1: Prosaposin Like 1</td>
<td>NS</td>
<td>–3.12#</td>
<td>–3.77^</td>
<td>anti-acne, anti-inflammatory</td>
<td>K</td>
</tr>
<tr>
<td>5. TRIB3: Tribbles Pseudokinase 3</td>
<td>NS</td>
<td>2.43#</td>
<td>3.92^</td>
<td>anti-acne, anti-inflammatory</td>
<td>F, K</td>
</tr>
<tr>
<td><strong>ANTI-AGING</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1. ADH1B: Alcohol Dehydrogenase 1B</td>
<td>NS</td>
<td>7.20#</td>
<td>8.93^</td>
<td>anti-aging, anti-pigmentation</td>
<td>F, K</td>
</tr>
<tr>
<td>2. CASP3: Caspase 3</td>
<td>NS</td>
<td>2.25#</td>
<td>2.84^</td>
<td>cell growth/renewal</td>
<td>F, K</td>
</tr>
<tr>
<td>3. COL1A1: Collagen type 1 alpha 1</td>
<td>NS</td>
<td>2.10</td>
<td>2.07</td>
<td>matrix structural protein</td>
<td>F</td>
</tr>
<tr>
<td>4. CST6: Cystatin E/M</td>
<td>NS</td>
<td>3.01#</td>
<td>6.06^</td>
<td>barrier integrity/hydration</td>
<td>K</td>
</tr>
<tr>
<td>5. DNASE1L2: Deoxyribonuclease 1 Like 2</td>
<td>NS</td>
<td>2.04#</td>
<td>3.31^</td>
<td>barrier integrity</td>
<td>K</td>
</tr>
<tr>
<td>6. DSC1: Desmocollin 1</td>
<td>2.14</td>
<td>3.29#</td>
<td>6.29^</td>
<td>anti-aging, matrix function</td>
<td>K</td>
</tr>
<tr>
<td>7. DSG1: Desmoglein 1</td>
<td>NS</td>
<td>2.49#</td>
<td>3.03^</td>
<td>anti-aging, matrix function</td>
<td>K</td>
</tr>
<tr>
<td>8. ELN: Elastin</td>
<td>NS</td>
<td>2.29#</td>
<td>2.55^</td>
<td>anti-aging, elasticity</td>
<td>K, F</td>
</tr>
<tr>
<td>9. GSDMC: Gasdermin C</td>
<td>NS</td>
<td>–2.11#</td>
<td>–2.84^</td>
<td>anti-aging, MMP expression</td>
<td>K</td>
</tr>
<tr>
<td>10. IGFL3: Insulin Growth Factor Like Family Member 3</td>
<td>NS</td>
<td>3.28</td>
<td>5.33^</td>
<td>cell growth/renewal</td>
<td>K</td>
</tr>
<tr>
<td>12. PFARCG1A: PPAR Coactivator 1 alpha</td>
<td>NS</td>
<td>3.01^</td>
<td>↑ Mitochondrial biosynthesis</td>
<td>F, K</td>
<td></td>
</tr>
<tr>
<td>13. S100A4: S100 Calcium Binding Protein A7</td>
<td>2.69</td>
<td>3.58#</td>
<td>7.22^</td>
<td>barrier function</td>
<td>K</td>
</tr>
<tr>
<td>14. SERPINF1: Serpin Family H member 1</td>
<td>NS</td>
<td>2.58#</td>
<td>7.06^</td>
<td>collagen biosynthesis, wound healing</td>
<td>K, F</td>
</tr>
<tr>
<td>15. SDR16C5: Short Chain Dehydrogenases/Reeductase</td>
<td>NS</td>
<td>2.08#</td>
<td>4.42^</td>
<td>anti-aging</td>
<td>K</td>
</tr>
<tr>
<td>16. TGM5: Transglutaminase 5</td>
<td>4.51</td>
<td>6.24#</td>
<td>12.63^</td>
<td>barrier function/hydration</td>
<td>K</td>
</tr>
<tr>
<td>17. TIMP 1: Tissue Inhibitor of Matrix Metalloproteinase 1</td>
<td>NS</td>
<td>2.31#</td>
<td>2.83^</td>
<td>breaks down MMPs</td>
<td>F, K</td>
</tr>
<tr>
<td><strong>ANTI-INFLAMMATORY (PAIN)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. CASP14: Caspase 14</td>
<td>NS</td>
<td>2.24#</td>
<td>4.11^</td>
<td>anti-inflammatory</td>
<td>K</td>
</tr>
<tr>
<td>5. CSF2: Colony Stimulating Factor 2</td>
<td>NS</td>
<td>–7.78^</td>
<td>anti-inflammation</td>
<td>F, K</td>
<td></td>
</tr>
<tr>
<td>6. DMKN: Dermokine</td>
<td>–4.49*</td>
<td>–6.38#</td>
<td>–12.91^</td>
<td>anti-inflammatory</td>
<td>K</td>
</tr>
<tr>
<td>7. IFNA1: Interferon Alpha 1</td>
<td>NS</td>
<td>–2.10^</td>
<td>anti-inflammatory</td>
<td>K</td>
<td></td>
</tr>
<tr>
<td>8. IL10: Interleukin 10</td>
<td>NS</td>
<td>NS</td>
<td>2.14^</td>
<td>anti-inflammatory</td>
<td>F, K</td>
</tr>
<tr>
<td>9. IL18: Interleukin 18</td>
<td>–3.50</td>
<td>–4.73#</td>
<td>–8.39^</td>
<td>anti-inflammatory</td>
<td>K</td>
</tr>
<tr>
<td>10. IL20RA: Interleukin 20 Receptor Subunit Alpha</td>
<td>–2.57</td>
<td>–5.89#</td>
<td>–6.75^</td>
<td>anti-inflammatory</td>
<td>K</td>
</tr>
<tr>
<td>11. IL20RB: Interleukin 20 Receptor Subunit Beta</td>
<td>–2.26</td>
<td>–2.09</td>
<td>–3.28^</td>
<td>anti-inflammatory, anti-psoriatic</td>
<td>K</td>
</tr>
<tr>
<td>12. IL23A: Interleukin 23 Subunit Alpha</td>
<td>6.72</td>
<td>7.31</td>
<td>9.43^</td>
<td>pro-inflammatory</td>
<td>F, K</td>
</tr>
<tr>
<td>13. IL24: Interleukin 24</td>
<td>2.43</td>
<td>3.03</td>
<td>3.63^</td>
<td>anti-proliferative</td>
<td>F, K</td>
</tr>
<tr>
<td>15. IFRF1: Interferon Regulatory Factor 1</td>
<td>2.95</td>
<td>5.79#</td>
<td>6.57^</td>
<td>tumor-suppressor</td>
<td>K</td>
</tr>
<tr>
<td>16. SERPINA12: Serpin Family A Member 12</td>
<td>5.53</td>
<td>9.54#</td>
<td>17.23^</td>
<td>anti-inflammatory, anti-microbial</td>
<td>K</td>
</tr>
<tr>
<td>17. SPINK5: Serine Peptidase Inhibitor Kazal Type 6</td>
<td>NS</td>
<td>3.21#</td>
<td>6.25^</td>
<td>anti-inflammatory</td>
<td>K</td>
</tr>
<tr>
<td>18. ST14: Suppression Tumorigenicity 14 (Maltriptase)</td>
<td>NS</td>
<td>–5.03#</td>
<td>–6.94^</td>
<td>anti-inflammatory</td>
<td>K</td>
</tr>
<tr>
<td>20. TNFSF10: Tumor Necrosis Factor Member 10</td>
<td>–5.00</td>
<td>–6.94#</td>
<td>–8.52^</td>
<td>anti-psoriatic</td>
<td>K</td>
</tr>
<tr>
<td>21. WDF5C5: WAP Four-Disulfide Core Domain 5</td>
<td>–2.46</td>
<td>–4.23#</td>
<td>–6.05^</td>
<td>anti-inflammatory, anti-psoriatic</td>
<td>K</td>
</tr>
</tbody>
</table>

Gene Expression (fold-change) influenced by CBC (0.3%) vs. Equol (0.5%) vs. CBD with Equol, which resulted in 2-fold or greater change (stimulation or inhibition) compared to Vehicle (Transcutol) Control values are displayed. The greatest numerical value (inhibition or stimulation) among the treatment groups is displayed in **bold**. Significantly differences were set at p ≤ 0.05. *= significantly greater (stimulation or inhibition) of CBD only compared to Equol only and/or CBD with Equol values. # = significantly greater (stimulation or inhibition) of Equol only compared to CBD only and/or CBD with Equol values. ^ = significantly greater (stimulation or inhibition) of CBD with Equol values vs. CBD alone and/or Equol alone. NS = not significant compared to vehicle control values. Skin cell type: F = Fibroblast, K = Keratinocyte. ↑ = increase.
Table 2. Human skin gene analysis: part 2.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>CBD</th>
<th>Equol</th>
<th>CBD + Equol</th>
<th>Skin function</th>
<th>Skin cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ANTIOXIDANT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. AHR: Aryl Hydrocarbon Receptor</td>
<td>NS</td>
<td>2.08</td>
<td>2.67^</td>
<td>activate Nrf2, ↑ other antioxidants</td>
<td>F, K</td>
</tr>
<tr>
<td>2. CAT: Catalase</td>
<td>NS</td>
<td>2.14</td>
<td>2.54^</td>
<td>antioxidant</td>
<td>F, K</td>
</tr>
<tr>
<td>3. DUOX1: Dual Oxidase 1</td>
<td>NS</td>
<td>-3.65</td>
<td>-4.10^</td>
<td>anti-inflammatory</td>
<td>F, K</td>
</tr>
<tr>
<td>4. EGLN3: Egl-9 Family Hypoxia Inducible Factor</td>
<td>NS</td>
<td>3.57#</td>
<td>4.29^</td>
<td>limits hypoxia factors</td>
<td>K</td>
</tr>
<tr>
<td>5. HAL: Histidine Ammonia Lyase</td>
<td>2.35</td>
<td>3.21</td>
<td>6.10^</td>
<td>antioxidant, anti-inflammatory, wound healing</td>
<td>F, K</td>
</tr>
<tr>
<td>6. HMOX1: Heme Oxygenase 1</td>
<td>-3.96</td>
<td>NS</td>
<td>3.48^</td>
<td>antioxidant, anti-inflammatory, wound healing</td>
<td>F, K</td>
</tr>
<tr>
<td>7. MT1A: Metallothionein 1A</td>
<td>3.67</td>
<td>4.57#</td>
<td>3.58</td>
<td>antioxidant</td>
<td>F, K</td>
</tr>
<tr>
<td>8. MT1G: Metallothionein 1G</td>
<td>42.78*</td>
<td>12.51</td>
<td>57.12^</td>
<td>antioxidant</td>
<td>F, K</td>
</tr>
<tr>
<td>9. NFE2L3: Nuclear Factor Erythroid Like 3</td>
<td>NS</td>
<td>-2.10</td>
<td>-2.53^</td>
<td>pro-survival, anti-proliferative</td>
<td>F, K</td>
</tr>
<tr>
<td>10. SLC30A1: Solute Carrier Family 30 Member 1</td>
<td>3.18</td>
<td>NS</td>
<td>3.55^</td>
<td>antioxidant</td>
<td>F, K</td>
</tr>
<tr>
<td>11. TXNRD1: Thioredoxin Reductase 1</td>
<td>NS</td>
<td>5.89#</td>
<td>7.71^</td>
<td>protects against free radicals/oxidative stress</td>
<td>F, K</td>
</tr>
<tr>
<td><strong>CIRCADIAN RHYTHM REGULATION</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. CIRT: Circadian Associated Repressor of Transcription</td>
<td>NS</td>
<td>NS</td>
<td>-2.21^</td>
<td>anti-proliferative</td>
<td>F, K</td>
</tr>
<tr>
<td>2. CLOCK: Clock Circadian Regulator</td>
<td>NS</td>
<td>NS</td>
<td>2.27^</td>
<td>DNA repair/hydration/barrier function</td>
<td>K</td>
</tr>
<tr>
<td>3. CRY1: Cryptochrome Circadian Regulator 1</td>
<td>NS</td>
<td>NS</td>
<td>2.79^</td>
<td>skin firmness/elasticity/antioxidant</td>
<td>K</td>
</tr>
<tr>
<td>4. NOCT: Nocturnin</td>
<td>NS</td>
<td>-2.68#</td>
<td>-3.13^</td>
<td>epidermis homeostasis/inverse CLOCK</td>
<td>K</td>
</tr>
<tr>
<td>5. PER2: Period Circadian Regulator 2</td>
<td>NS</td>
<td>2.02#</td>
<td>2.79^</td>
<td>CLOCK regulator, hair growth</td>
<td>K</td>
</tr>
<tr>
<td>6. RORA: RAR Related Orphan Receptor A</td>
<td>NS</td>
<td>NS</td>
<td>-3.05^</td>
<td>decrease acne</td>
<td>F, K</td>
</tr>
<tr>
<td><strong>PIGMENTATION</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. ABRB2: Adrenoreceptor beta 2</td>
<td>-3.02*</td>
<td>-2.10</td>
<td>-3.47^</td>
<td>anti-pigmentation</td>
<td>F, K</td>
</tr>
<tr>
<td>2. ALOX12B: Arachidonate Lipoxygenase 12R Type</td>
<td>-2.95</td>
<td>-30.27#</td>
<td>-33.74^</td>
<td>anti-pigmentation, brightening</td>
<td>K</td>
</tr>
<tr>
<td>3. BMP6: Bone Morphogene Protein 6</td>
<td>5.97*</td>
<td>4.25</td>
<td>9.63^</td>
<td>anti-pigmentation, scar formation</td>
<td>F, K</td>
</tr>
<tr>
<td>4. CALM3: Calmodulin Like 5</td>
<td>-2.81</td>
<td>-14.70#</td>
<td>-8.05^</td>
<td>anti-pigmentation</td>
<td>F, K</td>
</tr>
<tr>
<td>5. CRYBG1 (AIM1): Crystallin Beta-Gamma Domain 1</td>
<td>NS</td>
<td>NS</td>
<td>-2.75^</td>
<td>anti-pigmentation</td>
<td>F, K</td>
</tr>
<tr>
<td>6. CTSF: Cathepsin V</td>
<td>NS</td>
<td>-2.27#</td>
<td>-7.08^</td>
<td>anti-pigmentation</td>
<td>F, K</td>
</tr>
<tr>
<td>7. EDNRB: Endothelin Receptor Type B</td>
<td>3.32*</td>
<td>2.64</td>
<td>3.43^</td>
<td>pro-pigmentation</td>
<td>F, K</td>
</tr>
<tr>
<td>8. KIT: KIT Proto-Oncogene Receptor Tyrosine Kinase</td>
<td>-2.48</td>
<td>-2.23</td>
<td>-2.52</td>
<td>anti-pigmentation</td>
<td>F, K</td>
</tr>
<tr>
<td>9. KRT5: Kallikrein Related Peptide 5</td>
<td>NS</td>
<td>NS</td>
<td>-2.34^</td>
<td>anti-pigmentation</td>
<td>K</td>
</tr>
<tr>
<td>10. MC1R: Melocortin 1 Receptor</td>
<td>NS</td>
<td>-2.34#</td>
<td>-4.87^</td>
<td>anti-pigmentation</td>
<td>F, K</td>
</tr>
<tr>
<td>11. MITF: Melanogenesis Associated Transcription Factor</td>
<td>NS</td>
<td>NS</td>
<td>-2.66^</td>
<td>anti-pigmentation</td>
<td>F, K</td>
</tr>
<tr>
<td>12. PAQR7: Progestin, AdipoQ Receptor Family Member 7</td>
<td>-2.57</td>
<td>-2.94#</td>
<td>-2.02^</td>
<td>pro-pigmentation</td>
<td>F, K</td>
</tr>
<tr>
<td>13. PPARG: Peroxisome Proliferator-Activated Receptor G</td>
<td>NS</td>
<td>2.66#</td>
<td>3.36^</td>
<td>pro-pigmentation, anti-psoriatic</td>
<td>F, K</td>
</tr>
<tr>
<td>14. QPCT: Glutaminyl-Peptide Cyclotransferase</td>
<td>NS</td>
<td>-2.94#</td>
<td>-2.02^</td>
<td>anti-pigmentation</td>
<td>F, K</td>
</tr>
<tr>
<td>15. STAP2: Signal Transducing Adaptor Family Member 2</td>
<td>NS</td>
<td>NS</td>
<td>-2.27^</td>
<td>anti-pigmentation</td>
<td>F, K</td>
</tr>
</tbody>
</table>

Gene Expression (fold-change) influenced by CBC (0.3%) vs. Equol (0.5%) vs. CBD with Equol, which resulted in 2-fold or greater change (stimulation or inhibition) compared to Vehicle (Transcutol) Control values are displayed. The greatest numerical value (inhibition or stimulation) among the treatment groups is displayed in **bold**. Significantly different were set at $p \leq 0.05$. * = significantly greater (stimulation or inhibition) of CBD only compared to Equol only and/or CBD with Equol values. # = significantly greater (stimulation or inhibition) of Equol only compared to CBD only and/or CBD with Equol values. ^ = significantly greater (stimulation or inhibition) of CBD with Equol values vs. CBD alone and/or Equol alone. NS = not significant compared to vehicle control values. Skin cell type: F = Fibroblast, K = Keratinocyte. † = increase.
tment 2 clock genes displayed significant values over controls, but in the CBD with Equol treatment all 6 clock genes (CIRT, CLOCK, CYRI, NOCT, PER2 and RORA) were significantly greater (either stimulation or inhibition) compared to the CBD only or Equol only values, which included DNA repair/hydration, barrier function, skin firmness, elasticity, etc. (Table 2).

For genes associated with pigmentation, in the CBD only group 6 out of the 15 biomarkers showed significant changes (either stimulation or inhibition) compared to vehicle control values and 3 of these biomarkers displayed significantly greater levels compared to the Equol only group (i.e., ABRB2, BMP6 and EDNRB, see Table 2). In the Equol only group 11 out of the 15 biomarkers showed significant changes compared to vehicle control values or CBD only levels. However, in the CBD with Equol group 10 out of 15 biomarker displayed anti-pigmentation skin function results compared to the Equol only or CBD only values, suggesting support for skin lightening properties with this combination (Table 2).

Skin genes associated with hydration, tissue integrity and wound healing as influenced by the 3 treatments are displayed in Table 3. In the CBD only group, 2 genes showed significant changes (ALOX12B and BMP4) over vehicle control levels and in fact, BMP4 values were significantly altered (inhibition) compared to Equol only or CBD with Equol values. For the Equol only group, 4 out of the 5 genes showed significantly greater stimulation compared to the CBD only values. While in the CBD with Equol treatment 4 out of the 5 biomarkers displayed the greatest significant stimulation among the skin genes (ALOX12B, ALOXE3, AQP9 and EPHX3) compared to the CBD only or Equol only levels (see Table 3).

When the tissue integrity biomarkers were examined 4 out of 6 genes in the CBD only group showed significant stimulation over vehicle control values (for ABCG4, CD36, CDSN, FLG2). Also, in the Equol only group the same biomarkers displayed significantly greater stimulation compared to the CBD only levels. While in the CBD with Equol group all 6 out of 6 biomarkers (ABCG4, CD36, CDSN, FLG2, KRT1 and KRT10) displayed significantly greater stimulation compared to the CBD only or Equol only values, suggesting cell/tissue integrity, cell survival, collagen binding and cell adhesion characteristics (Table 3).

Finally, wound healing biomarkers were examined where 4 out of the 12 biomarkers were significantly stimulated in the CBD only group over vehicle control values (e.g., FAF2, FETUB, KUTKG, LEP). In the Equol only group, 3 out of the 12 biomarkers showed significantly increased stimulation (or inhibition) over vehicle control levels and with 2 genes (KITLG showed stimulation and PIP1 displayed inhibition) that showed significantly greater values compared to CBD only levels. For the CBD with Equol treatment, 11 out of the 12 biomarkers showed significantly greater stimulation or inhibition compared to the CBD only or Equol only values, suggesting enhanced wound healing, antioxidant effects and cell/tissue survival (Table 3).

4. Discussion

While CBD and Equol have been investigated individually as treatments, the purpose of this study examined whether CBD with Equol yielded better influences on human skin biomarkers compared to CBD or Equol alone. The transdermal delivery of CBD and Equol has been previously demonstrated via Franz cell diffusion testing [21–23]. In fact, the human transdermal delivery of Equol has been reported to accumulate in the epidermal layers (binding to estrogen receptors (beta)) then slowly released into the dermis over 28 hours after a single topical application [23].

Acne is the most common skin condition in the United States that affects approximately 85% of people 12 to 24 years of age [24]. Both CBD and Equol alone displayed some anti-acne influences on some of the skin biomarkers, but the greatest effect was observed when the CBD with Equol treatment was employed (in 4 out of 5 skin genes). The present data confirm the anti-acne effects of CBD, where previous reports suggest the anti-inflammatory mechanisms work upon acne by inhibiting the cytokines such as TNF-α and IL-1β [24,25]. The anti-androgen effects of Equol has been reported, where it binds the potent androgen 5α-dihydrotestosterone (5α-DHT) plus inhibits the 5α-reductase (type I) enzyme (in fibroblasts) responsible for converting testosterone to 5α-DHT that has pro-acne effects [11,13]. In this regard, Riyanto et al. [26] in 2015 reported that isoflavone administration of 160 mg per day for 12 weeks in a clinical study significantly reduced acne vulgaris. Presumably, the actions of CBD and Equol combined accounted for the anti-acne results reported herein, plus other beneficial anti-inflammatory, antioxidant and anti-androgen actions.

Aging of the skin is due to exposure to the sun (photaging) that damages dermal layers associated with the loss of collagen, elastin and the activation of matrix metalloproteinases (MMPs) that breakdown the structural components of the skin due to oxidative stress, inflammation and the lack of antioxidants to combat these assaults [11,27]. Previous in vitro, ex vivo and clinical studies demonstrated that CBD or Equol have anti-aging properties that boost dermal structural proteins (collagen & elastin) and antioxidants while inhibiting oxidative stress and inflammation [4–7,9–15]. Additionally studies that examined CBD showed that immune responses (e.g., the aryl hydrocarbon receptor (AHR) redox homeostasis, the capase-1-IL 1 beta axis) were significant in its skin rejuvenating properties [28–33]. In general, the present results confirm these skin protective and enhancement effects of CBD or Equol, but especially when CBD with Equol was utilized to examine the human skin biomarkers covering anti-aging, inflammation and antioxidants. For example, the collagen (COL1A1), elastin (ELN), calcium binding protein (S100 A7), ser-
Circadian rhythms are maintained by networks of molecular clocks throughout the core and peripheral tissues, including immune cells, blood vessels, perivascular adipose tissues, etc. [37]. Skin cells have specialized “clock” genes that when synchronized can maximize DNA repair by providing nutrients and compounds to assist in skin cell restoration at certain time intervals [38]. While the Equol treatment alone significantly stimulated two clock genes, CBD with Equol significantly stimulated 6 of 6 of the clock genes in vitro. 

The previous clinical studies on CBD or Equol that investigated anti-aging, inflammatory and antioxidant parameters were validated, in part, by the in vitro human skin gene expression results in the present report.
especially beneficial in aging individuals [39]. Also, the polyphenols, such as resveratrol and Equol have been studied via “clock” genes, where activation of SIRT1 by the PER2 and CLOCK genes have been shown to promote vascular health [37]. In brief, the present finding of the CBD with Equol treatment, when the 6 “clock” genes were analyzed, suggested a potential benefit to skin health.

For the 15 pigmentation genes examined in this study (while CBD alone or Equol alone demonstrated significant anti-pigmentation results) the best outcome was with the CBD with Equol treatment, where 10 out of the 15 biomarkers were significantly altered (Table 2). As reviewed by Baswan et al. [6] in 2020, where CBD pigmentation treatments were evaluated (upon citation of a few available studies) their conclusion was the complex CBD-melanocyte function warranted further research to determine targeted pigmentation disorders using cannabinoids. Conspicuously, Tanaka et al. [40] in 2021 reported from in vitro studies that equol metabolites (quinones) were cytotoxic to melanocytes. However, this notion is unlikely since large clinical studies where topical equol treatment has been shown to improve skin spots/discoloration in (a) women, (b) in a small pilot study in men (topical or oral) and (c) in high unit topical commercial cosmetic products used by women (>100,000 subjects) no adverse effects were observed [13,27,41]. Therefore the present human skin gene expression results may support, in part, regulation of pigmentation for increase skin radiance.

Hydration plays a vital role in the preservation and repair of skin integrity. Dehydration disturbs cell metabolism and wound healing. Moreover, adequate fluid intake is necessary to support blood flow to wounded tissues and prevent breakdown of skin components such as the stratum corneum [42,43]. The therapeutic potential of cannabinoids for integumentary wound healing [44] and the isoflavonoid, equol has estrogenic actions such as skin hydration, cellular/tissue repair and wound healing [23,27] have been reported elsewhere. Again, CBD or Equol alone displayed skin biomarkers that were positive for hydration, tissue integrity and wound healing, but the greatest significant stimulation of gene expression was seen in the CBD with Equol treatment group (Table 3), that suggested skin health-promoting actions among the biomarkers examined. However, despite the wide-ranging benefits of CBD on dermal health, Perez et al. [45] in 2022 reported that cannabigerol (CBG), which is the direct precursor to CBD and tetrahydrocannabinol (THC) to have greater anti-inflammatory, antioxidant (protecting against UV light or photo-aging), anti-acne and skin barrier properties compared to CBD. In fact, in vehicle-controlled clinical studies, Perez et al. [45] showed that topical application of CBG was safe and may be more effective for its anti-aging properties than CBD. Therefore, further research is necessary to determine if the combination of CBG and Equol (or other polyphenols like resveratrol, quercetin, etc.) would provide better improvements in skin biomarkers in vitro and a technology platform for advanced skin technology in vivo for clinical/commercial use.

5. Conclusions

The present data sets suggest that CBD or Equol alone has beneficial effects on the skin parameters tested, but the CBD with Equol treatment indicated greater efficacy across the 9 dermal function categories analyzed among the 97 biomarkers that were quantified. In ranking the treatments CBD < Equol < CBD with Equol, which had synergistic effects. This represents opportunities for technological advancements of this combination of active ingredients and other potential sources of natural ingredients as therapeutic applications for improved skin health, especially protecting against photo-aging.

Availability of Data and Materials

All applicable data and materials are included in the manuscript.

Author Contributions

EDL designed the research study, analyzed the data, wrote the manuscript, contributed to editorial changes in the manuscript, and read and approved the final manuscript.

Ethics Approval and Consent to Participate

This study used human skin tissues from a commercial supplier and the studies were performed by a contact research organization (CRO), thus the requirement for BYU university IRB approval was waived # IRB 100122019, therefore this approval is not applicable.

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Conflict of Interest

Edwin D. Lephart has no funding or sponsor conflicts of interest in the decision of the data/research presented in this report and regarding the publication of this manuscript. Edwin D. Lephart is an inventor on polyphenolic patents (US and worldwide) on various human health applications. Given the role as Guest Editor, Edwin D. Lephart had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Graham Pawelec.
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